Immunofixation offers the worker an economical means of physically locating a protein in an electrophoretic strip and is ideally suited to forensic medicine, genetic studies, or research. The method is as simple and economical as the commonly used one- or two-dimensional immunoelectricphoresis, yet yields considerably more information.

Many plasma proteins display molecular heterogeneity when viewed with special procedures. Perhaps the most direct and frequently used method is electrophoresis, in which the isoelectric points (IP) of the various polymorphic forms of an individual protein species is what principally dictates their locations within a strip of gel or on a separatory column in systems that do not also involve molecular sieving. Both molecular alteration and genetic variation affect a protein’s carbohydrate and peptide content, which, in turn, alters the IP of a given species, subspecies, or fraction. Therefore, electrophoresis inherently is a technique for demonstrating microheterogeneity, but because of the nonspecific means used to make visible the protein in the gel matrix (staining), resolution is poor.

Ordinarily, serum protein electrophoresis resolves six to eight major fractions that contain about a dozen major species and more than 100 minor fractions (Figure 1). The IP of many are close, hence the small number of fractions that are resolved in even the best agarose or membrane systems. Because of the many minor fractions that often represent degradation products or variants of completely unrelated species, high-resolution gels make identification even more confusing. Immunological identification offers the only sure means of detecting protein fractions that move in unusual positions because of genetic variation or biochemical alteration. Alper and Johnson (1) described a technique called “immunofixation,” which allows the worker to anchor, in situ, proteins of a single immunologic species by exposing the protein-containing gel to specific antibody after electrophoresis. Afonso (2) described a forerunner of true quantitative immunoelectrophoresis that was to be described two years later by Laurell (3). Afonso’s description made use of the basic concept of immunofixation, but with a polyclonal antiserum overlaid on microimmunoelectrophoresis films. The results were as difficult to interpret as the usual microimmunoelectrophoresis developed with polyclonal antiserum.

The purpose of this paper is to describe the easy application of a modified immunofixation method to two unmodified agarose electrophoresis systems that are in wide use (4).

Materials and Methods

Electrophoretic systems: System A, consisting of agarose gel bonded to plastic film, was obtained from Millipore Biomedica, Acton, Mass. 01720. The buffer system at pH 8.6 is used as supplied by the manufacturer. System B is a water-cooled, agarose (“Seakem
Agarose"; Marine Colloids, Inc., Rockland, Maine 04841) electrophoresis system as described by Laurell (3), without modification except that in casting the gel film no sample pockets are included.

**Gel preparation:** For system A, the plastic mask for sample application supplied by the manufacturer is stripped completely from the gel surface, in contrast to the recommended procedure. For both systems, a 2.5-cm wide strip of cellulose acetate membrane (supplier not critical) is applied smoothly over the application area for 10 to 15 s. Filter paper or blotter is not suitable for absorbing surface moisture. The slotted mask is then applied to the gel surface. Bubbles should be excluded carefully.

**Sample preparation and application:** Whole serum, plasma, other body fluids, or protein fractions are applied directly to the slotted mask, without mixing samples.

The amount of protein that diffuses into the gel can be controlled by either varying the duration of application, or by previously diluting the sample appropriately. Application time can vary from 30 s to 10 min, with a shorter time being used for samples with high protein concentration, longer times for those of low concentration.

If the dilution technique is used, a final concentration of 0.15–0.3 g/liter is desirable for discretely banded proteins and 2 g/liter for the heterogeneous immunoglobulins. Depending on the potency and avidity of the antiserum used, adjustment by trial and error may improve the final results.

**Electrophoresis.** Voltage is maintained at 300 V for system B, but because of manufacturer's engineering limitations, only 200 V can be applied to System A. Electrophoresis time is 10 to 20 min and 45 min, respectively. For proteins that have several closely migrating fractions, a relatively prolonged electrophoresis may be desirable.

**Immunofixation.** Cellulose acetate strips, 1 × 4 cm, are dipped into antiserum (Nephelometric grade; Atlantic Antibodies, Westbrook, Maine 04092) of the appropriate concentration, drained against the side of the container, and applied to the gel surface immediately after removal from the electrophoresis chamber. The strips are placed to cover the zone known to be occupied by the protein under study (Figure 2). Care should be taken to remove air bubbles from beneath the strips and to ensure that the adjacent strips containing different antibodies do not touch. In this approach, several different antisera can be used on a single gel film. If one protein is being studied in several samples, the strips can be wider and longer to cover as many samples as desired (Figure 2). The gel is incubated in a moist chamber for about 1 h. (The incubation temperature is not critical.)

**Processing.** At completion, the acetate strips are removed and discarded. Smooth filter paper of any variety is moistened with physiological saline and applied directly to the entire gel film. This is overlaid with 3 to 5 dry absorbent paper towels and covered with a plastic plate that will bear and distribute a weight of 5 kg over a 10 × 20 cm area. The plate is kept under this pressure for 10 min. After careful removal of the absorbent pads, the thinned gel is washed in a 1-liter agitated-saline bath overnight, dried in a stream of cool air, and stained with Amido Black or Coomassie Brilliant Blue in dilute acetic acid (50 ml/liter) exactly as for conventional protein electrophoresis.

**Results**

The improved resolution obtained with high-quality monospecific antiserum in this system presents convincing evidence that a given band or series of bands are a specific protein, regardless of the isoelectric point. The method described is easily applied to any protein present in a concentration exceeding 50 mg/liter. In the system described, similar results can be attained by adjusting the protein content by dilution of the sample and exposure of each dilution for a set period of time, or by exposure of each sample for periods of time ranging from 30 s to 10 min.

Electrophoresing excessive amounts of antigen decreases resolution and requires higher concentrations of antibody. If insufficient antibody is available the central portion of a protein band may remain unfixed through the process of antigen excess. For optimum
separation and sufficient intensity for visual detection or photometric recording, care must be taken in adjusting antibody content, sample concentration, time, and voltage. Table 1 lists conditions we found by trial and error to be optimal for several human serum proteins. Some final results are illustrated in Figure 3.

Consumption of high-quality antisera suitable for this technique is about 25 µl per square centimeter of cellulose acetate membrane, or, at the most, 100 µl per sample. With experience this can be decreased to 30 µl (by comparison, conventional immunoelectrophoresis requires 60 to 100 µl of antiserum).

**Discussion**

Microimmunoelectrophoresis and two-dimensional, crossed antigen-antibody electrophoresis have both been extensively used to study the microheterogeneity of human plasma proteins. Both have a common drawback not shared by immunofixation: long migration time with resulting low resolution. Immunofixation in the method described here combines excellent resolution produced by an extremely narrow application zone, rapid electrophoresis in a very thin gel, and anchoring of the protein by specific antibody within minutes after electrophoresis. The technique has the added value of allowing the worker to compare a band, as seen on immunofixation, with a band or zone seen with conventional protein electrophoresis in exactly the same system.

In the original technique (1), liquid antiserum was applied to the gel surface. In our experience, resolution was enhanced by the use of the membrane strip, which prevented diffusion of antigen upward and laterally in the liquid film. The membrane strips, also, considerably reduced the consumption of antiserum and prevented leakage of the material away from the desired area.

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**References**


