HOWARD K. SCHACHMAN

ULTRACENTRIFUGATION IN BIOCHEMISTRY

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1959

ACADEMIC PRESS • NEW YORK AND LONDON

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK 3, N. Y.

United Kingdom Edition
Published by
ACADEMIC PRESS INC. (LONDON) LTD.
40 PALL MALL, LONDON SW 1

Library of Congress Catalog Card Number 59-7688

PRINTED IN THE UNITED STATES OF AMERICA

Foreword

Over the past decade the use of the ultracentrifuge as a tool in biochemical research has developed in a dramatic fashion. Problems scarcely imagined ten years ago can now be investigated with brilliant success and with techniques only recently developed. Moreover the theoretical analysis of the processes occurring in sedimentation has been refined and deepened and placed upon a surer foundation. For a brief and clear perspective on these advances the reader may turn to the introductory chapter of this book. These developments have occurred with almost bewildering rapidity, and one who wishes to attain a comprehensive view of them has had to turn to many scattered papers and review articles. The classic monograph of Svedberg and Pedersen on the ultracentrifuge is still of vital interest for those who are concerned with research on sedimentation, and an important review of some of the theoretical aspects of sedimentation by Williams, van Holde, Baldwin, and Fujita, has recently appeared. There remains, however, need for an extended discussion of these more recent developments, combined with a statement of the basic and indispensable theory and a general description of the old and new experimental techniques.

I believe that these needs are admirably met by the present book. I cannot speak of it with complete detachment since, as one of the editors of *Advances in Protein Chemistry*, I had something to do with its origins. Dr. Schachman had, indeed, been invited by us to write a chapter for the *Advances*, dealing with the uses of the ultracentrifuge in protein chemistry. He responded with enthusiasm to the invitation, but soon found the scope of the discussion growing inevitably far beyond what he had originally contemplated; it evolved from a single chapter into an independent book, which is here presented.

This book is likely to become an indispensable companion to the laboratory worker who is planning and conducting an ultracentrifuge run for almost any purpose. It should also be of fundamental value to the thoughtful student or investigator who wants to know the present state of our knowledge in the field, both experimental and theoretical. No technique has done more than ultracentrifugation for the fundamental physical chemistry of macromolecules, and in recent years it has become increasingly applicable to small molecules as well. As a tool for the preparation and separation of proteins, viruses, and many other substances it is of great and growing importance. Moreover the processes occurring in a sedimentation experiment are complex and fascinating; it is important for the experimenter to under-

stand them, for many workers have gone astray through failure to interpret correctly the phenomena they observed. Dr. Schachman has, I believe, performed a major service by portraying the broad range of experimental techniques in sedimentation experiments and by providing a rigorous discussion of the underlying principles.

JOHN T. EDSALL

March, 1959

Preface

This monograph had its inception at the invitation tendered the author (by the Editors of the *Advances in Protein Chemistry*) to write a review dealing with recent advances in the application of the ultracentrifuge. To the author's chagrin it soon became apparent that the advances were occurring at a rate exceeding that achieved in the actual writing. Moreover, ultracentrifugal theory and practice had developed to such a degree that a description of them consumed space beyond the bounds of the typical review published in the *Advances in Protein Chemistry*. Consideration of this dilemma by the Editors and the Publisher led to the decision to publish this particular review as a separate monograph. This brief historical explanation has been presented here for two reasons. First, the author wishes to thank the Editors of the *Advances in Protein Chemistry* for providing the stimulus for this article, for their encouragement, and for the constructive criticisms they offered upon examination of the manuscript. Dr. J. T. Edsall, in particular, has been most generous with his time, and his suggestions have been of considerable value. Second, much has been added to the manuscript subsequent to the time it went to press as an article for the *Advances in Protein Chemistry*. Some of the inadequacies in organization can be attributed to the belated insertions and revisions. Though the author would like to be able to account for other deficiencies in a similar fashion, few valid justifications come to mind; he alone is responsible.

Despite the expansion of the manuscript while "in press," the goal initially set, i.e., to describe the principles of ultracentrifugation, remains unchanged. Many applications of interest and importance in biology are not discussed since they were considered to be outside the scope of this monograph. As a consequence the reader will not find in this volume detailed discussions, for example, of the behavior of serum albumin or the polydispersity of deoxyribonucleic acid or the properties of many other macromolecules of biological interest. When such materials are cited, only those aspects dealing with fundamentals of ultracentrifugation are stressed. Also missing from this volume are considerations of preparative centrifugation. Thus the reader will find no detailed treatment of the powerful methods of "zone centrifugation." Although one form of this technique—namely sedimentation equilibrium in a density gradient—is described, the emphasis is toward the theoretical side and the much more important practical aspects are scarcely mentioned. The results of several recent significant investigations were added while the monograph was "in press."

Further important contributions have since appeared, and the author regrets that it was not feasible to include a discussion of these later efforts. Already the monograph needs revision and extension. Perhaps others who have courage, energy, and patience may undertake such a task.

A considerable amount of hitherto unpublished work from the author's laboratory is described in this volume. Thus the reader will find more mention of the Rayleigh interferometer and examples of its application than could be justified by the existing literature. A similar situation exists with regard to the discussion of the application of the ultracentrifuge to the analysis of interacting systems composed of large and small molecules. In both instances the author was motivated not only by his own present research interests but also by the conviction that these areas are likely to become of paramount interest in the near future. Others no doubt would have emphasized different areas now under development.

Since the review was already set in print before the publication plans were altered, the original format containing sections, rather than chapters, was maintained. The first of these, *Introduction*, is a sketch of the field highlighting some of the principal developments. Following this is the section, *General Considerations*, in which ultracentrifugation is discussed in general terms and the division of the field into three major areas is described. This categorization is somewhat arbitrary, to be sure, but it has pedagogical value. The underlying theoretical considerations basic to all types of ultracentrifugal practice are presented in this section as well. The third section deals with developments of the experimental aspects of the field such as improvements in the instrument itself, cells, rotors, measurement and control of temperature, and the various optical systems. Included is a qualitative discussion of convection in ultracentrifuge cells. Despite the title, *Experimental Aspects*, no attempt was made to present here a guide for conducting experiments. For such material the reader will have to look elsewhere. The following three sections deal with the fundamental principles of the major divisions mentioned above: *Sedimentation Velocity*, *Transient States*, and *Sedimentation Equilibrium*. Much of the literature in the last mentioned area is concerned with nonbiological materials, and omission of pertinent discussions of some important developments is justified only by the decision to concentrate on ultracentrifugation in biochemistry. It is clear that these studies are rapidly becoming relevant to biochemists as they study nonideal solutions of materials like deoxyribonucleic acid. Future volumes will doubtless devote considerable space to consideration of the sedimentation equilibrium of charged macromolecules. Finally a section is included which deals with interpretation of sedimentation data in terms of hydrodynamic models, charge effects, and interactions in multicomponent systems.

Of the experimental work (cited in this monograph) which has been done

in the author's laboratory, much has been supported generously over a period of years by the Office of Naval Research under contract with the University of California. Substantial support has been received as well from the National Science Foundation. It is the hope of the author that the contributions of his former colleagues to the research activities of this laboratory have been adequately set forth. To each of them, P. Y. Cheng, Ann Ginsburg, W. F. Harrington, R. T. Hersh, S. Katz, E. G. Richards, V. N. Schumaker and J. Stenesh, and to Pearl Appel and Jean Miller for their able and enthusiastic technical assistance, he wishes to express his indebtedness and thanks. Also the author has had the benefit over many years of pleasant and stimulating discussions with R. L. Baldwin, G. Kegeles, E. G. Pickels, R. Trautman and D. F. Waugh on many diverse aspects of ultracentrifugation. Each of them has made available results of his own work prior to publication. Finally the indebtedness of the author to W. M. Stanley and M. A. Lauffer cannot be overemphasized: to the former for his continued support and encouragement over a long period of time and to the latter for the author's initial exposure to the field of ultracentrifugation and for the stimulation of his interest in the study of macromolecules of biological interest.

In the preparation of this monograph many valuable suggestions and criticisms have been made by R. L. Baldwin, S. Hanlon, W. F. Harrington, P. Johnson, E. G. Richards, and V. N. Schumaker and their help is gratefully acknowledged. Miss Jean Miller is largely responsible for the expert photographic work involved in transferring patterns from the ultracentrifuge plates to the printed page, without, I might add, the use of a paint brush. Considerable help has been given the author by Mary Abbott in the preparation of the manuscript. A major part of this review was written during the author's tenure at the Department of Microbiology, Washington University School of Medicine as a John Simon Guggenheim Memorial Fellow, 1957–58. The author would like to express his thanks to A. Kornberg and others in that department for their hospitality.

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I. Introduction

The past 10 years has been a period of revolutionary change in research with the ultracentrifuge—almost as dramatic as the era beginning in 1923 when Svedberg and his collaborators (Svedberg and Nichols, 1923) first began exploiting centrifugal fields for the study of macromolecules and colloidal particles. As recently as 1947, there were in operation throughout the world only about 8 oil turbine ultracentrifuges of the Svedberg design and about an equal number of air-driven ultracentrifuges of the Beams-Pickels type. Now in addition to those instruments, there are in widely separated laboratories over 300 electrically driven ultracentrifuges. Moreover preliminary results herald even more radical departures in instrument design and performance. As a consequence of this tremendous increase in research activity, a review of the field by a single author is a formidable task destined to fall short in some areas which workers consider of great significance and to be too detailed in other areas which to some are trivial. Fortunately there have been excellent reviews, in recent years, which add to the classic and definitive work of Svedberg and Pedersen (1940), so that the arbitrary concentration, in the present review, on certain aspects of the field should give not too distorted a picture of the growing discipline called ultracentrifugation. The reader is referred for other outlooks to Nichols and Bailey (1949), Kinell and Rånby (1950), and Williams (1954). A thorough treatment of the theoretical aspects of different types of ultracentrifugal problems has just appeared (Williams *et al.*, 1958).

Developments of note have included new types of ultracentrifuge cells which permit measurements hardly visualized by even the most farsighted research workers of the 1930's. Coupled with these radical modifications in ultracentrifuge cells has been the exploitation of multicell rotors so that many different samples can be examined simultaneously. The adaptation of optical systems hitherto unused in connection with the ultracentrifuge, tremendous improvements in older, familiar techniques, and the rebirth of discarded optical methods have all occurred in the past 10 years, and it is now safe to predict that at least three different optical systems will be in routine use on many ultracentrifuges in a short time. Factors hardly considered for many years, such as the precise measurement of temperature and the effect of pressure, are now either clarified or the subject of active and productive investigation. Older views emphasizing the instability of

ultracentrifuge boundaries in experiments at concentrations below 0.01% have required modification, and now meaningful, quantitative ultracentrifuge analyses on certain types of systems are being performed routinely at concentrations of several thousandths of a per cent.

Advances of a theoretical nature have been just as rapid. Interpretation of data from ultracentrifugal studies in terms of the properties of the sedimenting substance is now on a more firm basis. Because of theoretical developments, molecular weights can now be determined with accuracy in a single experiment of very short duration. Analysis of mixtures is now feasible. The effects of concentration have been carefully considered and reliable theories presented. We now recognize that many of the claims of the 1930's and 1940's regarding the homogeneity of proteins were ill-founded and must be modified in keeping with recent theoretical treatments. Despite this we can return, with greater confidence, to the view that the ultracentrifuge is one of the most powerful tools available for the study of the distribution of molecular weights within a given preparation. Moreover, considerations of polydispersity are no longer restricted to sedimentation rates or molecular weights. Ultracentrifuge data in conjunction with other measurements can provide valuable information about the variations in shape among a collection of macromolecules in a given preparation. Even without additional data, polydispersity with respect to density, and therefore with regard to chemical composition, is amenable to direct analysis by ultracentrifugation. The changes over the past years have been so extensive that even the term "sedimentation constant" is disappearing from the literature to be replaced by the more appropriate "sedimentation coefficient." New areas of research have led to the introduction of terms like the "second moment of the gradient curve," "differential sedimentation rate," and the "Archibald method."

In response to newer demands of the protein chemist, we seem to be approaching an era already reached in diffusion measurements where results with an accuracy of 0.1% can be expected. Thus the presence or absence of the contribution of a single amino acid to the molecular weight of a large protein molecule will be detected by ultracentrifugal methods already available or in process of development. Similarly, configurational changes in protein molecules in complicated solvent systems will be followed with the aid of the ultracentrifuge. Already techniques and theories have been developed for the determination of molecular weights of biologically active material, even in impure preparations and with 1-μg. quantities of material. All that is required is a sensitive and accurate biological assay specific for the entity under investigation and an ultracentrifuge cell capable of dividing the solution into two fractions at some desired time. It seems likely that enzymologists will employ these greatly

improved methods to an extent not hitherto contemplated. Most experiments can be performed rapidly and at low temperature, so that labile proteins can be examined with little risk of denaturation. For most substances, a complete study can be performed with as little as 10 to 20 mg., and in special circumstances microgram quantities will suffice. Newer ultracentrifugal methods are equally applicable to the examination of molecules considerably smaller than most proteins and can be used, therefore, in studies of coenzymes and substrates or the products of action of proteolytic enzymes or nucleases. As a consequence, it can be anticipated that the ultracentrifuge will find increasing application to studies of the mechanism of enzymatic action on large substrate molecules. In certain systems, the ultracentrifuge has already been extensively used in the study of protein-protein interaction, but surprisingly little use has been made of quantitative ultracentrifugal analysis in the examination of interactions between large and small molecules. Also little use of the ultracentrifuge has been reported in the study of the mode of synthesis of biologically important macromolecules or the path of labeled atoms of an infecting virus particle upon its invasion of a susceptible cell where more virus particles are produced at a later time. Many substances of interest to biochemists, such as the cytochromes, hemoglobins, nucleic acids, and nucleoproteins, have such high extinction coefficients either in a particular region of the visible spectrum or in the ultraviolet that the sedimentation of these materials is readily examined by absorption techniques even at concentrations of only 0.001%. Moreover, these substances have characteristic absorption spectra and can, therefore, be examined in the ultracentrifuge even in crude mixtures as long as light of the appropriate wavelength is available. Absorption methods are particularly valuable for the study of the interaction of dyes or coenzymes with specific proteins or enzymes. In structural studies of nucleoproteins, heme proteins, or cell particulates in which the macromolecules are being degraded by enzymes or chemical reagents, these absorption methods provide additional information because they can be used to determine the physical properties of a specific part of the macromolecule. The next few years are likely to produce more applications of the ultracentrifuge to these fascinating biological problems, while the application of the instrument to studies of purity and molecular size and shape are relegated to a more routine role.

Despite the phenomenal progress both experimentally and theoretically which has been outlined above, gaps in our knowledge still exist both with respect to understanding some effects already being studied and, even more, with regard to the potentialities of ultracentrifugation. This review deals principally with ultracentrifugation as it is currently being applied or, in the author's opinion, likely to be applied to biological systems. Of

necessity there will be speculation on the part of the author, and the treatment which follows will reflect his prejudices and interests. Some of the areas discussed are presently hot investigated from an experimental point of view, and doubtless revision of some of the ideas will be required as data become available. This course was chosen rather than an alternative one involving a compilation, perforce less critical, of the manifold applications of the ultracentrifuge to this or that protein. These examples are more profitably examined along with other physical and chemical data on a specific protein. Admirable reviews bringing together pertinent information on individual systems are now available (Edsall, 1953; Waugh, 1954; and Anfinsen and Redfield, 1956).

Much of the ultracentrifugal data in the literature require revision either because of improvements in experimentation or because theoretical developments have shown that the results are calculated or interpreted incorrectly. For this reason, we will concentrate on fundamental aspects of ultracentrifugation. Illustrations when given are to demonstrate a particular problem in ultracentrifugation, and diagrams frequently will be those obtained in the author's laboratory only because original ultracentrifuge patterns could not be obtained readily.

II. General Considerations

Ultracentrifuges can be used either to measure the velocity of movement of macromolecules in a solution under the influence of a centrifugal field or to determine the distribution of the macromolecules in a centrifuge cell which is rotating at comparatively low speeds. In the latter after a fixed period of time, an equilibrium state is established in which the concentration of solute at each level in the cell no longer varies.

The former method is known as the *sedimentation velocity method* and to date has been the more widely used. In a sedimentation velocity experiment, the ultracentrifuge rotor is operated at speeds up to 60,000 r.p.m. so that the solute molecules which initially were uniformly distributed throughout the solution in the ultracentrifuge cell are caused to settle at appreciable rates toward the periphery of the cell. This migration of the solute molecules leaves a region containing only solvent molecules in addition to the region in the cell where the concentration is uniform. Between the supernatant and the solution of uniform concentration, known as the "plateau region," there is a transition zone in which the concentration varies with distance from the axis of rotation. This transition zone is called the boundary, and the sedimentation velocity method is generally based on observations, by optical methods, of the movement of such boundaries, which in turn are a measure of the movement of the solute molecules in the plateau region. Sedimentation velocity data can be obtained, however, even in the absence of a complete boundary and this will be discussed presently. Figure 1A shows schematic drawings of the concentration of solute as a function of distance in the cell at different times. Since the more commonly used optical systems do not record concentration, but rather the change in concentration (more properly, the change in refractive index) with distance, the corresponding curves are drawn in Fig. 1B. It is such diagrams as illustrated in Fig. 1B, which are generally used to study the purity of a given preparation and to determine the sedimentation coefficient of the sedimenting material. It should be noted that the sedimentation velocity method permits the direct measurement of sedimentation coefficients which are related to both the size and shape of the sedimenting molecules. Other independent data are needed for the evaluation of molecular weights. Under certain circumstances fairly reliable diffusion coefficients can also be obtained from sedimentation

velocity experiments, and, therefore, molecular weights can be obtained solely from sedimentation velocity studies. Figure 1 shows the changes which occur for solute molecules which are more dense than the solvent and therefore migrate in a centrifugal direction leaving the newly created solvent region near the meniscus. For solutes which are less dense than

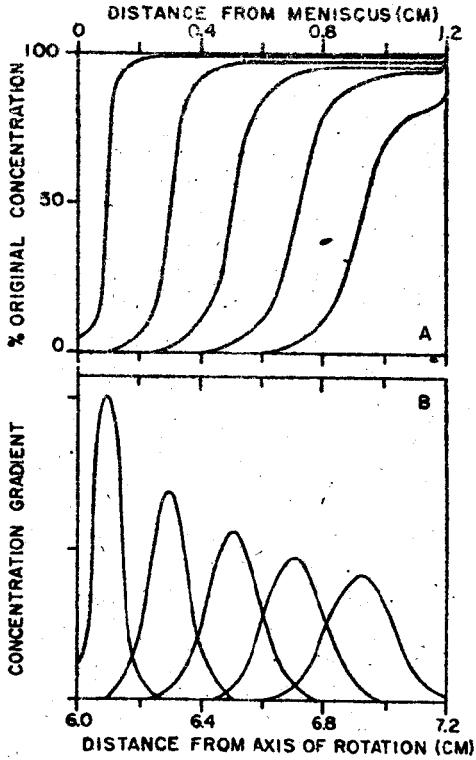

FIG. 1. Schematic diagram of the concentration (A) and concentration gradient (B) as a function of distance in the centrifuge cell during a sedimentation velocity experiment.

the solvent, such as lipoproteins in concentrated salt solutions, the solute molecules tend to float (migrating in a centripetal direction) leading to a region of pure solvent at the bottom of the cell. Sedimentation coefficients in the latter system would be negative. The concentration gradients (and generally the refractive index gradients) across the boundaries developed in such systems are negative, and the observed ultracentrifuge patterns, as a consequence, are inverted.

In the second application of the ultracentrifuge, known as the *sedimentation equilibrium method*, the ultracentrifuge is operated at relatively

low speeds (about 8000 r.p.m. for a protein with a molecular weight of 60,000). Under these conditions the transport of solute in a centrifugal direction due to sedimentation is sufficiently slow as to be counterbalanced by transport in a centripetal direction by the diffusion resulting from the concentration gradient created by the partial sedimentation of the macromolecules. During the first stages of a sedimentation equilibrium experiment, the concentration decreases at the meniscus and increases at the bottom of the cell, owing to sedimentation. As a consequence of back diffusion, however, a region devoid of solute is not created as in the sedimentation velocity method. Instead, the concentration will remain finite at the meniscus as long as the centrifuge is not operated at too high a speed. Under conditions which are ideal for precise experimentation, the concentration at the meniscus will approach a value about one-half its initial value. Similarly, sedimentation plus back diffusion near the cell bottom leads to a region in which the concentration is about twice the original concentration. In the sedimentation velocity method, the role of diffusion at the bottom of the cell is less important, and this region of high concentration is restricted to a very thin layer on the cell bottom, where the sedimented material is packed as a gellike pellet. During the early stages of a sedimentation equilibrium experiment, the concentration near the center of the cell is independent of position and practically the same as the initial concentration. As the run proceeds, the plateau region disappears, and there is only one position in the cell with a concentration equal to the initial concentration. Finally, after a considerable period of time, an equilibrium state is reached and no further changes in concentration occur with time. These variations in concentration with distance as a function of time are visualized in Fig. 2A and the corresponding gradient curves are illustrated in Fig. 2B.

In contrast to the sedimentation velocity method, measurement of the concentration distribution at sedimentation equilibrium gives directly the molecular weight of the sedimenting macromolecules (both methods require knowledge of the partial specific volume of the macromolecules). Despite this obvious advantage and the additional factor that the theoretical foundations for the equilibrium method are more firm than those for the sedimentation velocity method, there are only few recorded examples of the application of the sedimentation equilibrium method to the study of proteins. This seemingly anomalous situation has arisen both because of the lack of apparatus capable of sustained, continuous use for the long periods (days, in the case of proteins) required before equilibrium is reached, and because many proteins are not sufficiently stable to withstand such experiments. Apparatus is now available which is capable of continuous operation even at low temperature, but of greater importance,