METHODS OF BIOCHEMICAL ANALYSIS

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VOLUME VII

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PREFACE TO THE SERIES

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need for material achievement to keep in sight of the advance of useful ideas.

The current volume is the first of a series which is designed to try to meet this need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological and, if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

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IMMUNOELECTROPHORETIC ANALYSIS

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Important progress has been obtained in studies on proteins in general, and particularly on mixtures of proteins, through the use of various methods of electrophoresis. However, the possibility of resolving a mixture is limited by the sensitivity of the method used for the detection of the individual proteins of the mixture. It is also known that proteins possessing either similar or identical mobilities may exist. These considerations have provoked the idea of introducing a very sensitive method, that of specific precipitation by antibodies for the detection of various proteins after their elecphoretic

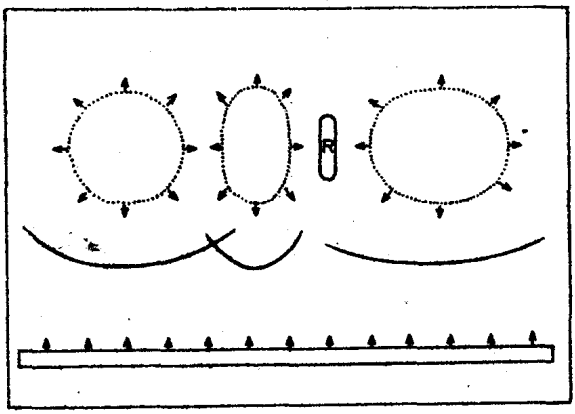

Fig. 1. Schematic representation of the precipitin arc formation.

separation. The principle of the method (17), which has been called "immunoelectrophoretic analysis" (IEA), follows: The mixture to be studied is subjected to electrophoresis in a gel. When the dispersion of the constituents by the electric field is judged to be sufficient, electrophoresis is stopped and a precipitating immune serum is allowed to diffuse perpendicularly to the axis of migration. The antibodies present in this serum and the antigenic constituents of the analyzed liquid diffuse into the gel (see the arrows, Fig. 1), and when they meet in proper relative proportions (corresponding to the optimal zone for their mutual precipitation) insoluble antigen-antibody complexes appear and arcs of specific precipitation become visible. Each antigen or hapten present in the analyzed solution reacts with its homologous antibody specifically and independently. Thus even substances possessing identical mobilities can be differentiated. The

great sensitivity of the specific precipitation reaction allows the detection of minute amounts of antigen, thus making it possible to perform analyses of very small amounts of mixtures or to detect the presence of traces of impurities.

IEA permits one to establish the minimal number of antigenic (or haptenic) constituents of a liquid and to identify them by their specific reactions with the homologous antibodies. This double definition can also be supplemented by other means of characterization, such as color reactions, enzymic actions, etc.

Since most proteins are good antigens, this method is particularly suited to the study of proteins and of complexes formed by proteins with carbohydrates, lipides, and metals. Moreover, some of the polysaccharides, although not always capable of eliciting the formation of antibodies, are haptens, capable of reacting with their specific antibodies and thereby detectable by the precipitin reaction. Therefore IEA can also be applied to the analysis of liquids containing polysaccharides or their complexes with proteins.

During the elaboration of this method, particular care has been taken to simplify it to the utmost and to avoid complicated apparatus in order to allow its application to diverse areas of research. Several of the factors which are involved can be varied at will, so that many modifications and variants of the original method can be envisaged. The different stages of the method will first be described and discussed. Then, a short description of some details of the standard technique as generally used in this laboratory, and of the micro technique of Scheidegger (33), will be given as examples. Finally the potentialities of IEA, the interpretation of the results obtained, and the advantages, disadvantages, and limitations of its use will be discussed.

I. PREPARATION OF THE GELS

1. Nature of Gels

For the purpose of IEA a gelified medium has a double advantage. Its very high liquid content allows an electrophoretic transport similar to that in a liquid medium, and its structure slows down the free diffusion of macromolecular substances during and after the end of the electrophoretic separation. For practical uses, the choice of a convenient gel is limited by the following considerations: (*a*) the sub-

stance which forms the gel should be a potent gelifier, i.e., it should be able to form a real gel even at very low concentration; (*b*) the gel should be formed in an aqueous medium, which is the normal biochemical one; (*c*) the gel-forming substance should be neutral, i.e., it should have as few ionized groups as possible in order not to be influenced by the electrical field; and (*d*) the gel should be transparent in order to allow the observation and photographic recording of specific precipitation arcs.

So far, the best results have been obtained with agar gels, and in the following descriptions of the technique only agar gels will be mentioned. Among other substances tested which are capable of forming true gels in water, one, pectin, gave satisfactory results (15). It even presents some advantages over the agar, such as formation of a gel at lower concentrations, the possibility of easy elution by means of enzymic breakdown of pectin, and formation of the gel by enzymic activity. However, the enzyme involved seems to be unstable and the proper quantity must be established each time by a series of preliminary experiments. Moreover, the necessity of introducing calcium ions entails some disadvantages. Therefore, pectin gels have not yet been widely employed.

Many other substances, often said to be capable of forming gels, in reality form only very viscous solutions. Devoid of elasticity, such solutions will flow and are therefore of limited utility in this case.

Some interesting results have been obtained with starch. Compared with agar, starch has the disadvantage of being opaque, and if it is used for immunoelectrophoresis, supplementary manipulations are required. Slater (37), after electrophoresis in starch, produces an agar gel in which the precipitin reactions take place at the side of the starch block. Other authors carry out the immunochemical reactions on eluates from starch electrophoresis. It seems simpler to avoid supplementary manipulations and to perform both the electrophoretic separation and the precipitin reaction in the same medium, as is done in the present method.

Another important factor that must be mentioned is the concentration of the substance used as support. The concentrations of starch necessary for formation of a suitable supporting medium are much higher than those of agar. The possibility of nonspecific interactions with the medium is thereby increased. It seems well established that the mobilities of some proteins in starch gels are significantly different

from those which these substances show in liquid media. This difference is probably due to an adsorption of the substance by the starch. In some instances this property may have value in separation of certain constituents with the same mobilities in liquid media.

Mixed gels containing a very low concentration of agar (e.g., $< 0.5\%$) and starch (e.g., 5%) seem to be useful; the elution from them is easy and it is possible to obtain nearly transparent films upon drying. To the author's knowledge only preliminary experiments have been performed with such supports (16,21).

Instead of gels, Kohn has proposed the use of cellulose acetate membranes for IEA and has obtained results which seem to be valid (24,25); but the relatively high content of dry substances in these membranes and the knowledge available of their structure induce some restrictions on their regular use. In fact, it is possible to imagine that parallel to the electrophoretic transport there may also be a certain filtering effect produced by the membrane itself; a partial retention or a formation of a "tail" may result in the case of particularly large or asymmetric molecules.

Such an effect, even in the case of agar gel of very low dry weight, has been envisaged (47), but in this laboratory it has not yet been observed, even with macroglobulins of great molecular weight. Their observed relative mobilities in the agar gels seem to be identical to those in liquid media, but it is not impossible that a certain "tailing" effect is actually taking place. A retention in the agar gel has been observed in the case of native fibrinogen (36) and the lysozyme of egg white. In the latter case it is probable that the retention is due to a sort of combination of this enzyme with the agar, which is a polysaccharide, because, when larger amounts of lysozyme are used, a certain mobility can be observed (18).

With the exception of these two instances, the agar gels appear to be superior to the other substances studied so far. Agar gives sufficiently rigid gels still containing 99% of liquid and the relative mobilities of many different substances are the same in them as in free electrophoresis. These gels are transparent and can be easily transformed into thin, dry elastic films which may be preserved for an indefinite time, and in which various substances may be made apparent with certain dyes.

2. Purification of Agar

Commercial agar preparations differ in origin and state of purity. A few brands of highly purified agar can be used without further purification, but their properties are not always constant from one batch to another and should be controlled. Others contain many impurities (dust, salts, low molecular weight components, and nitrogen-containing substances) which must be eliminated. In order to obtain convenient gels with a low concentration of agar, it is important to avoid its degradation by hydrolysis which is easily provoked by frequent or prolonged heating. At a concentration from 1 to 1.5%, a suitable preparation of agar will form an elastic gel and not a friable paste which will break up when compressed between the fingers.

Two methods of purification will be described, both applicable to agar of good quality, obtained either in fibers or in powder.

A. PURIFICATION BY HEATING AND WASHING

The agar is introduced into gauze sacks and washed for at least 24 hours in running water, or, better, in repeated changes of distilled water. For this purpose it is important to use neutral distilled water containing no detergents or traces of impurities such as are frequently encountered in water purified on resin columns when not correctly operated.

After this preliminary washing, the agar is rapidly melted in distilled water to give an approximate concentration of 7%. As soon as the agar is completely melted, the solution is poured into a cuvette or large cylindrical beaker. When the gel has solidified, it is cut into small cubes of about 0.5–1 cm. These cubes are washed for 3 days in distilled water, which is changed twice a day; at the end of this process they become white. These cubes are then melted in their own water. This material can be preserved in well-stoppered bottles at refrigerator temperatures for 1 or 2 weeks or more. Antiseptic or antibiotic substances may be added for prolonged storage. The dry weight of this preparation is determined in order to know its exact concentration.

All melting or heating must be kept as short as possible and carried out in a water bath, in order to avoid hydrolysis and caramelization of the agar.

B. PURIFICATION BY FREEZING AND ALCOHOLIC PRECIPITATION

The following unpublished technique has been worked out by Cl. Péaud-Lenoel, following suggestions by A. Coté. It consists of eliminating insoluble constituents by centrifugation, and nitrogen-containing substances by freezing and thawing (30,31). The agar is then fractionated by alcohol (22,26) to provide primarily a high molecular weight agar preparation.

High quality Japanese agar fibers (250–500 g.) are carefully separated without being broken and washed at 50°C. in a large vessel (at least 25 liters capacity) with running tap water for 1 hour. The washing is repeated with distilled water, also at 50°C., for 1 hour. The washed fibers are dissolved to give a concentration of approximately 3% in distilled water at the lowest possible temperature (such as rapid melting in an autoclave). Addition of 1 g. of activated charcoal per liter of solution and a 5 minute centrifugation at 60°C. eliminate insoluble particles. The supernatant should now be clear, and nearly colorless. After cooling, the solidified gel is cut into pieces about 5 cm. square, which are kept at −10°C. until completely frozen. These pieces are allowed to thaw at room temperature and the liquid is squeezed out through gauze sheets. The fibers thus obtained are washed once in distilled water, and again compressed on gauze. They are then suspended in distilled water, the total volume being the same as that of the initial solution, NaCl is added to provide a 0.8% solution, and the agar is dissolved by heating. The solution obtained is cooled to 65°C. and absolute alcohol at the same temperature is carefully added until precipitation begins (about 1.1 volume). This precipitate is centrifuged at 60–65°C. and discarded. To the supernatant is added with stirring 0.2–0.3 volume of hot absolute alcohol until a snow-like flocculation appears. The precipitate is allowed to settle overnight at 37°C., the somewhat cloudy supernatant is siphoned away, and the precipitate is centrifuged, washed with absolute alcohol, and dried at 37°C. The ground white precipitate can be preserved in well-stoppered flasks. It forms a very rigid gel at room temperature at a concentration of 1% and its nitrogen content does not exceed 0.013–0.015%.

3. Buffered Agar Gels

A small preliminary test will suffice to establish the precise concentration of the agar giving a resistant and elastic gel; usually 1–1.5%

will be satisfactory. A convenient quantity of agar purified by one of the methods described or a highly purified commercial agar is rapidly dissolved in a buffer to give the final concentration established in the preliminary experiment and the final concentration of the buffer chosen for electrophoresis (see Section II.2). If the resultant solution is not perfectly clear, it can be rapidly filtered by light suction on a Büchner funnel through a double layer of filter paper. After addition of an antiseptic substance (for example, merthiolate 1:10,000), the liquid is distributed in small flasks containing the volume just necessary for the preparation of one plate, or of any desired number of plates, of a chosen dimension, thus avoiding unnecessary supplementary heating.

4. Preparation of Plates

The electrophoresis is preformed in a thin layer of gel formed on a photographic glass plate. The adhesion of the agar to the glass is improved if the plates are carefully washed; it can be increased if a few drops of melted agar (1%) are first spread on the surface and dried at 80°C., as is done for tubes (29), or in open air.

A uniform layer of agar gel on these plates is then formed. The simplest way to obtain a perfectly horizontal base for this operation is to pour a layer of crude melted agar into a photographic tray firmly fixed on a table and allow it to harden. The prepared glass plate is placed on this surface; two strips of chromatographic filter paper 4 cm. wide and as long as the glass plate are placed on the ends of the plate, the papers extending about 3 cm. beyond the glass (Fig. 2). The purified melted agar solution in buffer is poured into the tray; it covers the glass and paper strips which later will serve as the electrical connections of the gel. The volume of the agar solution used for one plate is calculated to form a layer of 4 mm.

When the gel has hardened, the plates are lifted from their base. For this purpose, the gel around the glass plates and the filter paper is first circumscribed with a spatula. A light incision is made in the gel over the border of the plate where it is covered by the paper strips (line *l–l'* in Fig. 2), and the strips are bent to a 90 degree angle. Melted agar is poured on the cut (*D* in Fig. 3) to fill it and form a continuous agar layer (Fig 3).

Either before or after this last operation (i.e., the lifting of the

plate), one or more holes are made in the gel. These holes serve as reservoirs for the sample to be analyzed. The forms and dimensions of the holes may vary as a function of the volume of this sample.

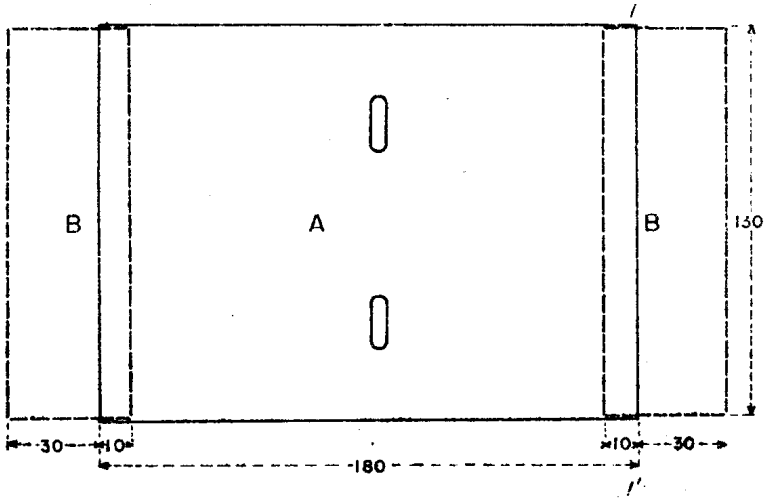

Fig. 2. Preparation of an agar plate for the IEA. A, plate; B, filter paper strips. Dimensions in mm.

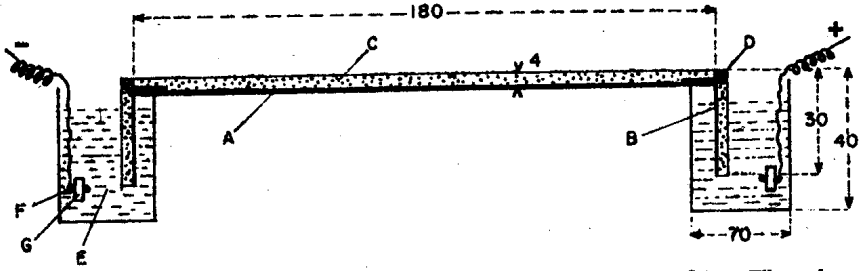

Fig. 3. Schematic representation of the apparatus for IEA. The glass plate (*A*) with filter paper strips (*B*) is covered with the agar gel (*C*); the incisions in *D* are filled with melted agar. *E*, buffer solution in the electrode trays. *F*, platinum wire electrode fixed on a plastic support. *G*, Dimensions in mm.

An elongated trough, perpendicular to the migration axis, allows the use of a larger volume than a round hole of the same width on this axis. Simple punches, easily manufactured from copper tubes, are used to make these holes (Fig. 4).

At the end of the electrophoresis, long wells parallel to the migration axis are cut out of the gel with a dental spatula. These wells serve as reservoirs for the immune sera. They are in most instances 5 mm.

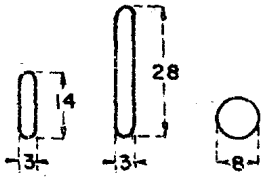

Fig. 4. Different forms of reservoirs for the solution to be analyzed. Dimensions in mm.

wide and occupy nearly the whole length of the plate. The holes should be sealed at the bottom with a drop of melted agar.

The placement of the reservoirs will depend upon the purpose of the

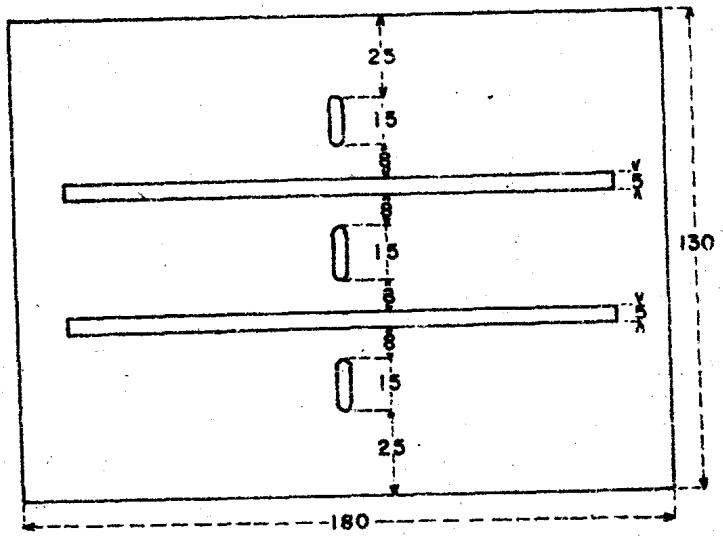

Fig. 5. Example of placement of reservoirs in an agar plate. Dimensions in mm.

analysis. In most cases, the hole for the sample is made equidistant from the cathodic and anodic ends of the gel, in order to compensate the electroendosmotic flow (see Section II.3). Figure 5 illustrates the

locations of the reservoirs in a 13 × 18 cm. plate. The distances (see Fig. 5) between the central hole and the immune serum wells may also vary with the concentration of antibodies in the particular immune serum and with the free diffusion rate of the antigens (see Section III.2), but in most instances it is 5–10 mm. In order to facilitate the preparation of these holes, a drawing of their disposition can be made on the opposite side of the glass plate with glass ink; one can also prepare a drawing on a graph paper and place it under the agar plate. The sides of the wells must be regular and clear-cut; the use of a ruler is recommended.

The solutions to be analyzed, mixed with melted agar of such concentration that the final mixture possesses the same agar concentration as the rest of the plate, are introduced in the central reservoir. The agar solution is maintained in a melted state in a water bath at 42–45°C.; a measured volume of the solution to be analyzed is rapidly mixed with a known volume of this melted agar and the mixture is placed in the reservoir. When this sample is solidified, a few drops of pure melted agar solution are poured over it to consolidate its junction with the agar gel on the plate. This technique usually allows one to obtain uniform results. With some substances, however, even this brief period of heating must be avoided. In these cases, the solution to be analyzed can be mixed with a suspension of small particles of an agar gel. Then this mixture, which must have a consistency of a paste, is placed in the reservoir and covered with a few drops of melted agar.

Another possibility has been proposed by Bustamente and Wunderly (cited in 51), who use a narrow filter paper strip saturated with the liquid to be analyzed, which is placed on the surface of the gel. After 15 minutes, it is taken off and the surface of the gel is washed with saline. This technique allows one to avoid heating, but the diffusion rate of the constituents of the liquid may not be equal and therefore a certain disparity of the quantitative relationships among the constituents may result.

In some cases it may be desirable to slow down the diffusion of the antibodies; for this purpose the immune serum is also mixed with melted agar before being introduced into the lateral wells. Generally, however, the immune serum is poured into these wells in liquid form, so that diffusion is more rapid and the appearance of the precipitation is accelerated.