Methods in ENZYMOLOGY

IV

METHODS IN ENZYMOLOGY

Edited by

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VOLUME IV

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METHODS IN ENZYMOLOGY

VOLUME IV

I: Preparation and Assay of Enzymes

II: Preparation and Assay of Enzymes

III: Preparation and Assay of Substrates

IV: Special Techniques for the Enzymologist

PREFACE

The first three volumes of this series have dealt with the commonly employed methods for the preparation and assay of enzymes, coenzymes, and substrates. The present volume deals with certain special techniques which are considered to be of interest to those in the field of enzymology. Descriptions of many of these procedures are available in monographs dealing respectively with the physical chemistry of the proteins, with specialized methods for the measurement of metabolism, or with procedures for the synthesis, degradation, and measurement of isotopically labeled compounds. It nevertheless appeared worthwhile to have these descriptions written with the special problems of the enzymologist in mind and to have them brought together in a single volume.

With the publication of this volume we bring to completion our original plans for a treatise on Methods in Enzymology. It has been our policy to select a recognized authority for the description of each laboratory procedure, with the aim of obtaining a reliable collection of reproducible methods. For the extent to which we have succeeded in this aim, we are grateful to all those investigators who have contributed. It would of course have been desirable that all procedures described in these four volumes be checked in an independent laboratory prior to publication. However, it is obvious that with such a system of checking, the publication of an organized treatise of this scope would have been impossible within any reasonable time period.

Users of these volumes will undoubtedly find that certain articles are already in need of revision and that certain important new subjects are not covered at all. In an attempt to correct these unavoidable deficiencies, an organized single supplementary volume is planned, which will include material not heretofore covered, as well as revised procedures, to supplement all four of the published volumes. We welcome any suggestions concerning the content of this supplementary volume, which will be published approximately two years from this date.

Baltimore, June 14, 1957

SIDNEY P. COLOWICK
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Section VII. Determination of Inorganic Compounds

Erratum for Volume I

P. 140, line 3: should read "19.21 g." instead of "21.01 g."

Erratum for Volume II

P. 432, line 2: should read "6.0 ml." instead of "60 ml."

Errata for Volume III

P. 663, line 3: should read "0.35 γ" instead of "35 γ."
P. 843, line 2 from bottom: should read "KH_2PO_4" instead of "K_2HPO_4."
P. 948, line 6: should read "31" instead of "0.031."
P. 1055, entry in author index: Abraham, S., 64.

VOLUME IV

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Section I

Techniques for Characterization of Proteins (Procedures and Interpretations)

[1] Electrophoresis

By ARNE TISELIUS

Enzymes, as well as proteins in general, are ampholytes, and therefore their electrochemical properties in relation to the pH of the medium are useful for their characterization. The mobility in an electric field at defined pH values and particularly the pH value at which the migration is zero (the isoelectric point) are widely used for this purpose, as these properties can be determined accurately and conveniently with the methods now available. The name electrophoresis for such phenomena used to refer chiefly to colloids and substances of large molecular weight. However, as the methods gradually have been extended also to low molecular weight material, the new term of "ionophoresis" has been introduced for this case. As it is very difficult to draw a limit here, and as the phenomena involved and the experimental methods applied are practically the same, the author has preferred to use the term electrophoresis throughout this paper.

A particularly interesting and valuable feature of electrophoresis is that the differences in rates of migration for the various constituents of a mixture lead to a certain separation, each component migrating at its own characteristic rate. Thus an electrophoretic analysis is possible which can give valuable information concerning the composition of complex mixtures in solution and the homogeneity or purity of a preparation. Another advantage is that this separation is such a gentle procedure, as it involves only the migration in a medium of approximately constant composition. For preparative purposes this may be very important, especially the recent developments in this field which have provided some very useful methods with many possible applications in enzymology as well in protein chemistry in general. A considerable experience collected during the past twenty years shows that the electrophoretic mobility is a highly specific property, and thus even closely similar proteins often may be differentiated by electrophoresis. This is true, however, only if a not too narrow range of pH is investigated, as in certain pH regions mobilities of different substances may differ very little. It should also be noted that the method, on account of its gentleness, will not resolve complexes of different substances unless they are extensively dissociated in solution under the conditions of the experiment. Many cases are known in which enzymes or other proteins, even after repeated crystallization, give inhomogeneous electrophoretic diagrams. Protein crystals tend to adsorb foreign material, but this phenomenon is probably less marked in electro-

phoretic separation where all components are in solution. Thus positively and negatively charged proteins may migrate almost independently of each other in buffered solutions. This is by no means universally true, however, and cases are known where interaction occurs.[1–3] Fairly stable complexes may be formed between dyes and proteins in solution, and these are not separated by electrophoresis. The demonstration of the existence of such complexes may, however, be of great interest from an entirely different point of view, if they are found in extracts of biological material which have not been subject to drastic operations. They give an indication of the presence of such associates in the original material which for many problems in enzyme chemistry may be significant. Complex formation may also be utilized to aid in the separation. The best-known example is perhaps the electrophoretic fractionation of uncharged carbohydrates which have been complexed with boric acid (Consden and Stanier[4]). No doubt there are many interesting possibilities of increasing the specificity of electrophoretic analysis by such methods (see also Cann *et al.*[4a]).

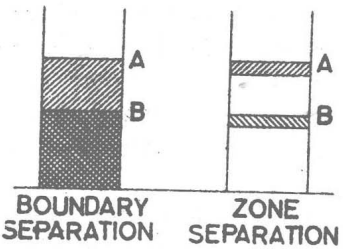

FIG. 1. Boundary and zone separation.

With methods which depend on differential migration through a medium it is convenient to distinguish between boundary methods and zone methods, as entirely different techniques are used (Fig. 1). In boundary separation the migration of the boundaries is observed, and the experiment is usually performed in a U-shaped tube where the protein solution is layered under a buffer solution. Thus the system is stabilized by the density differences, and the migration can take place in free solution without the addition of a stabilizing medium. In this case, however, only the fastest and slowest components can be isolated in substance. Intermediate components overlap, but although their separation is observed it cannot easily be utilized for a real isolation. In this respect zone separation is superior. A zone of the sample will, during its migration, split up into as many different zones as it contains differently migrating components, each zone consisting of a single component, which is easily isolated. As the zones have a higher density than the medium, however,

[1] L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.* **25**, 507 (1942).
[2] W. Grassmann, *in* "The Chemical Structure of Proteins" (Wolstenholme and Cameron, eds.), pp. 55, 195. Churchill, London, 1953.
[3] M. Lautout and M. de Serge, *Compt. rend.* **240**, 1282 (1955).
[4] R. Consden and W. M. Stanier, *Nature* **169**, 783 (1952).
[4a] J. R. Cann, J. G. Kirkwood, R. A. Brown, and O. J. Plescia, *J. Am. Chem. Soc.* **71**, 1603 (1949).

they are not stable, and the important advantage of zone methods has to be gained at the price of stabilizing the system by using a suitable filling material, such as cellulose, starch powder, filter paper, or a density gradient. This introduces a new factor which may influence mobilities and isoelectric points in a way which is difficult to predict. But if the separation is the main purpose, these methods are very useful and have found many applications during the last years.

Paper strip electrophoresis has become particularly popular as a micro-method and will be discussed in the following section by Dr. H. Laurell (Vol. IV [1A]). Zone electrophoresis in packed columns has recently been developed to a high degree of perfection. It bears the same relation to paper strip electrophoresis as column chromatography does to paper chromatography, and it allows the separation of considerable quantities of material. This method will therefore also be discussed in some detail.

There are some special modifications of electrophoretic analysis without a clear-cut zone or boundary separation which have gained preparative importance. This is particularly true of the electrophoresis convection method and of methods depending on electrophoresis through membranes. The literature on electrophoresis is very extensive. A selection of references to books or articles of a more general interest is given below.

1. General principles and boundary methods.[5–19]
2. Theory.[14,20]

[5] H. A. Abramson, L. S. Moyer, and M. H. Gorin, "Electrophoresis of Proteins." Reinhold Publishing Corp., New York, 1942.
[6] H. A. Abramson, E. J. Cohn, B. D. Davis, F. L. Horsfall, L. G. Longsworth, D. A. MacInnes, H. Mueller, and K. Stein, *Ann. N.Y. Acad. Sci.* **39**, 105 (1939).
[7] H. J. Antweiler, "Die quantitative Elektrophorese in der Medizin." Springer-Verlag, Berlin, 1952.
[8] D. R. Briggs, *in* "Biophysical Research Methods" (F. M. Uber, ed.), pp. 271–300. Interscience Publishers, New York, 1950.
[9] H. B. Bull, "Physical Biochemistry," 2nd ed., pp. 161–190. John Wiley & Sons, New York, 1951.
[10] L. G. Longsworth and D. A. MacInnes, *Chem. Revs.* **24**, 271 (1939).
[11] L. G. Longsworth, *Chem. Revs.* **30**, 323 (1942).
[12] J. A. Luetscher, *Physiol. Revs.* **27**, 621 (1947).
[13] K. G. Stern and M. Reiner, *Yale J. Biol. and Med.* **19**, 67 (1946).
[14] H. Svensson, *Arkiv Kemi Mineral. Geol.* **22A** (1946).
[15] E. Wiedemann, *Scientia Pharm.* **17**, 45 (1949).
[16] A. Tiselius, *Scientia* **45**, 163 (1951).
[17] A. Tiselius, *Naturwissenschaften* **37**, 25 (1950).
[18] A. Tiselius, *Trans. Faraday Soc.* **33**, 524 (1937).
[19] A. Tiselius, *Nova Acta Regiae Soc. Sci. Upsaliensis* [4]**7**, No. 4 (1930).
[20] L. G. Longsworth and D. A. MacInnes, *J. Am. Chem. Soc.* **62**, 705 (1940).

3. Zone methods in general and preparative electrophoresis.[21–25]
4. Paper electrophoresis (see Vol. IV [1A]).

Boundary Electrophoresis

For boundary electrophoresis experiments a U-shaped tube is generally used in which the sample, dissolved in a suitable buffer solution, is allowed to form a layer under a buffer solution of the same composition, so that the boundaries will migrate in a medium of approximately constant composition. The electric current is introduced with reversible electrodes, separated from the boundaries by a sufficient volume of buffer solution to prevent changes in the composition produced at the electrodes to reach the boundaries. The boundaries are stabilized by the differences in density, and thus no special stabilizing medium is required as in zone electrophoresis. Consequently the "free" electrophoretic migration of the components can be measured, and the true mobilities can be calculated directly from the experiments. To secure a sufficient stability the concentrations should not be too low, and it is generally difficult to obtain reliable results at concentrations below 0.01% (protein) unless special precautions are taken (low current). The heat produced by the current may cause convections, and when studying systems of high conductivity (e.g., serum) or low protein concentration it is essential to minimize this disturbance by running the experiment at a temperature just above 0°.[18] As water has a density maximum around +4°, the density differences produced by temperature changes in this region are very much smaller than at room temperature, for example. As low temperature is of advantage for other reasons in protein and enzyme work, most experiments of this type are nowadays being performed in refrigerated baths.

To facilitate optical observation of the boundaries by the methods described below, the electrophoresis cell is given a rectangular cross section. The U-tube is assembled by one or several such cells, mounted with plane-parallel plates which are greased so that they can slide horizontally a sufficient distance to cut the contents of the tube into a number of fractions. This sliding arrangement is also used when the boundaries

[21] H. G. Kunkel, *in* "Methods of Biochemical Analysis," Vol. I, pp. 141–170. Interscience Publishers, New York, 1954.
[22] H. J. McDonald, "Ionography. Electrophoresis in Stabilized Media." Year Book Publishers, Chicago, 1955.
[23] H. Svensson, *Advances in Protein Chem.* 4, 252 (1948).
[24] R. L. M. Synge, *in* "General Methods of Separation: Electrical Transport Methods in Modern Methods of Plant Analysis, I" (Paech and Tracey, eds.), pp. 55–65. Springer-Verlag, Berlin, 1956.
[25] A. Tiselius and P. Flodin, *Advances in Protein Chem.* 8, 461 (1955).