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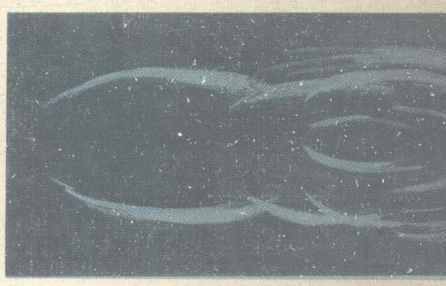

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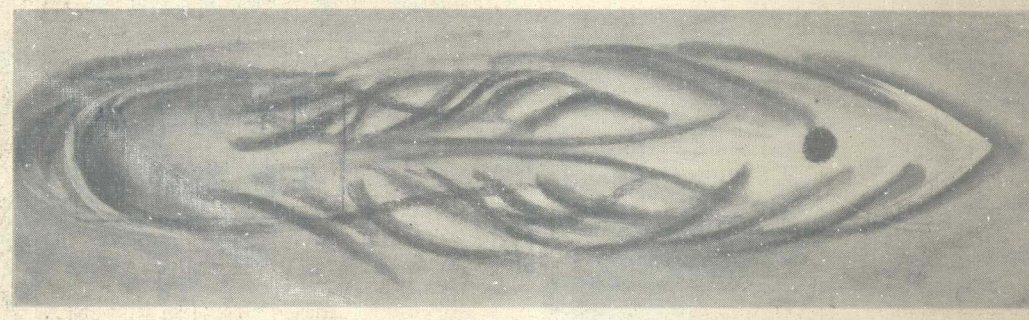

A Practical Laboratory Manual

ELECTROPHORESIS

A Practical Laboratory Manual

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Preface

This workshop is an outgrowth of one held at Children's Hospital, San Francisco, in 1959. At that time so many requests to attend the workshop were received that the course was repeated in spite of the fact that physical accommodations were poor. It was generally agreed that there was a real need and desire for "time at the bench"—an opportunity to personally use the necessary equipment and to have the experience of carrying out new and complicated procedures from beginning to end under actual working conditions and with supervision. This need has been expressed repeatedly not only by technologists but by biochemists and pathologists. That this type of training is practical is reflected by the almost immediate use of the learned procedures in the students' own laboratories. Although the need was great, nothing was done about it until Dr. Roger Wilson of the University of California postgraduate department accepted the challenge. His enthusiasm and leadership are largely responsible for the presentation of this course.

The specific techniques and the equipment were developed largely at Children's Hospital, and particular care has been taken to simplify both to the utmost. Most of the apparatus used in this course is simple to construct from easily obtainable materials. For this reason, drawings are included to enable those persons who are "handy" with tools to construct their own apparatus if desired. The principles, however, can be directly applied to equipment of various manufacturers.

In presenting new developments in the field of clinical pathology, as in the present course of electrophoresis, a practical difficulty was encountered—the difficulty of finding experienced personnel to teach and supervise the procedures. We met this difficulty by training our own (Children's Hospital) personnel. This proved to be distinctly beneficial since it pointed up possible areas of confusion and directly aided in clarifying certain aspects of the course. Nevertheless, we appreciated and used the suggestions and criticisms of the participants in order to improve the presentation of the material and to plan future courses at the workbench in other fields.

S. T. Nerenberg, M. D., Ph.D

Acknowledgments

The large number of pieces of equipment needed for the course presented a practical problem until solved by the ingenuity and kindness of the Hyland Laboratories and their personnel, particularly Mr. Charles Schultz who is in a large part, responsible for furnishing equipment and supplies, including all of the antisera used for immunoelectrophoresis, abnormal hemoglobins (hemoglobinopathies), agar plates, etc. The unique preparation of the "ready to use" gel plates for agar electrophoresis and immunoelectrophoresis, and the lyophilized control abnormal hemoglobins should prove to be a boon to the busy technologist and pathologist. We are also grateful to the Hyland Laboratories for the use of their expert personnel in presenting the material on immunoelectrophoresis.

In addition, I would like to emphasize that many of the techniques presented in this manual were worked out with the help of Mr. George Pogojeff, a member of the laboratory staff at Children's Hospital, who also is responsible for a great number of the drawings found in this manual.

Finally, I would be remiss if I did not mention the superb job done on the manuscript by Mrs. Marye Rose, secretary.

S. T. Nerenberg, M.D., Ph.D.

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PART II. ADVANCED TECHNIQUES

Introduction

The course in zone electrophoresis* has been divided into two parts as delineated below:

The first part is a basic course intended to acquaint the student with the principles of electrophoresis and to familiarize him with the preparation of specimens to be used with such common supporting media as cellulose acetate membranes, agar, starch, and acrylamide gels.

The second part represents an advanced course in which the student will apply the principles and techniques learned in the basic course, to the detection and quantitation of complex substances as described in the manual.

An outline of the course, as it is to be presented, follows: The student is asked to peruse the listed references, *before class,* to help him obtain the maximum amount of information. He should feel free to question the instructors about any aspect of the course on which additional information may be needed.

The techniques to be taught are those actually used at Children's Hospital at San Francisco. Variations in technique, as described in the literature, will be mentioned (with references†) to give a more rounded view of the field.

Emphasis will be placed primarily on accuracy and only secondarily on ease of performance. For this reason a discussion on accuracy, sources of error, and "quality control" are included in the manual.

In the material that follows, methodology has intentionally been physically separated from the discussions in order to provide extra methodology sheets while working at the bench. This arrangement will prevent undue dirtying of the manual which can then be studied between laboratory sessions. This arrangement has necessitated some repetition.

Any equipment described in the manual may be obtained (made to order) by contacting:

Arthur Larsen, Jr.
306 Hemenway Street
Marlboro, Massachusetts

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* The term "zone electrophoresis" was introduced by Tiselius to indicate a difference from "moving boundary electrophoresis." In the latter technique only partial separation of different fractions results. In zone electrophoresis, the use of solid material as a supporting medium allows the separation of a mixture into discrete zones.

† With a few exceptions, references will not be referred to in the body of the text. An extensive bibliography will be found in the appendix of this manual. References are listed under subject headings.

Part I

Basic Course

I. Theory and General Principles of Electrophoresis

A. GENERAL INFORMATION

Electrophoresis is one of the most powerful analytic techniques in biochemical research. Its scope of application has been broadened tremendously in recent years by simplification of the apparatus required for its use, and more recently by the availability of purified supporting media which have shortened markedly the time of analysis. For example, an electrophoretic run of serum proteins which formerly took 16 hours, using paper as a supporting medium, is now done more easily, better, and more satisfactorily in 20 to 30 minutes using cellulose acetate.

The combination of electrophoresis and immunodiffusion into the analytical procedure known as immunoelectrophoresis has provided both the specialist (immunologist) and non-specialist with an analytical tool that allows detection of serological reactions undreamed of only a few years ago. More than 30 proteins have been identified in human serum by this technique; more than a hundred are known to exist.

The theory underlying electrophoresis is relatively simple. Direct current is used to separate components of serum blood, and urine (or other solutions), by means of an electric charge placed on the various components of the solution. The greater the charge on a substance the faster it will move in comparison to components having a lesser charge. In this way, the components having little or no charge remain relatively stationary, while the charged components move off in the direction of the pole having the opposite charge. Obviously, if a substance (such as sucrose) cannot be charged to form ions it will not move when a charge is placed on it. Such substances cannot be separated by electrophoresis.

Since proteins are composed of amino acids which have both acidic ($COOH^-$) and basic (NH_3^+) groups, they can be (1) charged positively (more NH_3^+ groups), (2) charged negatively (more $COOH^-$ groups), or (3) remain neutral (same number of $COOH^-$ as NH_3^+).

The net electrical charge on a protein depends on the buffer pH in which it is dissolved. At its isoelectric point (same number of NH_3^+ as $COOH^-$) the protein is electrically neutral. As the pH is raised, the NH_3^+ groups are progressively neutralized by the alkali used in the buffer so that the $COOH^-$ groups are predominant, giving the protein a net negative charge. The reverse occurs if the pH is acidic.

As an example, albumin which has an isoelectric point (pI) of 4.7 will be charged strongly negative in a buffer of pH 8.6 as compared to gamma globulin which has a pI of 7.2. The albumin, then, will migrate much further than the gamma globulin when a charge is placed on these two components.

TABLE I. Classification of plasma proteins

System	Plasma			Protein			Components
Electrophoresis				Increasing electrophoretic mobility →			
	Gamma (γ)			Beta (β)	Alpha-2 (α_2)	Alpha-1 (α_1)	Albumin
	γ_2	γ_1					
Ultracentrifugation	7 S	19 S or B_2A (Macroglobulin)					
Immunoelectrophoresis	Formed in plasmacyte and lymph cells						
	γG or I_g G	γA or I_g A	γM or I_g M				
Molecular wt. (approx.)	160,000	1,000,000	160,000 and up	500,000 to 20,000,000	145,000-800,000	45,000-400,000	69,000
Specific protein fractions	Antibodies Agglutinins			Lipoproteins Transferrin Factor VIII Fibrinogen Siderophyllin	Glycoprotein Plasminogen Prothrombin Ceruloplasmin Macroglobulin Haptoglobin 2-2 Haptoglobin 2-1	Orosomucoid Glycoprotein Haptoglobin 1-1 Lipoproteins Factor V	Function: 1. Osmotic pressure regulation 2. Transport of fatty acids and other compounds

Given in chart form (Table I) is an electrophoretic classification of the five chief proteins found in the serum along with some of their properties.

It is important to note that a mixture of pure amino acids can also be separated by electrophoresis for the reasons given above.

The ionicity of a buffer solution is important in electrophoresis since low ionic strength buffers permit fast migration rates and low heat development. High ionic strength promotes the sharpening of zones, but causes high heat production and low migration rates.

The ionic strength of a buffer solution is given by:

$$I = 1/2 \Sigma C Z^2$$

where

C = molality of a particular ion ·
Z = ionic charge
Σ = summation of all ions in solution

In practice, solutions of ionicity between 0.025 and 0.075 for the veronal and 0.12 and 0.03 for the Tris* buffers are the two most commonly used buffers. More concentrated buffers are not used because of heating effects and slowness of migration. In other words, satisfactory resolution is accepted in lieu of the best possible resolution in order to achieve faster migration rates with fewer heating effects. Faster migration rates greatly decrease the time needed to separate the solution components which in turn decreases the production of heat; heat causes many artifacts (drying and other distortions).

Very often the two buffers, veronal (barbituric acid) and Tris borate,* are used in a discontinuous system.

A continuous system indicates that the same buffer is used in the buffer chambers as is used in the supporting media (cellulose acetate, starch, etc.), whereas a discontinuous system indicates that the buffer used in the chambers (such as TEB†) is different from the buffer used in the supporting media (such as veronal).

Methods of preparation and constituents of the more commonly used buffers, their pH's and ionicities, are listed in chart form in the appendix of this manual.

B. APPLICATION OF SAMPLE

Of critical importance in zone electrophoresis is application of sample. The width of application should be kept as narrow as is practicable to give the best possible resolution. For this reason the sample applicator is of great importance. It makes little difference how long or how deep (as with the gels) an application is, as long as the width of thickness is kept to a minimum. Linearly oriented applications are

* TRIS (or Trizma Base): Tris (hydroxymethyl) amino-methane (formerly called Sigma-121).

† Tris-Ethylene-Diamine-Tetra-Acetic Acid-Borate.

much preferable to spot (round) applications since the latter tend to overlap in those zones where there is little physical space separating the zones, leading to a great loss of resolution. An applicator such as that shown in Figure 1 is recommended. It is made of very fine wire* arranged in the form of an extremely narrow rectangle; the long sides of the loop are in close enough proximity to hold the sample by surface tension. The wire loop has been soldered to a pencil clip. The application is made by contacting the supporting medium.

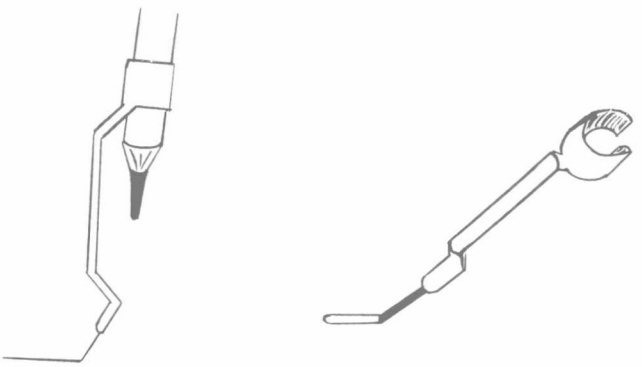

FIGURE 1. Pencil applicator.

It is important to avoid over-loading an application since this also leads to loss of resolution. The volume of an application and therefore the applicator must be fitted to a particular supporting medium. For example, less of a sample can be applied to the cellulose acetate supplied by the Millipore Co. (Beckman) than can be applied to the Oxoid product.

Applications to gels utilize a different approach. Either a piece of filter paper cut to a size to fit a slit in the gel (the sample is then applied to the filter paper) or an applicator with a hole at one end to hold the sample by surface tension is used. Use of these applicators will be described under the appropriate chapters.

It is often convenient to follow the progress of an electrophoretic run. This is most simply done by adding a "marker," such as bromphenol blue (a tiny crystal), directly to the serum. The dye attaches only to the albumin; not to the non-denatured globulins.

Another method is to use a jaundiced control serum, run concomitantly with the unknown serums. The bilirubin which is bound firmly to the albumin (1 gm albumin binds 16 mg of bilirubin) can be easily observed during an electrophoretic run because of its strong yellow color.

It is preferable, *before* making a sample application to cellulose acetate, to allow the apparatus first to come into hydrostatic equilibrium for five minutes with the power on; otherwise the sample will be quickly displaced by flow of the buffer from

* Made from V-10852 probes, Aloe Scientific Co.

the buffer chamber through the cellulose acetate. In the analysis of urine for amino acids it is sometimes better to dry the applications with a hair dryer.

All supporting media become charged when in contact with electrolyte solution, leading to an electro-osmotic (endosmotic) flow of the buffer to the electrode having the same charge as the supporting medium. This is in the opposite direction to the movement of the sample. A common method of measuring the rate of this flow is to use an uncharged substance like dextran which is passively carried by the buffer.

The greater the endosmotic flow, the broader the resulting zones obtained with the substance being analyzed, leading to a loss of resolution. One method of keeping the endosmotic flow to a minimum is to utilize a *countercurrent effect* by elevating the buffer chamber (increased hydrostatic pressure) to which the endosmotic current is directed. Presumably, vertically oriented systems, as with gels, use this arrangement to obtain their excellent resolution.

In the separation of proteins the adsorption of the latter to the support leads to "tailing" which manifests itself as an immobile trail. This is particularly marked in the case of albumin when using paper as a support. Cellulose acetate manifests much less of a tailing phenomenon, although it is still present. This phenomenon is particularly disturbing with the use of radioisotopes; for example, in the determination of labeled thyroglobulin binding, the labeled substance travels attached both to the albumin and to the protein between Alpha-1 and Alpha-2. When albumin leaves a "tail," with its associated radioactivity, it becomes mixed with the radioactivity of the fractions (Alpha-1 and Alpha-2) that follow closely, leading to erroneous results. Probably the best and simplest method of avoiding this phenomenon completely is to use two-dimensional electrophoresis (see below).

The adsorption of negatively charged proteins to the supporting medium is usually considerably less than positively charged ones. It is for this reason that alkaline pH buffers are utilized (above the isoelectric point) instead of acidic buffers (below the isoelectric point). It is interesting to note that amino acids show no adsorption effects whatsoever, indicating that adsorption is solely a protein effect.

C. QUANTITATION

The simplest and cheapest way to quantitate proteins resolved by zone electrophoresis is to stain and treat them as histological specimens. Quantitation is then obtained photometrically by measuring the quantity of the dye attached to the proteins. The dye may be measured while still attached to the protein by using (1) densitometry, or (2) the dye may be separated from the protein by elution, or (3) by dissolving the supporting medium (such as cellulose acetate) in a suitable solvent. By preparing adequate blanks and controls, quantitation becomes a practical laboratory procedure.

II. Equipment - General Discussion

A. GENERAL PRINCIPLES OF ELECTRICITY AND ELECTRONICS

To use the equipment properly a few, simple basic facts of electricity and electronics must be understood.

As noted under the discussion of theory, direct current (DC) is used to drive various charged particles in the supporting medium. This direct current is furnished by a power supply which converts (rectifies) the alternating current obtained from the wall plug into pulsating DC current which is filtered to get rid of 98 per cent of the "ripple." This is expressed diagrammatically in Figure 2.

FIGURE 2. Diagram—rectified and filtered AC current.

The filtered direct current is then conducted to the negative electrode (usually platinum or carbon) supplying free electrons to the buffer solution which then flows through the supporting media into the positively charged buffer solution, through the positive electrode, and back into the power supply (completing the circuit).

The power (rate of doing work in terms of watts) supplied to the circuit is expressed by:

$$P = EI = I^2R$$

where

E = voltage
I = current
R = resistance

The power put into a system is directly proportional to the voltage or to the square of the current. The heating effect is more markedly increased by increasing the current. If current is increased at the expense of voltage (since the relationship