Methods in Enzymology

Volume XXVII

Enzyme Structure

Part D

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Preface

This is the second of two volumes of "Enzyme Structure" devoted to physical methods. (Part C, Volume 26 of "Methods in Enzymology," appeared recently.) Although coverage of the various techniques is not exhaustive, it is hoped that the intent of presenting a broad coverage of currently available methods has been reasonably fulfilled.

These volumes present not only techniques that are currently widely available but some which are only beginning to make an impact and some for which no commercial standard equipment is as yet available. In the latter cases, an attempt has been made to guide the reader in assembling his own equipment from individual components and to help him find the necessary information in the research literature.

In the coverage of physical techniques, we have departed somewhat in scope from the traditional format of the series. Since, at the termination of an experiment, physical techniques frequently require much more interpretation than do organic ones, we consider that brief sections on the theoretical principles involved are highly desirable as are sections on theoretical and mathematical approaches to data evaluation and on assumptions and, consequently, limitations involved in the applications of the various methods.

The division of the material between the two parts is arbitrary. Thus, there is a considerable amount of overlap between general categories, and, at times, the descriptions of closely related techniques are found divided between Parts C and D. We do not believe, however, that this should hinder the reader in his use of these volumes for, in every case, each chapter is a completely self-contained unit.

We wish to acknowledge with pleasure and gratitude the generous cooperation of the contributors to this volume. Their suggestions during its planning and preparation have been particularly valuable. We also wish to thank the staff of Academic Press for their many courtesies.

C. H. W. Hirs
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METHODS IN ENZYMOLOGY

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II. Preparation and Assay of Enzymes
III. Preparation and Assay of Substrates
IV. Special Techniques for the Enzymologist
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Section I

Molecular Weight Determinations and Related Procedures

[1] Ultracentrifugal Studies with Absorption Optics and a Split-Beam Photoelectric Scanner[1]

By H. K. SCHACHMAN and STUART J. EDELSTEIN

I. Introduction

As in other areas of research on the ultracentrifuge during the past 50 years, we have witnessed remarkable progress in the development, adaptation, and application of a variety of optical methods for viewing sedimentation processes. Emphases and goals have changed markedly as new problems in biology were recognized, the demands of research workers became more exacting, and the developments in technology opened new avenues for further explorations. In describing the present use and application of the photoelectric scanning absorption optical system, it behooves us to note that the first optical system employed by Svedberg and his colleagues 50 years ago was based on the absorption

[1] This research was supported in part by U.S. Public Health Service Research Grants GM 12159 to H.K.S. from the National Institute of General Medical Sciences and HL 13591 to S.J.E. from the National Heart and Lung Institute, and by National Science Foundation Research Grants GB 4810X to H.K.S. and GB 8773 to S.J.E.

of light by the sedimenting macromolecules.[1a,2] Their absorption optical system, which seems inconvenient, inaccurate, and unwieldy by today's standards, was replaced within 15 years by the schlieren optical system.[3-5] This latter system provided direct viewing of the movement and distribution of molecules in a centrifugal field. But this extraordinarily convenient schlieren optical system gave way in part about 15 years ago because of the pressing demands for enhanced accuracy. Hence, many sedimentation experiments, and particularly sedimentation equilibrium studies, are analyzed today by means of interference optics.[6] Meanwhile, the requirements for greater sensitivity and the need of biochemists to distinguish among the various chemical species present in solutions led to the rebirth of the light absorption optical system which had been discarded prematurely and ignored too long.[7,8]

Accompanying the renewed and widespread use of absorption optics for the study of nucleic acids was a growing frustration with a system which had been denounced variously as "inflexible," "inaccurate," "inconvenient," "laborious," "time-consuming," and even "impossible." Hence efforts were initiated in the late 1950's to incorporate into the optical system some of the products of the technological revolution which had occurred since Svedberg and his co-workers developed and employed absorption optics. The resulting photoelectric scanner has been used widely during the past decade for many types of sedimentation studies. Meanwhile the requirements of the workers have increased again, and the scanner in the form used in most laboratories is no longer considered satisfactory. Thus major changes in it are occurring. In this article we first review the advantages of the absorption optical system in relation to the schlieren and interference systems. This comparison in the next section highlights the principal defect, insufficient accuracy, of absorption optics. Following that, we consider the basic principles of split-beam scanners and the virtues and deficiencies of instruments based on the use of double-sector ultracentrifuge cells. Later sections deal with the applications of existing techniques for a host of sedimentation velocity and equilibrium studies. Both interacting and noninteracting systems are

[1a] T. Svedberg and J. B. Nichols, *J. Amer. Chem. Soc.* **45**, 2910 (1923).
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illustrated. Experimental aspects, and particularly pitfalls and remedies, are treated in the following section. Finally we discuss the recent development of scanners connected to on-line computers which, though not widely tested as yet, show considerable promise in yielding greatly enhanced accuracy.

II. Comparison of Absorption Optics with Other Optical Systems

The ideal optical system for the ultracentrifuge should be sensitive, convenient, discriminating, versatile, and accurate. All these demands cannot as yet be met by any single system, but the absorption optical system shows considerable promise in fulfilling satisfactorily most of the criteria which research workers would agree upon.

A. Sensitivity

Sensitivity was apparent even in the original optical system devised by Svedberg and his co-workers.[2] Since many biological macromolecules absorb appreciable amounts of light in the near or far ultraviolet region of the spectrum, their migration or redistribution in a centrifugal field can be measured readily by an absorption optical system equipped with a monochromator.[9] For nucleic acids the absorbance at 260 nm is so great that solutions containing only a few micrograms per milliliter can be analyzed readily.[7,8] Comparable absorbances with protein solutions can be achieved with light of wavelength about 220 nm, with the result that proteins can be studied now at these same great dilutions.[10,11] These same macromolecules when added to dilute aqueous solutions cause such small increments in refractive index that neither schlieren optics nor interference optics can rival the absorption method in terms of sensitivity. For some biopolymers, such as polysaccharides, this sensitivity does not prevail since there is little absorption of light by the polymer in a wavelength range which is readily accessible for experimentation. Thus sensitivity must be gauged in terms of the spectral properties of the macromolecules and the solvent. Although some substances could be detected and analyzed readily with infrared light, the experimentation may not be feasible because the solvent itself may absorb most of the light.

[9] H. K. Schachman, L. Gropper, S. Hanlon, and F. Putney, *Arch. Biochem. Biophys.* **99**, 175 (1962).

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B. Convenience

Convenience has been achieved only recently with the development of the photoelectric scanning system.[12-18] Prior to the construction of the scanner, the absorption system was woefully inadequate. Not only were the operations time-consuming and laborious but there was, in addition, the overwhelming psychological drawback that the research worker was unable to observe the sedimentation process during the experiment. The tedium and the delay in analyzing experiments were eliminated when the photoelectric scanner replaced the photography and the required densitometry.[2] Even in its earliest, primitive form the scanner produced rapidly and directly plots of concentration (really absorbance) and concentration gradient versus position in the cell. Subsequent developments which permit multiplexed operations have yielded even greater convenience since many different samples can be studied in a single ultracentrifuge experiment. Since the electrical pulses from the photomultiplier are digitized and interfaced conveniently to dedicated computers[19-21] the scanner has the added convenience of automation. Developments in this area are just beginning, but already the results with on-line computer operations are so promising that the absorption optical system compares favorably with the schlieren and interference optical systems.

C. Discrimination

The absorption optical system has the great advantage of discrimination since different components can be distinguished one from another by way of variations in their absorption properties. In contrast, the schlieren and interference optical systems are inadequate since these methods are responsive to changes in refractive index only and since most solutes cause approximately equal increments in refractive index. Hence schlieren and interference optics afford no possibility for distinguishing or identifying different chemical species in solution. By judicious choice of the wavelength of light with the absorption system the research worker

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