METHODS OF PROTEIN SEPARATION

Volume 2

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PREFACE

This open-end treatise on methods concerning protein separation had its beginning in an American Chemical Society symposium entitled "Contemporary Protein Separation Methods" which was held in Atlantic City, New Jersey in September 1974. The purpose of the symposium—and subsequently of the present work—was to review the available modern techniques and underlying principles for achieving one of the very important tasks of experimental biology, namely the separation and characterization of proteins present in complex biological mixtures. Physicochemical characterization was covered only as related to the parent method of fractionation and therefore involved mostly mass transport processes. Additionally, the presentation of methods for gaining insight into complex interacting protein profiles was considered of paramount importance in the interpretation of separation patterns. Finally, specific categories of proteins (e.g., chemically modified, deriving from a specific tissue, conjugated to different moieties, etc.) require meticulous trial and selection and/or modification of existing methodology to carry out the desired separation. In such cases, the gained experience provides valuable guidelines for further experimentation.

Although powerful techniques exist today for the separation and related physicochemical characterization of proteins, many biological fractionation problems require further innovations. It is hoped that the description in the present treatise of some of the available separation tools and their limitations will provide the necessary integrated background for new developments in this area.

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SCANNING GEL CHROMATOGRAPY 1

GARY K. ACKERS

I. INTRODUCTION

Gel permeation chromatography is widely used both as a preparative separation technique and as an analytical tool for the estimation of molecular size and the study of interactions between macromolecules. In this latter category it is unsurpassed among physical techniques in sensitivity, precision, and flexibility. The migration rates and detailed shapes of zones containing interacting solutes permit the determination of reaction stoichiometry and equilibrium constants over a very broad range of conditions. In addition to these nonequilibrium transport methods, the technique of equilibrium gel permeation provides a powerful means to study thermodynamic properties of multicomponent interacting solute systems, such as macromolecule–ligand binding (see Ackers, 1975, for a general review). A new and particularly promising development is the technique of active enzyme chromatography (Jones, 1975; Jones *et al.*, 1976), in which a small band of enzyme is passed down a column previously saturated with substrate and the product of the enzymatic reaction is monitored by successive optical scanning of the column during transport. By determining the "rate of motion" of the chromophoric product being formed, it is possible to infer the molecular size of the active species.

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The most commonly employed experimental arrangement for analytical applications of gel chromatography is the conventional elution experiment in which concentrations of solute are monitored (e.g., by spectrophotometry) in the effluent as a function of time. This has the virtue of requiring an absolute minimum in equipment. However, a great deal of information is lost by such procedures wherein the solute profile is sampled at only a single point in space (i.e., that corresponding to the end of the column). An alternative approach to the elution mode of data acquisition lies in direct optical scanning of the solute profiles at a sequence of stages during transport along the column (Brumbaugh and Ackers, 1968). In this way, a large amount of detailed information regarding shapes and positions can be determined rapidly during the course of an experiment. It is the purpose of this chapter to review the technique of scanning gel chromatography and to describe the applications of this technique to analysis of macromolecular solutes. The theoretical background of gel chromatography has been brought to a rather complete stage of development elsewhere (for a recent review, see Ackers, 1975) and will not be discussed further here.

II. INSTRUMENTATION

A. Scanning Gel Chromatographs: General Description

The instrumentation required for direct optical scanning of gel columns is basically very simple in design, although considerably higher performance is required than for scanning continuous slabs or cylinders of gel. The high photometric performance is required because of the great amount of light scattered by the gel particles. The diagram in Figure 1 shows the general features of such a system, which is representative of several scanning gel chromatographs developed in our laboratory. In these instruments the column is made to move while the optics remain stationary, although other arrangements are possible. Columns consist of precision-bore quartz and are fitted at top and bottom with porous polyethylene discs. The column and its adapter are mounted on a precision screw with a scanning range determined by limiting microswitches at each end. A beam of light from a stable deuterium or tungsten source is passed through the monochromator (and any additional filters used) and subsequently through a horizontal slit 1 mm high. Thus a 1-mm "slice" of the column is sampled by a beam which is 2 mm wide or less, depending on the (vertical) monochromator slit width. This beam of parallel monochromatic light, rectangular in cross section, is passed through the

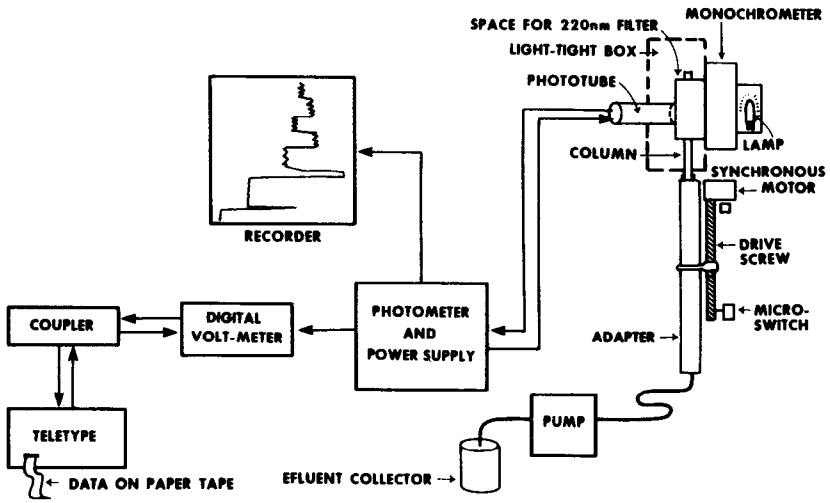

FIGURE 1. Schematic diagram of scanning gel chromatograph with digital data acquisition system.

column. Light exiting from the column (again through an identical 1-mm horizontal slit) is measured by an end-on photomultiplier positioned close to the column track. Columns of approximately 1 by 15 cm with gel bed volumes of 10 ml are most commonly used in applications to be described here. Samples are added manually to the column. Signals from the photomultiplier are fed into an amplifier with a digital voltmeter-coupler, and the output voltages, proportional to transmittance, are recorded on a teletype equipped with a paper tape punch (380 data points for a 10-cm scan) as well as on a strip chart recorder. The recorder is used primarily as a visual monitor on the quality of data being produced in successive scans. The punched paper tapes are analyzed on a minicomputer by an interactive program that permits great flexibility in editing, averaging, etc. The criterion used for reproducibility of baselines and repetitive scans of other kinds is that they must be within 0.0015 absorbance unit—a level of precision easily achieved with our instrument even at wavelengths of 220 nm. Scans are performed while the column is flowing. The flow is controlled by an external pump on the output side of the system and is always set at a value less than the column's natural flow rate. Typical flow rates range between 1 and 6 ml/hr and typical scanning times are on the order of 3 min.

B. Optical and Photometric Requirements

For accurate quantitative work, the requirement of high photometric accuracy and linearity must be met at high absorbances (e.g., up to 3.0 absorbance units) because of the need to work above the high light-scattering background of the gels. This background may be as high as 1–2 absorbance units, as shown in Figure 2 for a series of Sephadex gels at different wavelengths. For studies with proteins at very low concentration, it is necessary that the photometric requirements be met at wavelengths below 280 nm, in the region of the peptide bond absorption (which has a maximum near 190 nm). The instruments we have developed operate successfully on the side of this band, at wavelengths down to 210 nm, and routinely at 220 nm,

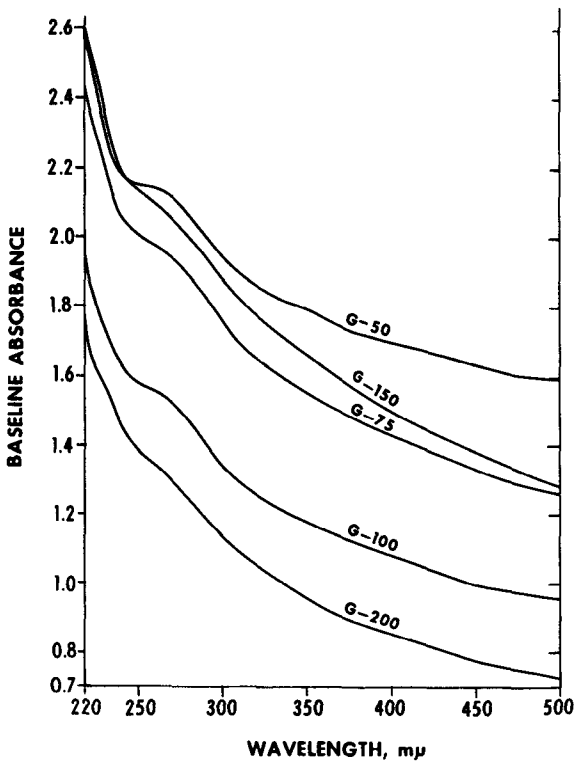

FIGURE 2. Apparent absorbances due to light scattering by Sephadex gels as a function of wavelength (pathlength 0.945 cm). These values define the baselines above which absorbances by chromophoric solutes must be measured. From Ackers (1970).