IMMUNO-ELECTROPHORETIC ANALYSIS

APPLICATIONS TO HUMAN BIOLOGICAL FLUIDS

Edited by

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PREFACE TO THE FRENCH EDITION

Numerous difficulties are encountered in the study of natural macromolecular substances, which often are labile as exemplified by the proteins. Among the modern physico-chemical methods, the most widely used has probably been electrophoresis and it has resulted in a considerable development of our knowledge of these materials. On the other hand, the specificity of the antigen–antibody precipitation reaction has been known for a long time and the studies by the immunochemists have facilitated its application to the study of the proteins.

In the elaboration of the immuno-electrophoretic method of analysis, we have sought to utilize the possibilities provided by both of these means of investigation: the definition of a substance by its electrophoretic mobility, on one hand, and by its immunochemical specificity, on the other. Since these two definitions are based on distinctly different properties (electrochemical for the mobilities, and structural for the immunochemical reactions) their conjunctive employment permits the defining of a protein or the proving of the homogeneity of a preparation with more certainty than by a single method. Immuno-electrophoretic analysis accomplishes a double or sometimes even a triple definition of a substance in a single operation. A very small amount of the material suffices for the analysis, even though it be a natural very complex mixture, which in addition may not be subjected to any preliminary treatment because of the danger of damaging alteration. The method has already been employed by many workers in a wide variety of studies. But the greatest number of investigations have certainly been devoted to the analysis of human biological products.

The accounts of the results already obtained are scattered among various scientific periodicals and therefore we have thought it would be worthwhile to collect the principal facts in this present monograph. We do not claim that we have assembled all of the findings and all of the references to the literature, which has been carried up till 15th December 1959. We beg the indulgence of the authors of papers that have not been cited.

Our warmest thanks are due to all those whose cooperation in writing the various chapters of this monograph has alone made it possible to assemble this text, chapters which deal very generally with the researches conducted by these writers themselves. We take this opportunity to express our sincere thanks also to our students or collaborators who have participated in the researches mentioned in this monograph, such as Mr. M. Challeil and Miss J. Courcon, whose technical assistance over a period of many years has been especially valuable. The secretarial collaboration of Miss C. Forestier has been of the utmost assistance.

Paris, 1960 P. GRABAR

PREFACE TO THE ENGLISH EDITION

This monograph appeared originally in 1960 in French. The text was revised extensively and brought up to date before being passed to the translator. Certain imperfections of style in the first translations forced us to enlist the aid of several English speaking colleagues present in our laboratories at the Institut Pasteur or at the Institut de Recherches Scientifiques sur le Cancer at Villejuif. For their attempts to improve it, and for their helpful corrections and criticisms, we would like particularly to thank Dr. Samuel B. Aronson, Dr. Howard C. Goodman, Dr. Calderon Howe, Dr. Philip MacMaster, Dr. Robert C. Skarnes, Dr. Norman Talal and Dr. Tom Webb.

The time lost in these extra corrections has made the text of certain chapters less up to date than would otherwise have been the case. For this we ask the reader's indulgence.

Paris, 1964

P. GRABAR AND P. BURTIN

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PART I

METHODS

Chapter 1

THE IMMUNO-ELECTROPHORETIC METHOD OF ANALYSIS

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The principle of the method hereafter termed immuno-electrophoretic analysis (I.E.A.) is the electrophoretic separation of mixtures in a gel medium followed by the diffusion of precipitating antibodies into the same gel at right angles to the direction of the electrophoresis.

Although many years ago immunologic reactions with products separated by electrophoresis were performed by Tiselius and Kabat[30] and more recently by others using a preliminary separation on starch or paper, we prefer to use the term immuno-electrophoretic analysis only for the method described above. In I.E.A. the precipitating antibodies unite with their corresponding antigens to produce arcs of specific precipitate in the gel. Each antigen produces an independent arc as it precipitates with the antibody. Consequently one may count the number of antigenic constituents of a mixture and define them according to their mobilities, as well as by their specific reactions with antibody. This double definition may be supplemented by other means of characterization such as enzymic reactions, histochemical stains etc.

The specificity and sensitivity of the precipitation reactions allow one to distinguish even between substances possessing similar or identical electrophoretic mobilities, as well as to detect antigens present only in minute quantities, even when micro-techniques are employed.

Although numerous variations in the details of this simple method have been used in many laboratories, we will describe below the method in a general manner, and then briefly consider the techniques used in our laboratory, the micro-method devised by Scheidegger[27] and the technique of double migration of Blanc[1].

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1. ELECTROPHORESIS IN A GEL

The great advances in our knowledge of macromolecular chemistry and physics are partly due to the development of numerous electrophoretic techniques. As a medium for electrophoresis, a gel offers several advantages.

The electrophoretic migrations are analogous to the mobilities in a liquid medium, but the structure of the gel limits the development and speed of currents, thus keeping the molecules nearly stationary after electrophoresis. However, relatively free diffusion due to molecular motion does occur, thus enabling immunologic reactions to take place. Gels formed in salt and water are admirably suited for the study of such biologic materials as proteins, carbohydrates, and lipoproteins for they are readily soluble in the liquid, and denaturation due to the solvent is generally minimal or nonexistent.

To obtain satisfactory results, certain properties of the gel are important. The gel must maintain a certain degree of elasticity even when the supporting material is in low concentration to prevent the appearance of artifacts. Further the gel should be made in an aqueous medium and with a substance as nearly neutral as possible to limit the electroendosmosis (see below) as well as other effects due to the action of ionizable groups in an electric field. Finally, to render the precipitin arcs readily visible, it should be transparent.

Among the different substances that have been tried, really satisfactory results have been obtained with gels of agar-agar and pectin. The latter even shows some advantages, but the formation of pectin gels is a function of several factors which cannot always be readily controlled[9]. It seems that the new synthetic product called 'Cyano-gum' (Lederle & Co.), which gives transparent aqueous gels, can also be used. Starch gels[29] permit certain interesting separations, but these gels are not transparent and give electrophoretic mobilities different from those observed in liquid media.

Membranes constructed of cellulose acetate can take the place of gels[18] but in view of the structure of these membranes it is not impossible that they may exert a certain 'filtering effect' and hence macromolecular materials may be retarded in their electrophoretic transport.

In the following discussion, we will deal only with the use of agar gels since all the results mentioned in this monograph have been obtained

with them. Some 'filtering effects' have been observed, but they seem to occur only in very rare cases with agar gels. For example we have never observed any retardation in the migration of macroglobulins with high molecular weight. By contrast, a retention has been found in the case of native fibrinogen[28] and of the lysozyme of egg white[15]. Agar yields gels which are sufficiently strong though containing up to 99% of liquid, and in them the relative mobilities of known substances are the same as those in liquid media. Furthermore, these gels are perfectly transparent and they can be converted into dry elastic films which keep indefinitely.

(a) Preparation of agar gels

The agars available from the dealers come from various sources and they differ in their degree of purity. Some commercial preparations have been well purified and may be used as purchased, whereas others require a supplementary purification. Several methods of purification may be employed (see below). However, it is always necessary to melt the agar one or more times; we recommend to avoid unnecessary heating and to do it in a water bath to limit hydrolysis and carmelization of the agar. In general high molecular weight agar yields better gels.

Experience has shown that the movements of various substances in agar gels are not exactly the same when agars of various origins are used, and sometimes different lots of the same commercial brand yield somewhat different results. Consequently, it is advisable always to use the same brand and to run comparison control studies when a new lot of agar is employed.

To prepare the gels, preliminary trials are made to determine the concentration of agar sufficient to produce an elastic and firm gel; usually 1–1.5% is about right. The desired amount of agar is then dissolved in the appropriate buffer solution (see below); if the resulting solution is not clear enough it is filtered rapidly through a Buchner funnel fitted with fast filter paper. The desired amount of disinfectant is added to the solution or filtrate (for instance 1 : 10,000 merthiolate) and the agar solution is divided among small bottles or flasks, each accomodating enough of the solution to prepare a known number of plates of the desired dimensions. Repeated harmful heating of the entire volume of the solution is thus avoided.

The electrophoresis is carried out in gels formed on glass plates, preferably on photographic plates which are free of defects. The gel adheres

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better to the glass if the latter is cleaned with much care, and a drop of melted agar gel is spread over the glass surface and then dried either at 80°, as is the case in tubes[23] or simply in the open air.

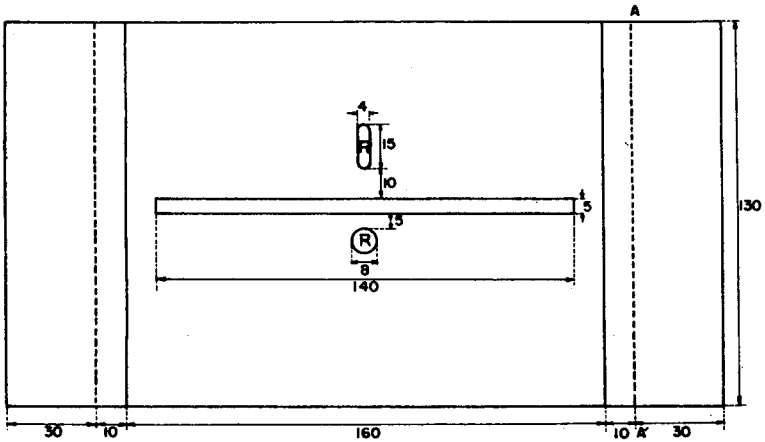

Fig. 1. Schematic representation of the arrangement of the filter paper, the reservoirs for the sample to be analyzed (R) and the trough for the immune serum on a plate of agar 130 × 180 mm.

To produce a uniform layer of agar on the glass plates, they must be placed on a horizontal surface in a tray. Such surface may readily be made by pouring 5% melted agar into a porcelain photographic tray, which is firmly fixed to a table. The size of the trays should be selected in accord with the number of plates it is desired to prepare. The photographic plates are placed on the level surface prepared in the trays; strips of chromatograph filter paper (for example Arches No. 302) are placed at each end of the glass plates; the length of the strips corresponding to the width of the glass. The width of the strips is 4 cm of which 1 cm rests on the glass (Fig. 1). The strips of paper serve as junctions between the gel and the electrode compartments and it is well to cover or coat them with the gel to prevent losses of current. The warm buffered solution of agar is poured into the tray avoiding the formation of bubbles. The volume of agar solution used should be sufficient to give a layer about 4 mm thick on the glass plate and the paper strips.

The plates are detached from their support as follows: after cooling, an incision is made with a spatula around the glass and the filter paper

strips, and then a slight cut is made in the gel at the junction of the glass with the paper (along the line AA', Fig. 1). The glass plate is raised and the paper is folded at a right angle, and then molten agar gel is poured into the incision (into A, Fig. 2) to insure the continuity of the gel.

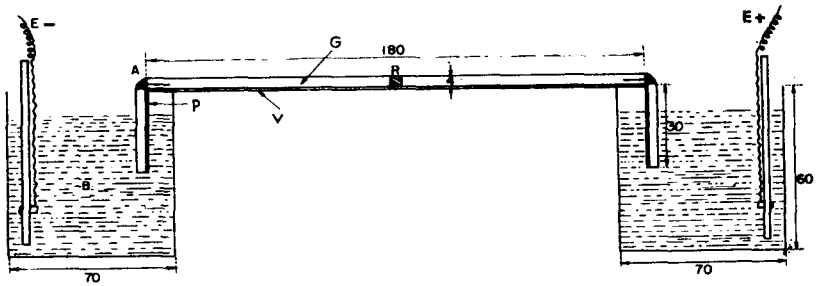

Fig. 2. Diagram of the electrophoresis apparatus in agar gel. B = electrode compartments; E = electrode; V = glass plate; G = gel; P = filter paper covered with gel serving as junction between the agar plate and the electrode compartments; R = reservoir for the solution to be analyzed; A = agar poured in to fill the space formed by the folding of the filter paper.

The sample for electrophoresis may be placed in a small reservoir (R) made by cutting out a bit of the gel near the center of the plate (Fig. 1). The shape and dimensions of the reservoir may be varied, but generally is oblong with the long axis perpendicular to the direction of electrophoresis, for such wells have a greater volume than round or square holes possessing the same dimension parallel to the axis of migration. With the aid of a small brass punch, a uniform size may always be obtained. The antibody is put in long troughs cut out of the gel after the electrophoresis, by means of knife and dental scapula, and the surface of the glass sealed to the gel with a minimal amount of melted agar. Such troughs usually run nearly the length of the plate, parallel to the direction of the electrophoresis, are about 5 mm wide, and lie at a suitable distance from the reservoir, which is determined by preliminary trial and error, but generally is in the range of 5–10 mm. Cutting the reservoir and the troughs in the right position may be facilitated by placing the glass plate on a piece of paper bearing a pattern.

The distance between the reservoir and the trough is selected to give the maximum number of independent arcs, which is a function of the concentration of antibody, versus that of the antigen.

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To avoid cracking the gel around the reservoir of antigen and to assure the even movement of the antigens through the gel, the antigen solution with an ionic force near that of the buffer of the gel, is usually mixed with an equal volume of molten 2% gel at 42–45°, and pipetted into the reservoir. Once it has solidified a drop of melted agar is put on top of the well to ensure continuity and proper height of the gel.

It has been found that certain substances, and notably some constituents of serum (probably the complement)[35] may undergo modifications under the effect of this brief heating of the mixture with the molten gel. To avoid this, the operator may either introduce the liquid as such into the central reservoir and not seal the latter until after the electrophoresis has gone on for a time, or mix the liquid sample in the cold with agar in paste form and then cover the whole with several drops of molten agar gel. Bustamente and Wunderly introduce the liquid into the gel by placing a small piece of filter paper impregnated with this liquid in contact with the gel for 15 min and then wash the surface of the gel[3]. This technique avoids any heating, but there is a possibility that the rate of diffusion of the various constituents in the gel (which is not necessarily identical) may change the ratios of the concentrations of these constituents.

When the electrophoresis is terminated, the immune serum is poured into the lateral troughs; it is usually added in liquid form, but sometimes we have used a mixture of the immune serum with agar to retard the diffusion of the antibodies.

(b) The electrophoresis

Methods of electrophoresis in jellified media have been described by various workers, quite some time ago, and for instance Gordon *et al.* used an agar gel for this purpose[6]. We prefer to designate these various methods as 'simple electrophoresis', *i.e.* without involvement of immunological reactions. The technique which we will describe may also be used as 'simple electrophoresis'; it yields results analogous to those obtained in electrophoresis on filter paper, but the transparency of the gel and the possibility of obtaining a transparent film present several advantages over the use of paper. The spots, which may be stained, provide the possibility of making quantitative estimations of the principal fractions of the mixture being studied in parallel trials in which immuno-electrophoretic analysis is employed, and consequently more complete information may be gathered[31].

In order to obtain a maximum dispersion of the constituents of the mixture it obviously would be preferable to apply high differences of potential; they also afford the possibility of shortening the electrophoresis time and hence curtail the free diffusion of the constituents of the mixture being examined. However, the operator is limited by the deleterious effect of the heat released in the gel because of the passage of the current at a high potential. Experience has shown that by choosing rather low salt concentrations, it is feasible to apply to the gel potential differences of the order of 3–6 V/cm* without being obliged to take special cooling precautions; it is not necessary to work at other than laboratory temperatures. The slight drying of the gel during the passage of the current does not alter the reproducibility of the results. But it is well to protect the agar plate against too pronounced evaporation by using, for instance, baffles which prevent or divert air currents. In some cases, and especially when studying materials that are markedly unstable, the electrophoresis may be conducted in a cold chamber, or it may be advisable to use a refrigeration system (for example in the apparatus described by Frentz[5]).

As was pointed out above, we ordinarily use relatively low salt concentrations to avoid heating the gel. However, to obtain a regular migration and to maintain a constant pH in the interior of the gel, it is necessary to employ buffer solutions. Satisfactory results are obtained by using solutions whose ionic strength is of the order of 0.025–0.05. If the electrophoresis time is to exceed 5 h, under the usual conditions, the migrations will be found to slow down and the gel will suffer a loss of electrolytes (reduction in the conductivity). In cases of this kind, we have reestablished suitable conditions by immersing the gel for 15–20 min in a buffer solution; for we have found that this is long enough for the gel to absorb again the salts. If the ordinary ionic strength is too low to keep certain constituents in solution, this strength may be increased, but in such instances it is preferable to lower the voltage, which will slow down the electrophoretic migration, or to work in a cold chamber.

The choice of the buffer solution depends on the experimental conditions. Agar gel is stable between pH 5 and 9. The use of phosphate solutions requires a better purification of the agar. Ordinarily we use veronal or borate buffers and pH values which do not exceed 8.2 since a higher alkalinity can bring about irreversible changes in certain groups of pro-

* The voltage should be measured inside the gel to avoid errors due to the loss of current in the junctions.

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