Gel Electrophoresis in Capillary Electrophoresis

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Immunoelectrophoresis

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Development

The discovery that antigen–antibody interaction could be produced not only in liquids, but also on gel media, such as agar or agarose gels, with the formation of insoluble immunoprecipitates, opened the door to the development of the gel diffusion techniques for immunoprecipitation analysis. The first of these techniques, known as double diffusion, was introduced by Ouchterlony in 1948. In this technique, the antigens (proteins) and the corresponding antibodies (immunoglobulins, Ig) are located on a thin agar gel, in small and separated wells. The simple diffusion of the antigen and the antibody produce precipitation lines between the two wells where the interaction of these molecules occurs.

Advances in gel immunoprecipitation techniques occurred in 1953, when Grabar and Williams described immunoelectrophoresis (IE), in which the high resolution of electrophoresis and the specificity and sensitivity of the immunological reactants are combined. The immunoelectrophoretic techniques had a very rapid development during the 1960s and early 1970s, because they are adequately suited to the analysis of complex mixtures of proteins. The first attempts to improve the immunoelectrophoretic technique pursued two main objectives: to increase its speed (IE was rather slow, mainly due to the time-consuming immunodiffusion step) and to achieve quantitative methods (IE is basically qualitative).

In a short period of time new techniques based on crossed immunoelectrophoresis (CIE), rocket immunoelectrophoresis (RIE), counter IE, crossed-affinity IE (CAIE) and charge shift IE, were introduced.

This article describes all the techniques in which electrophoresis and immunoprecipitation steps performed in agar or agarose gels are combined. Diffusion-type techniques, such as double immunodiffusion and the quantitative radial immunodiffusion, will not be described. Other immunochemical techniques, such as enzyme-linked immunosorbent assay (ELISA) and Western blotting, in which immunoprecipitation is not produced, will be considered elsewhere.

Conventional Immunoelectrophoresis

The scheme in **Figure 1**A shows the principles of the techniques, such as it was originally introduced and generally used by many workers. The first step consists of electrophoresis in 1% agar–agar or agarose gels, prepared in different buffers at pH ranging from 8.2 to 8.6 (Veronal 0.025 mol L^{-1}, pH 8.2 is one of the buffers frequently used). The sample to be analysed, usually a complex mixture of proteins, is applied in small wells located in the middle of agar–agar gels, or partially displaced towards the extreme nearest to the cathode when agarose gels are used. Most of the proteins, for example in blood sera or plasma, have a negative net charge in buffers with pH higher than 7.0. Thus, when a continuous electrical field is applied to the gel, the tendency of these proteins is to move towards the anode with a migration rate which mainly depends on charge-to-size ratio. However, the migration rate is in part reduced by the electroendosmotic effect due to the negative charges in polymeric molecules of the gel. This electroendosmotic effect is greater in agar–agar than in agarose gels. The extension of the electrophoretic run can be precisely fixed by controlling the migration of a marker. One of the markers routinely used is the protein stain Amido black. The second step is a double immunodiffusion performed in the same gel plate (Figure 1A). For this, longitudinal channels are cut parallel to the direction of the electrical flow and separated 4 mm from the original wells where the samples were applied. The channels are filled with the

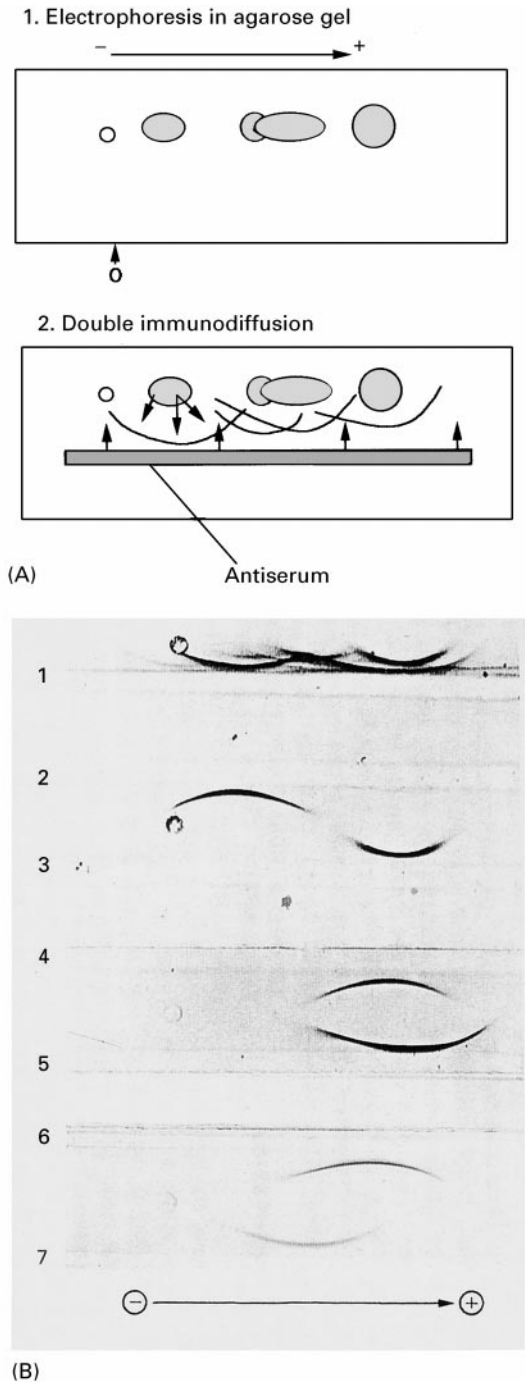

Figure 1 (A) Schematic representation of the principle of conventional immunoelectrophoresis. 1, Electrophoresis of the sample in agar or agarose gels; 2, double immunodiffusion of the separated proteins and the corresponding antiserum. (B) Application of the technique to the study of specificity of various antisera. In all wells, fetal pig serum was applied. In the channels, the following antisera were assayed: 1, anti-fetal pig serum, 2, anti-transferrin; 3, anti-albumin; 4, anti-α-fetoprotein; 5, anti-α_1-antitrypsin; 6, anti-α_1-acid glycoprotein; 7, anti-fetuin. (With permission from Lampreave, F, González-Ramón N, Martínez-Ayensa S, Hernández MA, Lorenzo HK, García-Gil A and Piñeiro A (1994) *Electrophoresis* 15: 672–676.)

corresponding antiserum and the plate is maintained in a humid, sealed box, for a period of between 24 and 48 h. Under these conditions, the combination of the radial diffusion of the proteins from the circular or ovoid spots obtained after the electrophoretic run, with the uniform diffusion of the antibodies, occurring in a perpendicular direction to the channel, produces arcs of precipitation in the different electrophoretic zones.

The IE patterns can be directly visualized or photographed, in the wet gel, by dark-field illumination. In this method the oblique light from a circular source placed below the plate is directed through the gel and transmitted at an angle of about 25°. The immunoprecipitates are visible as white lines on the dark background. To preserve the plates and before staining the immunoprecipitates, it is necessary to wash the gel plates extensively so that all the unprecipitated materials are removed. The wash is accomplished by immersing the gel plates in a buffered saline solution ($0.01\ mol\ L^{-1}$ phosphate, NaCl $0.15\ mol\ L^{-1}$ buffer, pH 7.4) for 1–3 days, and with several changes of liquid. Alternatively, a quick procedure can be carried out by placing several paper towels on the gel plate and applying a moderate pressure for some minutes. In this way, the liquid and all the soluble material are removed from the gel and absorbed by the paper towels. Then, the plate is submerged in the saline solution until the gel almost recovers its original thickness. Repeating this drying/soaking cycle, it is possible to achieve effective and rapid washing of the plates. Finally, the plates are dried and the immunoprecipitates stained, commonly with Coomassie blue or Amido black. The sensitivity of the method, using these conditions, allows the detection of proteins with concentrations ranging from 3 to 20 $\mu g\ mL^{-1}$. Specific staining methods have been introduced to facilitate the identification of single proteins. For example, Sudan black can be used to detect lipoproteins and periodic acid (Schiff reagent) for polysaccharides and glycoproteins. There are also specific staining procedures for some proteins, such as ceruloplasmin, hemopexin, etc.

IE has been used to analyse complex mixtures of proteins from tissue extracts; to detect impurities during the monitoring of protein purifications; to detect differential expression of protein during growth and differentiation; to study the expression of single proteins during pathological situations and to detect protein polymorphisms. Figure 1B shows an example of the application of the IE technique. In the plate, the specificity of different antisera against six proteins isolated from fetal pig sera (albumin, α-fetoprotein, α_1-acid glycoprotein, α_1-antitrypsin, fetuin and transferrin) is analysed by attaching them to

a fetal pig serum by this technique. As reference, the same fetal pig serum is analysed against a polivalent anti-fetal pig antiserum.

Counter Immunoelectrophoresis

This technique is a modification of conventional IE, that is performed in agar–agar gels at pH 8.0. Under these conditions, the antibodies are positively charged whereas the antigens present a negative net charge. Antigen and antibodies are applied in wells of 3 mm diameter, as indicated in **Figure 2**. Antibodies are placed on the well nearest to the anode and the sample in that close to the cathode. By applying a voltage across the gel the antigen and the antibodies move towards each other, forming lines of precipitation between the two wells. For this technique, the utilization of agar–agar gels is convenient since the medium, at the pH commonly used, generates a significant electroendosmotic flow that increases the cathodic movement of the antibodies. This technique can be used to detect both antigen and antibodies; rapidity is the main advantage.

Crossed Immunoelectrophoresis

Crossed IE is another approach in IE which is well suited for qualitative and quantitative analysis. The method, which was originally named two-dimensional electrophoresis or antigen–antibody crossed IE, offers not only higher resolution and simpler interpretation of the results than in conventional IE but it can also be standardized for quantitative analysis.

The principles of this technique are presented in **Figure 3A** and consist of two electrophoretic runs, both in 1% agarose gel plates. Veronal 0.05 mol L^{-1} pH 8.6, 1 mmol L^{-1} calcium lactate is a buffer that is frequently used. Samples are applied in wells of around 3 mm diameter and a current applied for about 3–4 h at a potential gradient of 10 V cm^{-1} (to achieve a total electrophoretic run of around 6 cm). Afterwards, a longitudinal strip of 1 cm width, which includes the separated protein fractions, is cut off and transferred to a plate with the second agarose gel (prepared in the buffered solution as above) that contains the antiserum corresponding to the material to be tested.

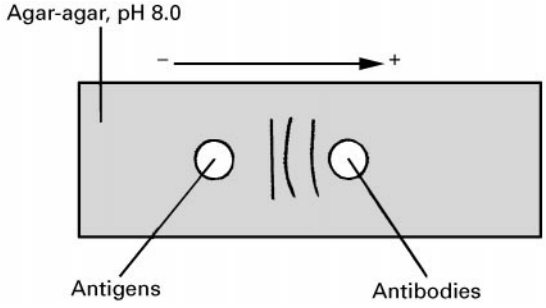

Figure 2 Principle of counter IE. This technique is developed in agar–agar gels, at pH 8.0. Antigens move towards the anode and antibodies to the cathode, due to its charge and to the electroendosmotic effect. (With permission from Lampreave F and Piñeiro A (1992) Concentration of major plasma proteins in serum and whole-tissue extracts of porcine fetuses during development, *Journal of Reproduction and Fertility* 95: 441–449.)

The second run is carried out in a perpendicular direction to the first separation, at a potential gradient of around 5 V cm^{-1}. The time of this electrophoretic step varies depending on the mobility of the antigens. For complex mixtures, and depending on the relative proportions between antigens and antibodies, periods of around 6–10 h may be needed. Under these conditions, precipitation peaks are produced in which the height prevails over the width. Furthermore, the area of each peak is now related to the amount of protein contained in the sample.

Identification of proteins in the crossed immunoelectrophoretic patterns, though simpler than in the patterns obtained by conventional IE, presents difficulties when complex mixtures of proteins are analysed. For that reason, some modifications to crossed IE, such as fused rocket IE, line IE, tandem crossed IE, crossed line IE and crossed IE with intermediate gel, have been introduced.

Crossed IE techniques enable the easy comparison of the content of a determined protein in different samples. Figure 3B shows an example of the potential of this technique, applied to the study of the acute-phase proteins in pigs. The comparison of the crossed immunoelectrophoretic patterns of blood serum from the same pig before (left) and 48 h after the induction of acute inflammation by turpentine injection (right, acute-phase serum), permits detection of important differences in the concentration of some plasma proteins. Especially notable is the increase of peak 9, that corresponds to the major acute-phase protein in pigs, called Pig-MAP, which could be detected through this technique.

Crossed IE can also be applied to the study of membrane proteins. For this it is convenient to solubilize them with nonionic detergents, such as Triton X-100, that better preserve the structural and functional properties of these proteins. The detergent does not cancel out the antigenic properties of the membrane proteins, nor does it impede the antigen–antibody reaction. In the characterization of membrane proteins it is important to know whether the proteins studied possess hydrophobic domains that anchor them to the hydrocarbon interior of the bilayer, or whether they are externally bound to the membrane. Charge-shift immunoelectrophoresis permit the user to easily obtain that information even in

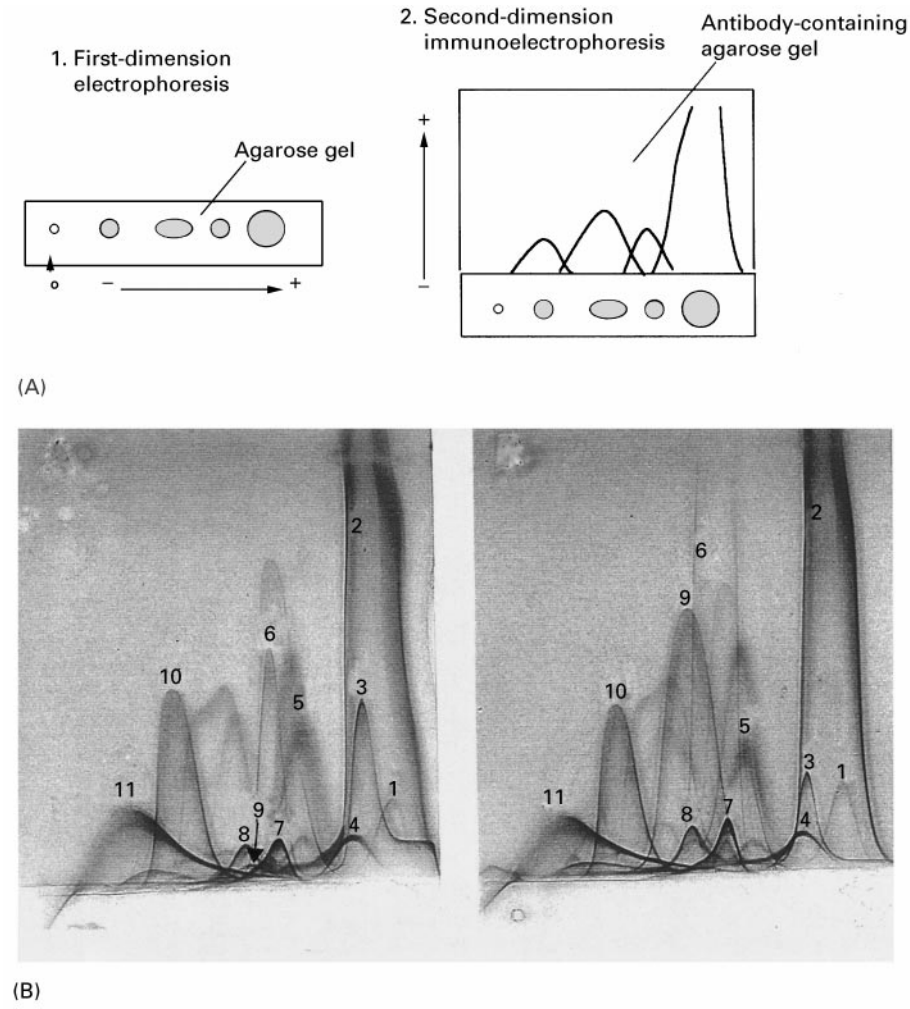

Figure 3 (See Colour Plate 41). (A) Schematic illustration of crossed IE. 1, Agarose electrophoresis of the sample (O = origin, corresponding to the well where the sample is applied); 2, a longitudinal strip of the first-dimension gel is transferred into a second-dimension gel containing a polyvalent antiserum. The second-dimension IE is performed perpendicularly to the first-dimension run. (B) Example of use of the crossed IE technique. The blood serum from the same pig is analysed (left) before and (right) 48 h after turpentine injection. The changes in the protein concentrations, induced by inflammation, can easily be studied by analysing the area (or height) of the different-numbered peaks. (With permission from Lampreave, F, Alava MA and Piñeiro A (1996) Trends in Analytical Chemistry 15: 122–129.)

complex mixtures. Membrane proteins are solubilized either with Triton X-100 alone or mixed with other detergents, for example, Triton X-100/sodium deoxycholate (an anionic detergent) and Triton X-100/cetyltrimethylammonium bromide (a cationic detergent). Afterwards, these three membrane extracts are analysed comparatively by crossed IE. The detergent-induced shift in mobility provides a method to distinguish between hydrophilic and amphiphilic proteins.

Rocket Immunoelectrophoresis

This immunochemical method, introduced by Laurell in 1966, is suitable for the quantitative estimation of proteins. In this case (**Figure 4**A) there is only one electrophoretic run carried out in 1% agarose gel (plates of uniform thickness of about 1.5 mm) in Veronal buffer, pH 8.6 as described before, but including an appropriate quantity of an adequate antiserum. The samples are applied in separated wells of 3 mm diameter, located near the extreme of the plate in contact with the cathode. To obtain quantitative results, different dilutions from a primary standard (a solution of the purified protein of known concentration) or a secondary standard (a serum previously evaluated using a primary standard) are applied on the gel. The electrophoresis is accomplished over periods ranging from 4 to 6 h depending on the charge of

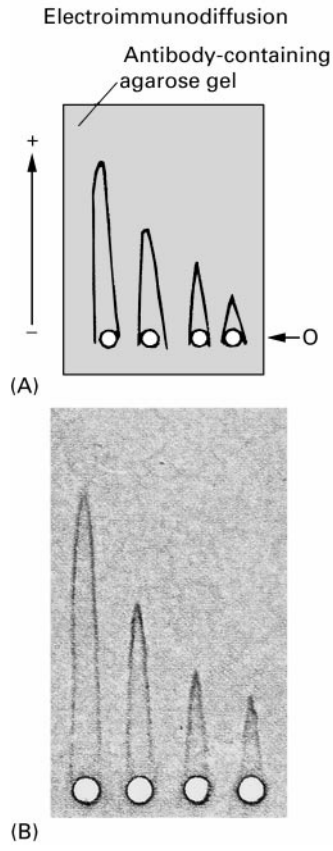

Figure 4 (A) Schematic representation of rocket IE. The electrophoresis is carried out in agarose gel containing specific antiserum against the protein to be determined. The area of the rockets (or its height) is proportional to the content of protein in the sample. (B) Application of this technique to the quantification of human α-fetoprotein. To improve sensitivity, the first immunoprecipitates (developed with rabbit antiserum to human α-fetoprotein) were treated with glucose oxidase-labelled sheep antibodies to rabbit immunoglobulins. From left to right, solutions containing α-fetoprotein concentrations of 720, 360, 180 and 120 ng mL^{-1} were assayed.

the antigen and on the relationship between the amount of antigen and antibodies included in the gel plate. The immunoprecipitates formed in this case have the shape of a rocket, the area (or height) of which is proportional to the concentration of the antigen applied in the well. The concentration of the protein in unknown samples can be determined by reference to the calibration line obtained by representing the height versus the concentration of each standard.

This technique can be applied to any protein with a net charge that differs from that of the antibodies. Some proteins, such as, for example IgG, hardly move during electrophoresis using a pH 8.6 buffer. In these cases, the mobility of the antigen to be determined can be increased by carbamylation of the sample with KCNO.

Rocket IE is the most sensitive of the conventional immunoelectrophoretic methods and allows one to accomplish the quantification of proteins (using stains to visualize the immunoprecipitates) up to concentrations close to 1 μg mL^{-1}. This sensitivity can be increased by combining immunoenzymatic methods. **Figure 4**B shows an example of one of these methods applied to determine human α-fetoprotein. During the electroimmunodiffusion, the first antigen–antibody reaction is produced using a rabbit antiserum containing the specific antibodies (Ig). In a second step, the plate is incubated with glucose oxidase-labelled sheep antibodies to rabbit Ig. Finally, the glucose oxidase bound to immunoprecipitates is revealed by incubating the plate with a solution that contains glucose, MTT-tetrazolium and phenazide methasulfate. The immunoprecipitates stain blue-violet over a fainter background of similar colour. This method provides reproducible results and allows the quantification of α-fetoprotein at concentrations as low as 50 ng mL^{-1}.

Crossed-affinity Immunoelectrophoresis (CAIE)

Affinity electrophoresis refers to any technique in which two or more components specifically interact during an electrophoretic run. Affinity electrophoresis in agarose gels, combined with subsequent immunochemical detection (CAIE), was introduced by Bog-Hansen in 1975 as a useful tool for the characterization of biospecific macromolecular interactions. This technique permits, among other things, the demonstration of ligand-binding proteins, enumeration of binding sites and estimation of binding constants, with the added advantage of being adequate for determination of very small quantities in impure materials.

An important field of application of CAIE has been the study of the interaction of glycoproteins and lectins. Lectins are animal and mostly plant-derived proteins that specifically interact with the carbohydrate components of glycoconjugates. There is a considerable amount of information in the literature about the biochemical properties of numerous lectins and on the type of glycan structures that they can recognize. Using different lectins in the first electrophoresis run (affinity electrophoresis step), CAIE permits the analysis of the heterogeneity of glycoproteins in complex mixtures. The serum protein glycoforms, after fractionation by lectin affinity electrophoresis, can be revealed with specific antibodies and finally quantified.

Figure 5A shows a schematic illustration of the CAIE technique. First, the serum proteins are subjected to electrophoresis (generally at pH 8.2–8.6)

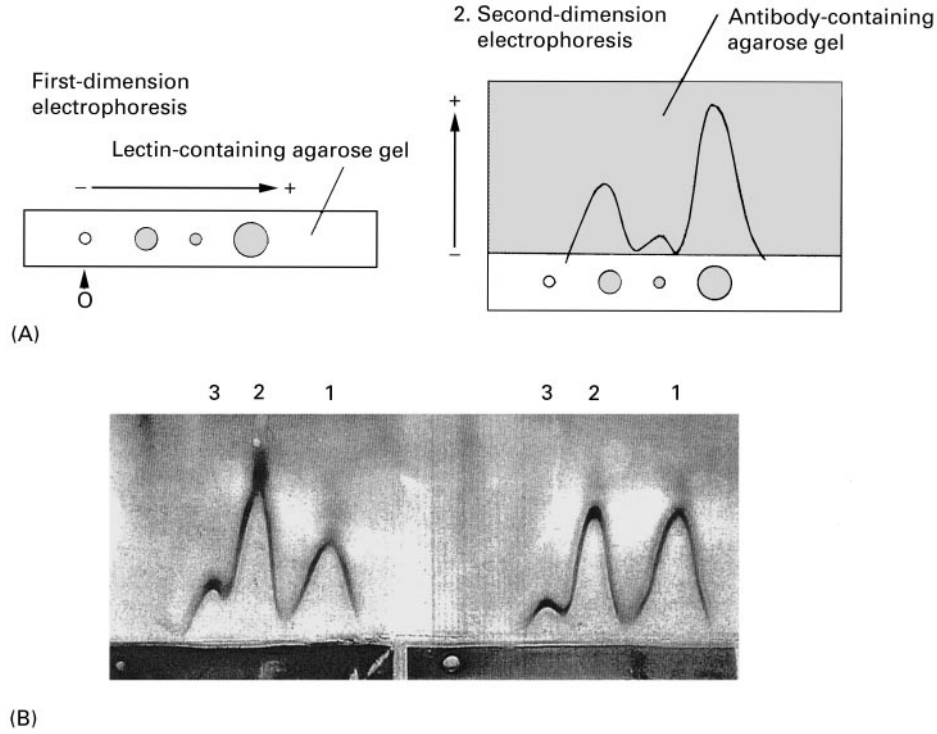

Figure 5 (A) Schematic representation of the CAIE technique. O, origin, corresponding to the well where the sample is applied in the first-dimension electrophoresis. (B) CAIE patterns of human α_1-acid glycoprotein from (right) healthy individuals and (left) patients with inflammatory processes. The numbered peaks correspond to microforms of α_1-acid glycoprotein in decreasing order of mobility.

in lectin-containing agarose gels (first-dimension gel). The lectin is added to the melted agarose (at around 55°C) before pouring the gel. Under these conditions most of the lectins used in CAIE do not have sufficient mobility. The proteins are fractionated in their different glycoforms, whose mobility depends on their corresponding affinity for the lectin. The first-dimension gel is then transferred into a second-dimension agarose gel containing specific antibodies against the protein to be analysed. The second-dimension electrophoresis produces for many serum glycoproteins fused precipitating peaks, which corresponds to the different microforms present in the sample. The CAIE patterns can be visualized by the methods described in the previous immunochemical techniques. The amount of each glycoform is related to the corresponding immunoprecipitating area that can be calculated by planimetry or by triangulation.

Figure 5B shows, as an example, the CAIE patterns of α_1-acid glycoprotein from human serum. In the first dimension gel, 1 mg mL^{-1} of the lectin Concanavalin A was included. The second-dimension gel contained specific rabbit antiserum against human α_1-acid glycoprotein. The different peaks have been labelled in decreasing order of mobility. Peak 1 corresponds to glycoforms of the human α_1-acid glycoprotein that did not react with the lectin and contains tri- and tetra-antenary glycans; peak 2 corresponds to weakly reactive glycoforms of the protein containing one bi-antennary glycan; finally, peak 3 corresponds to strong reactive glycoforms containing at least two bi-antennary glycans.

Equipment and General Methods

Agarose gels (1% w/v) are prepared in the described buffers on glass plates of different size according to need. For example 9×12 cm plates are suited for conventional IE (they permit the analysis of eight samples or seven antisera) and also for RIE (in this case, about 20 samples can be applied). For the CIE and CAIE techniques, 4×9 cm and 7.5×10 cm glass plates can be used for the first and the second electrophoretic run, respectively. In all cases, one of the surfaces of the glass plates is coated with an agarose solution (1% w/v, in distilled water) that, after being dried, serves as an anchor for the agarose gel. To ensure regular thickness throughout the gel plate, which is essential for the reproducibility of quantitative methods, the melted agarose is poured between two glass plates separated by rigid spacers of around 1.5 mm thickness.

When antibodies, purified proteins or lectins are to be added to the gel, the agarose solution must be previously equilibrated around 55°C in a thermostatized water bath to avoid protein denaturation. The

growth of microorganisms is avoided by the addition of preservatives to the agarose solutions, for example 0.1% sodium azide or 0.01% sodium merthiolate.

Simple equipment is commercially available, though much can be made in-house. The electrophoresis system only requires two electrophoretic tanks (provided with platinum wire and connected to the electrodes), and an adjustable power supply delivering voltage up to 400 V at 400 mA. The connection between the agarose plates and the buffer in the tanks can be accomplished through filter-paper wicks previously wetted in electrophoretic buffer (the same as that used for preparing the gel). To avoid excessive warming of the gel during electrophoresis, it is convenient to cool the agarose plates using a system connected to tap water. This allows the electrophoresis to be run at room temperature in the laboratory, alternatively, it can be carried out in a cold room at 5°C.

A great number of polyvalent and specific antisera prepared in goat, sheep or rabbits can be obtained from different suppliers or obtained in-house.

Present and Future Developments

Though most immunoelectrophoretic techniques described here were developed at least 25 years ago, they still enjoy great popularity and continue to be excellent tools for biochemists and immunologists. IE and CIE are very useful techniques for the characterization of complex mixtures of proteins and for the study of certain pathological situations that evolve with changes in plasma protein patterns. CAIE is a powerful technique for detecting glycoprotein microforms using different lectin specificities. Advances in the characterization of new lectins with restricted specificity represent a future development in this field. CAIE can also be applied to many affinity systems, including the important contribution of monoclonal antibodies in the affinity electrophoresis step.

Immunoelectrophoretic techniques are time- and antisera-consuming techniques. These limitations could be improved by including the capillary methods commonly used in capillary electrophoresis systems.

Further Reading

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Isoelectric Focusing

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Isoelectric focusing represents a unique electrokinetic method in that it is based on steady-state patterns attained by amphoteric species (mostly proteins and peptides) along a pH gradient under the influence of an electric field. Due to a continuous balancing of diffusion away from the p*I* (isoelectric point) and p*I*-driven electric forces, extremely sharp zones are obtained, characterized by a very high resolving power. This article will consider conventional isoelectric focusing (IEF) in soluble, amphoteric buffers; and immobilized pH gradients (IPG) in insolubilized,