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Dean A. Arvan[a]; Leslie M. Shaw[a]
[a] THE WILLIAM PEPPER LABORATORY DEPARTMENT OF PATHOLOGY, UNIVERSITY OF PENNSYLVANIA SCHOOL OF MEDICINE, PHILADELPHIA, PENNSYLVANIA

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Two-Directional Immunoelectrophoresis: Technique and Applications in the Clinical Laboratory*

DEAN A. ARVAN and LESLIE M. SHAW

THE WILLIAM PEPPER LABORATORY
DEPARTMENT OF PATHOLOGY
UNIVERSITY OF PENNSYLVANIA
SCHOOL OF MEDICINE
PHILADELPHIA, PENNSYLVANIA 19104

Abstract

Some recent applications of "crossed electrophoresis" (also called "electro-immunodiffusion" and "rocket electrophoresis") are reviewed, particularly in the clinical laboratory.

INTRODUCTION

The development and application of high-resolution immunochemical techniques during the past two decades has been uniquely responsible for the vast, and at times spectacular, advances in our understanding of the structure and metabolism of human proteins. The widespread use of these techniques for the study of protein changes in health and disease

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has contributed equally to the impressive accumulation of new information in this area.

In the modern clinical laboratory, microimmunoelectrophoresis as developed by Scheidegger in 1955 (*1*) and radial immunodiffusion as popularized by Fahey and McKelvey 10 years later (*2*) comprise the basic analytical tools for the specific identification and measurement of proteins in a variety of biological fluids.

It is now widely appreciated that immunoelectrophoresis is, at best, a procedure giving only semiquantitative results and, although considerable information can be derived from the examination of patterns using antisera with a variety of specificities, interpretation is at times difficult primarily because of overlapping bands. Conversely, although radial diffusion is a quantitative method of acceptable precision, it suffers from the major limitation that only a single protein can be measured at any one time. Early attempts to develop a technique which yielded qualitative and quantitative data simultaneously were thus not unexpected (*3–6*). It was Laurell and his colleagues, however, who, in 1966, introduced the so-called "crossed electrophoresis" (*7, 8*) (otherwise known as "electroimmunodiffusion" (*9*) or "rocket electrophoresis") technique, setting the stage for the subsequent development of two-directional immunoelectrophoresis by Clark and Freeman (*10*).

The purpose of this paper is to review some of the recent applications as well as our own experience with this analytical technique which is receiving steadily increasing attention in the literature. Since "electroimmunodiffusion" or "rocket electrophoresis" involves migration of protein antigens in antibody-containing agarose during the second stage of two-directional immunoelectrophoresis, it is appropriate to begin by briefly describing the basic principles in this step.

ELECTROIMMUNODIFFUSION

Figure 1 diagrammatically shows the principle involved in electroimmunodiffusion. A glass slide (or plate) is first coated with agarose gel of uniform thickness containing a monospecific antiserum (e.g., antialbumin, antitransferrin). Known amounts of antigen are placed in wells of uniform diameter, cut toward the cathodic end of the slide, and an electric potential is applied. The antigen in the well is thus forced into the gel at a rate dependent upon its net electrophoretic mobility. The electric field causes migration of the antibody as well, but since the

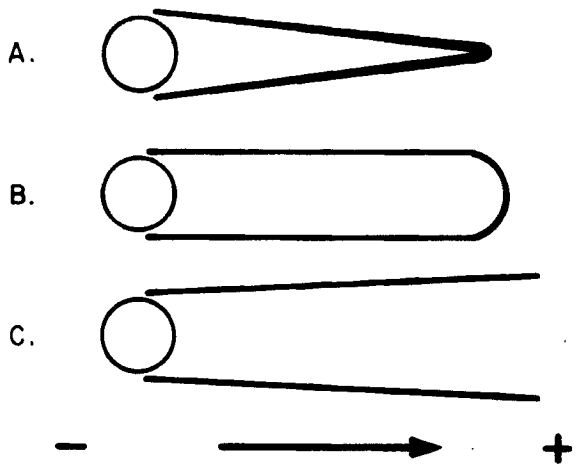


FIG. 1. Diagrammatic illustration of the principle of electroimmunodiffusion. A known amount of antigen is placed in each well. An electric field forces the antigens into the antibody containing gel, and in the face of temporary antigen excess the antigen–antibody complexes partly redissolve, and continue to move forward. When optimal antigen–antibody ratios exist, cone-shaped precipitates form at the completion of the run as in *A*. Patterns *B* and *C* are obtained in the face of moderate or marked antigen excess, respectively.

latter is a gamma globulin, its mobility in agarose relative to that of most antigens is slight and, because of electroendosmosis, is usually toward the cathode. Antigen and antibody react with one another to form precipitation zones shaped like "rockets." As electrophoresis progresses, excess antigen within the cone moves into the area of the precipitation creating temporary antigen excess and leading to partial dissolution of the antigen–antibody complexes. Thus the precipitation front is continuously displaced forward, and its final position is reached when all of the antigen is consumed in the formation of a stable precipitate. Once the antigen–antibody reaction goes to completion, no further migration is possible. This slide also demonstrates diagrammatically the configuration of the precipitation cone when test conditions are suboptimal. When an excess of antigen relative to the antibody is present, the precipitation front becomes rounded and less sharp, as in (*B*), or in the presence of marked antigen excess it redissolves (*C*), its lateral boundaries diverge and become less distinct.

There are several very important interrelated factors which influence the results obtained by this method: (a) antigen concentration, (b) antibody concentration, (c) quality of antiserum, (d) duration of electrophoresis, and (e) electrophoretic mobility of the antigen.

For each antigen–antibody system there is an optimum range of antigen–antibody ratios which influences very markedly the sensitivity, reproducibility, and quality of immunoprecipitates. The quality of cones, in turn, depends to a certain extent upon the strength and animal source of the antiserum. Horse antisera, for example, give very sharp bands but only within relatively narrow antigen–antibody ratios. Beyond these ratios they have a tendency to yield soluble precipitates.

If the electrophoretic mobility of the antigen is slow and the duration of the electrophoretic run is short, the patterns will not reach equilibrium, thus inaccuracies will result.

Once these variables are studied and optimized for any given antigen–antibody system, the application of standardized conditions will yield reproducible results.

Figure 2 illustrates the effect of one of these variables, i.e., quality of antiserum, on the appearance of the "rockets."

This, then, is a brief description of the principle of "single-directional crossed electrophoresis" of Laurell (or electroimmunodiffusion). Because of its sensitivity it has been used quite extensively for the rapid quantitation of individual protein fractions in dilute biologic fluids (*9*). As already mentioned, it is employed in the second direction of two-directional immunoelectrophoresis, although in the latter method a polyvalent antiserum (e.g., antiwhole human serum) is usually employed. For electrophoresis in the first direction, the proteins are initially fractionated on a high-resolution medium, e.g., agarose (*11–15*), acrylamide gel (*16*), and starch gel (*17–19*).

TWO-DIRECTIONAL IMMUNOELECTROPHORESIS

The remainder of this paper will deal with both the technical and interpretative aspects of two-directional immunoelectrophoresis with emphasis on its application to certain clinical problems.

A convenient apparatus developed in our laboratory and specifically designed for two-directional analyses has been previously described (*15*) and it allows the simultaneous development of two patterns.

The conditions for two-directional immunoelectrophoresis in our system are outlined below.

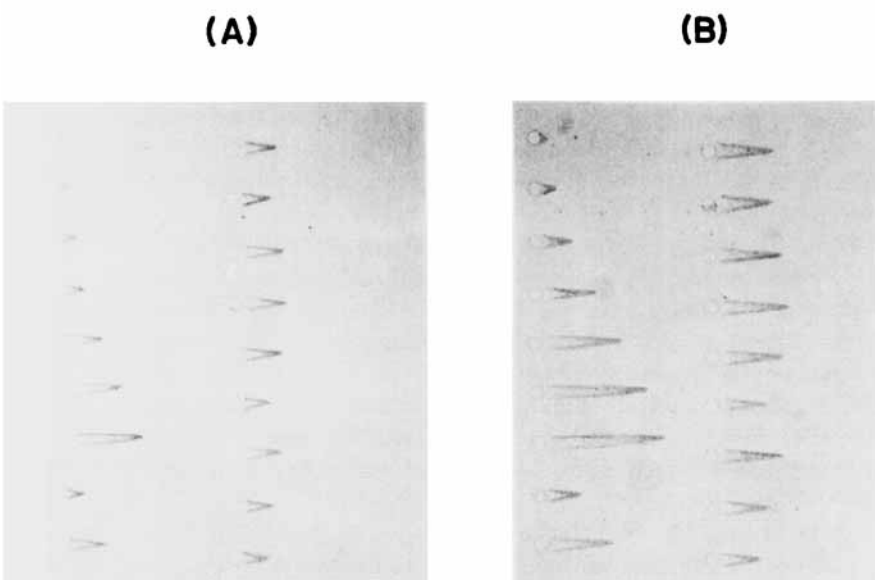


FIG. 2. Effect of quality of antiserum on the appearance of the "rockets". Goat (*A*) and rabbit (*B*) antiserum to human immunoglobulin G (IgG) obtained from two different manufacturers were used. The samples are identical in each frame. Note that the corresponding patterns in *B* are longer and somewhat fainter than in *A*. Based on principles outlined in the text, this suggests that antiserum *B* has a lower precipitation titer than *A*.

A. First direction.
   1. 10 ml of 1% agarose in 0.0125 *M* barbital, pH 8.6, evenly poured over a standard 3 × 4 glass slide.
   2. 0.35 μl of sample, applied with a Hamilton syringe.
   3. Electrophoresis conditions, 15°C.
      a. 0.025 *M* barbital buffer, pH 8.6.
      b. 250 V, 1½ hr.

B. Second direction.
   1. Overlay slide with 20 ml of 1% agarose (w/v) containing 2% (v/v) antihuman serum (horse).
   2. Electrophoresis, 15°C.
      a. 100 V, 20 hr.
      b. Barbital buffer, 0.025 *M*, pH 8.6.

Note that, except for those instances in which data for the construction of a calibration curve are desired, ordinarily only 0.3–0.4 μl of serum

is used, an amount which is obviously quite easily obtainable by fingerstick.

It should be emphasized that these conditions are not necessarily applicable to other systems; we, ourselves, are considering several revisions in the future in order to improve the efficiency and reduce the cost of the measurements. Indeed, a somewhat different system has recently been described (*20*).

Using a normal serum sample as the antigen and antihuman serum for the second direction, the pattern shown in Fig. 3 is obtained in which the various proteins are numbered for identification purposes. From 15 to 20 different proteins can be identified and quantitated. The conditions of the test are such, however, that optimum conditions are not achieved for the immunoglobulins or albumin. Difficulties are encountered in the quantitation of the former because of the slow mobility and the presence of multiple species with different mobilities and in the latter because of a large antigen excess.

An important question that may be raised at this point is: how does one identify individual proteins in such a complex mixture, since with with high quality antisera some 30–40 peaks may be observed. There are several ways in which this could be done:

(1) Substituting one or a limited number (e.g., 3–5) of monospecific antisera of known composition and strength in place of antiwhole human serum and employing identical analytical conditions so as to keep electrophoretic mobilities constant.

(2) Adding a small amount of the purified protein to the serum (if one is available) and observing the increase in peak height (e.g., transferrin and albumin) (*11*).

(3) Differential staining for a specific protein (e.g., ceruloplasmin and lipoproteins).

(4) Adding monospecific antiserum to antiwhole human serum and observing a decrease in peak height (*11*).

Figure 3 also illustrates one of the shortcomings of this method, namely that the immunoglobulins, particularly the IgG, cannot be adequately evaluated because both antigen and antibody have similar electrophoretic mobilities and do not give sharply demarcated, undirectional peaks.

One additional point needs to be amplified here, namely, that the time required to obtain a stable peak is related to the electrophoretic mobility of the specific protein, its concentration, and the concentration of the

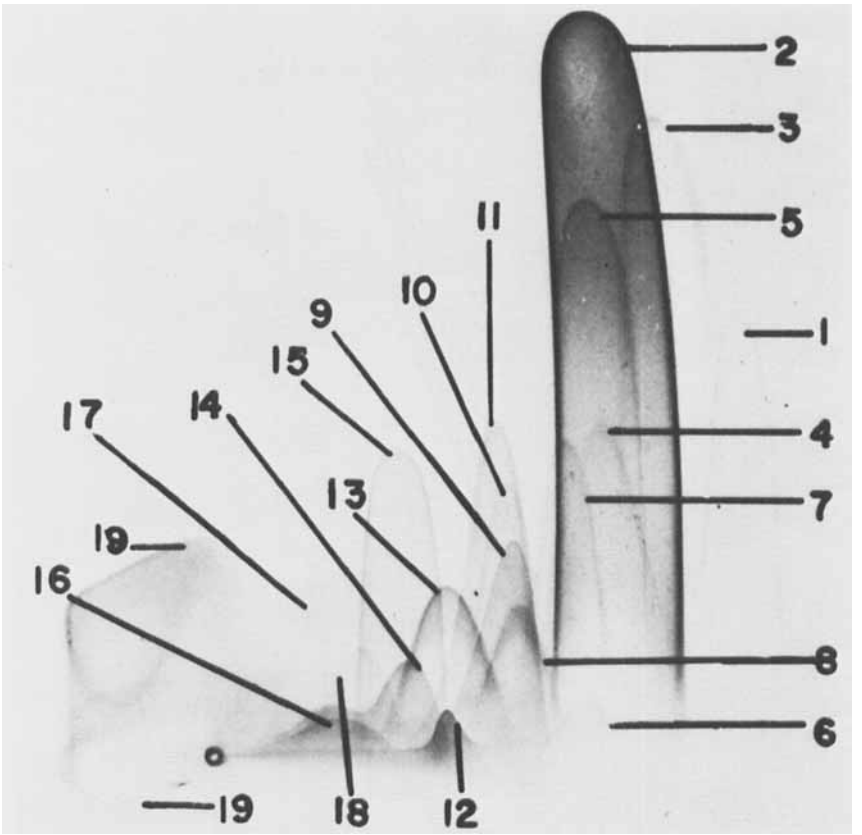


FIG. 3. Two-directional immunoelectrophoretic pattern of a normal human serum. The anode is to the right. The antiserum is antiwhole human serum (horse, Hyland Laboratories). 1, prealbumin; 2, albumin; 3, α_1-acid glycoprotein; 4, α_1-lipoprotein; 5, α_1-antitrypsin; 6, unidentified (? α_1-easily precipitable glycoprotein); 7, Gc-globulin; 8, α_2-HS glycoprotein; 9, α_2-macroglobulin; 10, ceruloplasmin; 11, haptoglobin; 12, β-lipoprotein; 13, hemopexin; 14, complement (β_{1C}/β_{1A}); 15, transferrin; 16, IgM; 17, IgA; 18, unidentified; 19, IgG.

antibody in the gel. Thus, for equivalent protein concentrations, the slower the mobility of the antigen the longer the time to reach equivalence (e.g., transferrin vs. α_2-macroglobulin) (*13*).

The next question that should be considered relates to the standardiza-

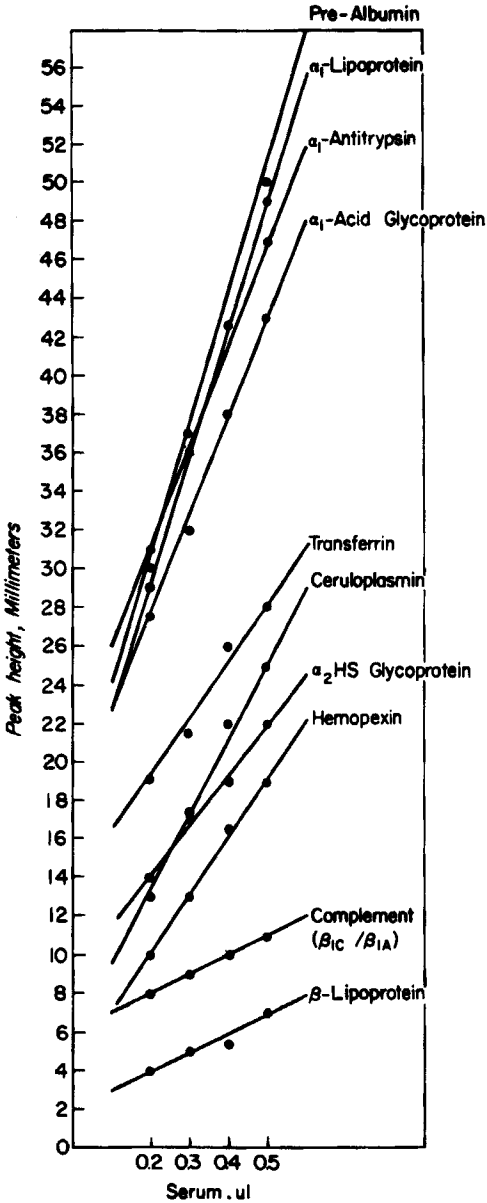


FIG. 4. Calibration curves for several serum proteins. Increasing amounts of a normal serum were applied in four different plates. Similar curves were obtained with several additional proteins not shown in this figure.

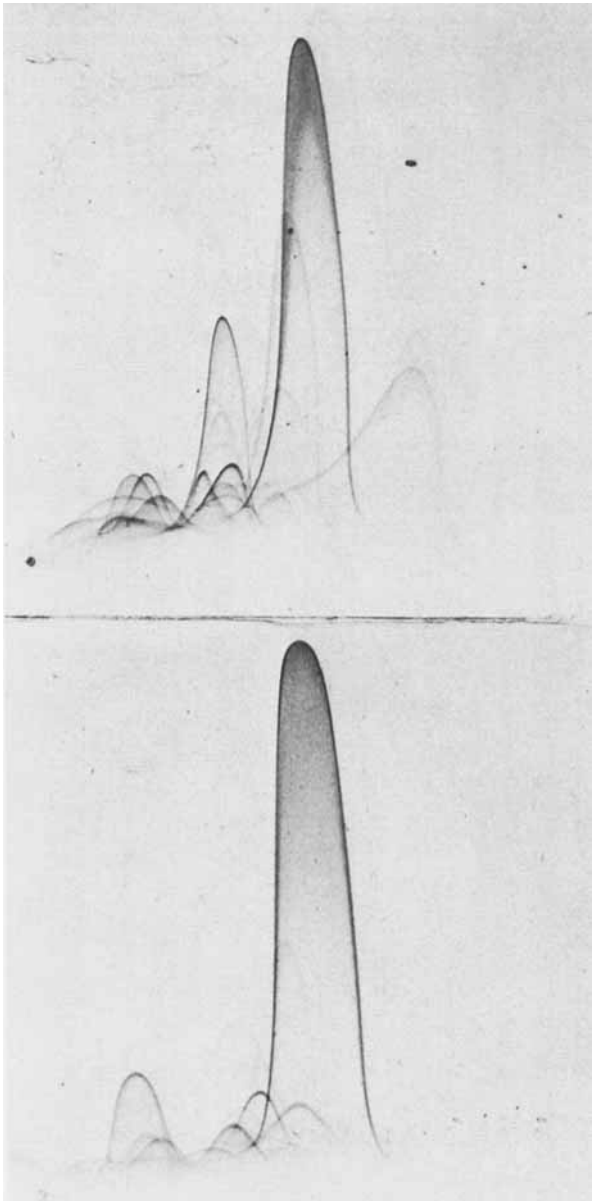
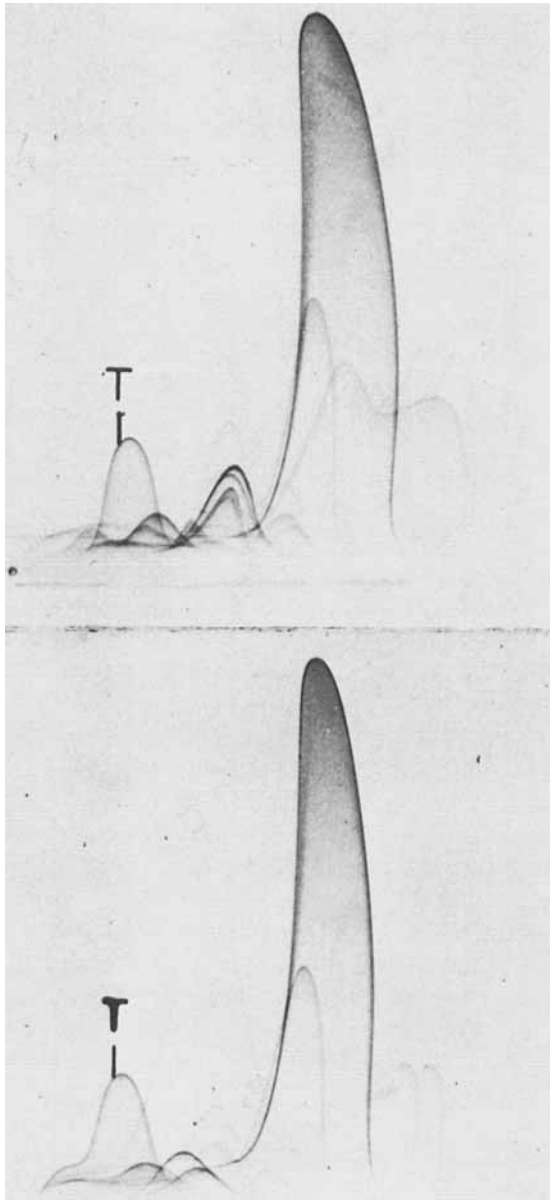


FIG. 5. Two-directional immunoelectrophoretic patterns obtained with the serum and urine of a patient with the "nephrotic syndrome," a type of renal disease in which large amounts of serum proteins are excreted in the urine. Upper frame: serum. Lower frame: urine. The urine was concentrated several times prior to application. (Reproduced from Ref. *15* by permission of the publisher.)



tion of the technique and its use for the acquisition of quantitative data. It is obvious that using standardized test conditions from one day to the next is an important prerequisite for acceptable quantitative measurements. With regards to quantitation itself, we have been using peak heights for this, although other workers have measured the area under the peak by either manual or electronic planimetry (*10*).

In Fig. 4 a series of calibration curves for some of the protein fractions are shown applying different amounts of serum from a normal individual. A linear relationship between peak height and the amount of protein is obvious for the indicated range. Since a primary protein standard is not yet available, a number of different methods for calculating relative or absolute concentrations have been employed. Clark and Freeman have used the mean values obtained with a repeatedly analyzed pooled normal human serum and express the results of the unknown as a percentage of the "normal pool." We, as well as others, have used Standardized and Stabilized Human Serum (Behring Diagnostics) for which the concentrations of several proteins are given. Other proposed secondary reference standards are carbamylated transferrin (*21*) and acetylated albumin (*22–25*). Known amounts of either of the latter two preparations are quantitatively added to each unknown serum, and on electrophoresis unique and easily identifiable peaks are obtained. The height or area of all other peaks can then be expressed in relation to one of these.

Table 1 shows the day-to-day precision for several components in the previously mentioned SS Human Serum. It should be noted that using a constant amount of antigen (0.40 μl), the CV is better than 10% in all instances and usually less than 8%, showing a reproducibility similar to that of many other quantitative immunochemical methods. We have recently obtained virtually identical precision values on a larger series of measurements.

Before turning to some of the applications of this technique, it is necessary to point out that caution must be exercised with regard to possible pitfalls in reading of peak heights or areas. Errors may be caused by (a) alterations of electrophoretic mobility of some protein fractions as a result of storage of the specimen, e.g., α_1-lipoprotein and complement

FIG. 6. Two-directional pattern of serum (upper) and cerebrospinal fluid (lower) from an essentially normal patient. Note the peculiar configuration of the transferrin (*T*) in the cerebrospinal fluid in comparison with the serum, a normal finding. (Reproduced from Ref. *15* by permission of the publisher.)

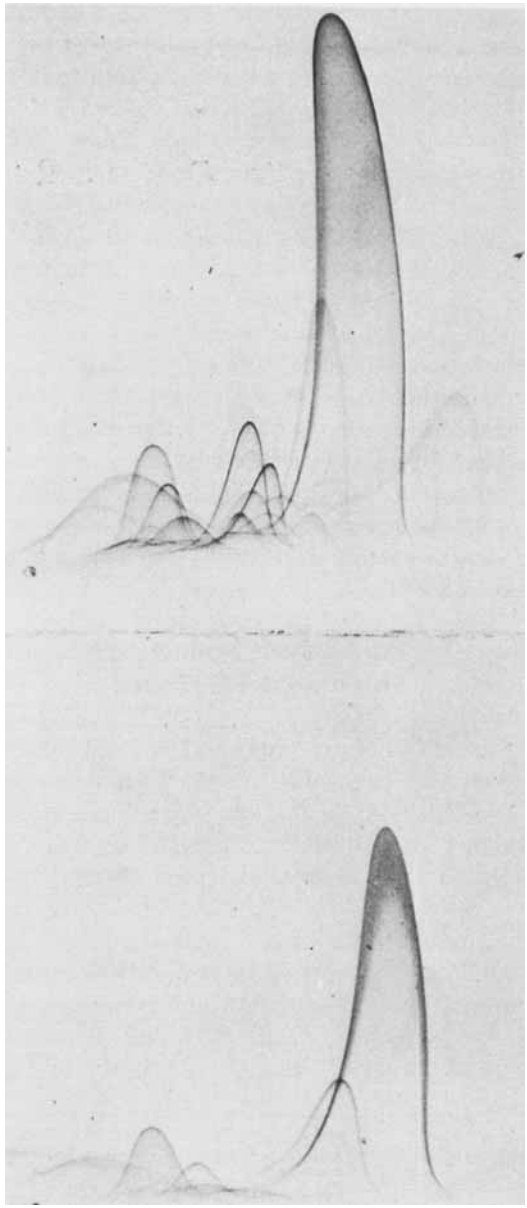


TABLE 1

Day-to-Day Precision in the Measurement of Peak Heights (in millimeters) by Two-Directional Immunoelectrophoresis[a]

	Mean[b]	SD	CV (%)
Prealbumin	43.3	2.93	6.8
Albumin	50.5	4.15	8.2
α_1-Acid glycoprotein	42.0	3.08	7.3
α_1-Antitrypsin	36.0	2.88	8.0
Unidentified	29.9	2.78	9.3
Unidentified	9.6	0.49	5.1
Haptoglobin	26.4	2.15	8.2
α_2-HS glycoprotein	14.7	1.25	8.5
α_2-Macroglobulin	12.2	1.08	8.9
Transferrin	22.7	1.57	6.9
Hemopexin	13.0	1.30	10.0
Complement (β_{1C}/β_{1A})	9.7	0.61	6.3

[a] 0.4 μl of Standardized Stabilized Human Serum (Behring Diagnostics, batch #469) was used for all measurements.
[b] $n = 6$.

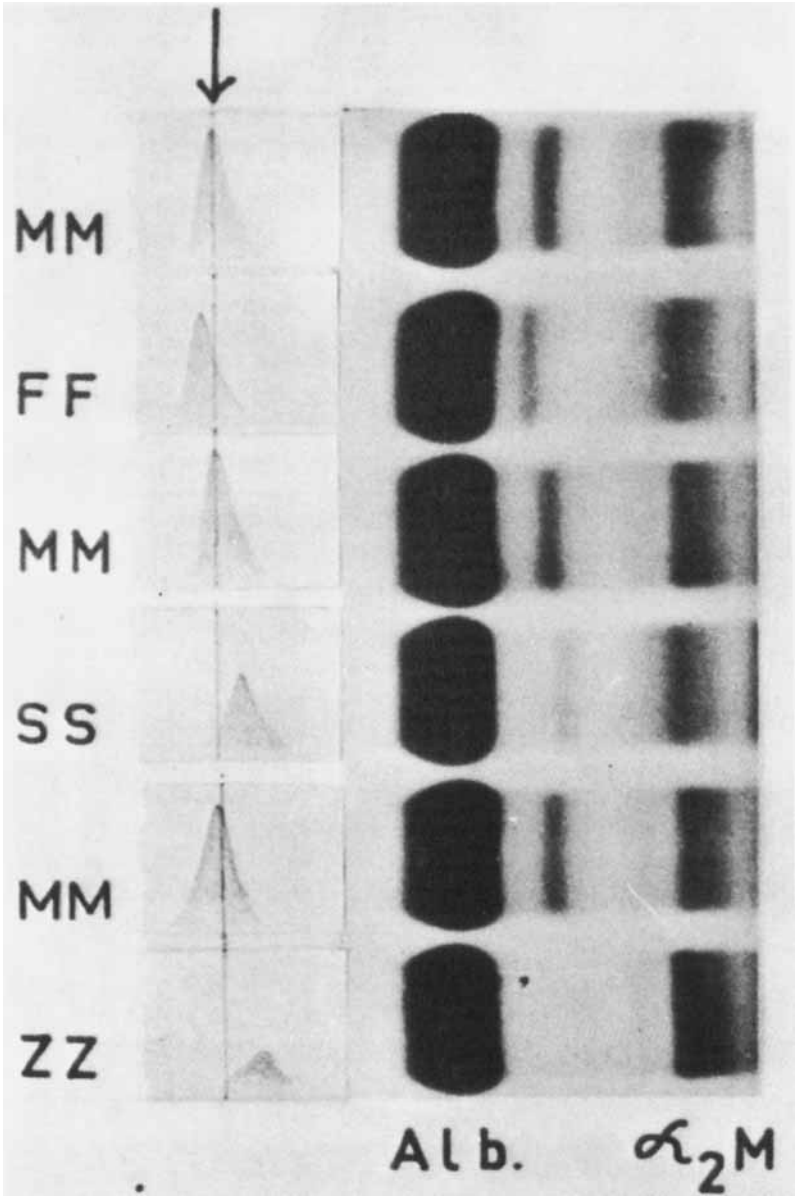
(β_{1C}/β_{1A}), and (b) the presence of genetic variants. These must always be kept in mind when analyzing a particular pattern.

Finally, as mentioned earlier, proteins can be readily fractionated in the first direction on some other high-resolution medium, e.g., acrylamide gel, starch gel, or other appropriate mixtures using either a continuous or a discontinuous buffer system. Agarose, however, has been used by virtually everyone for electrophoresis in the second direction. It should be pointed out that use of such media requires identification of the various fractions by one of the procedures mentioned previously.

GENERAL APPLICATIONS

In this section consideration will be given to a few of the clinical and research applications of two-directional immunoelectrophoresis.

FIG. 7. Comparative changes in the serum (upper) and concentrated bronchial washings (lower) from a patient with "pulmonary alveolar proteinosis," a rare disorder of the lungs in which the air spaces are filled with a proteinaceous material of obscure origin. (Reproduced from Ref. *15* by permission of the publisher).



Proteins in Biologic Fluids

The first area that comes to mind is in the study of comparative changes of various proteins in biologic fluid. It is quite possible that in some disease processes the physician or investigator may wish to know whether or not changes in various serum protein components are accompanied by similar changes in other biologic fluids. In the investigation of the relative protein clearances in renal disease, for example, one could apply this technique to measure the changes simultaneously in several protein fractions in accurately timed urine specimens and compare these to serum. This information may be of value to the physician in assessing the degree of impairment in glomerular selectivity.

Figure 5 illustrates this point. The top frame shows the two-directional pattern of the serum and the bottom frame of the concentrated urine from a patient with renal disease. Note that many serum components are clearly low but particularly affected are the low molecular weight proteins, albumin and transferrin.

Similarly, Fig. 6 compares the protein changes in cerebrospinal fluid to serum and Fig. 7 in this series shows the proteins in lung washings from a patient with pulmonary alveolar proteinosis, an obstructive lung disease of unknown etiology characterized by the accumulation of proteinacious material within the alveoli. Thus, when the degree of concentration of various dilute biologic fluids is known, relative clearances can be calculated. In addition protein components unique to this particular disease entity could be identified.

Polymorphic Variants

This technique appears to lend itself particularly well to the investigation of polymorphic variants of certain proteins such as α-1-lipoprotein

FIG. 8. Fractionation of polymorphic variants of α_1-antitrypsin by two-directional immunoelectrophoresis in agarose. The sera are first subjected to routine gel electrophoresis (right half of the frame) with which an α_1-globulin band is obtained (first band to the right of the albumin) containing α_1-antitrypsin as well as other α_1-globulins. A section of the gel pattern is then removed, placed in a layer of agarose containing monospecific antiserum to α_1-antitrypsin and electrophoresed at right angles to the first direction (left frame, arrow). This allows identification of phenotypic variants as well as their quantitation. MM and FF are normal variants whereas ZZ is a homozygous deficient variant associated with low serum α_1-antitrypsin levels and familial pulmonary emphysema (Reproduced from Ref. *18* by permission of the publisher.)

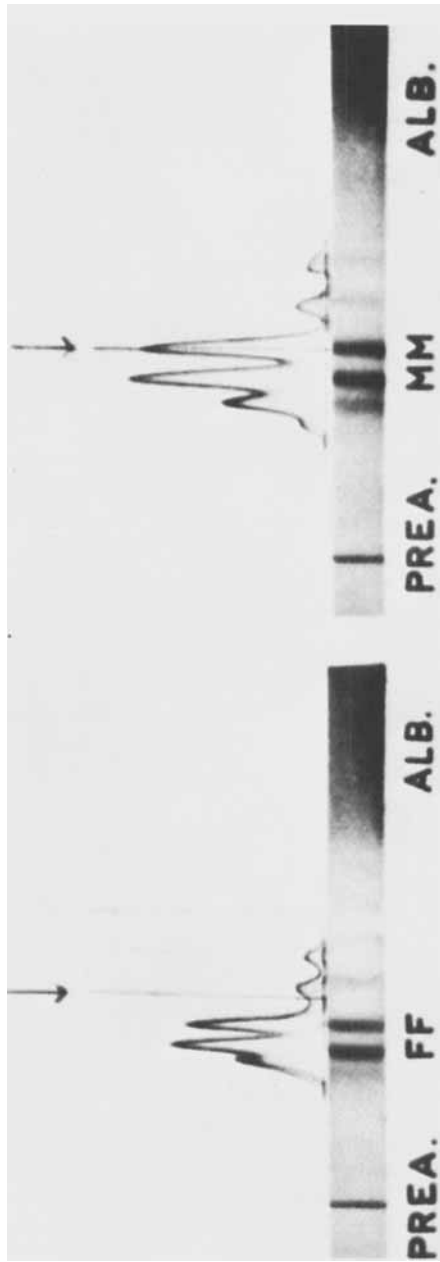


FIG. 9. Two-directional immunoelectrophoresis in the fractionation of α_1-antitrypsin variants using starch gel electrophoresis (discontinuous system using buffer at pH 4.95 in the gel and at pH 9.0 in the vessels). Note the improved resolution of two of the variants (MM, FF) shown in Fig. 8. (Reproduced from Ref. *18* by permission of the publisher.)

or α-1-antitrypsin. In Fig. 8, for example, which is taken from the work of Fagerhol and Laurell (*18*) illustrates genetic variants of α-1-antitrypsin, a normal serum protein component whose function appears to involve inactivation of trypsin and other proteolytic enzymes. To resolve these variants even further, these investigators fractionated the proteins first in a discontinuous starch–gel system at an acid pH, followed by crossed electrophoresis in the second direction in agarose gel, and this is

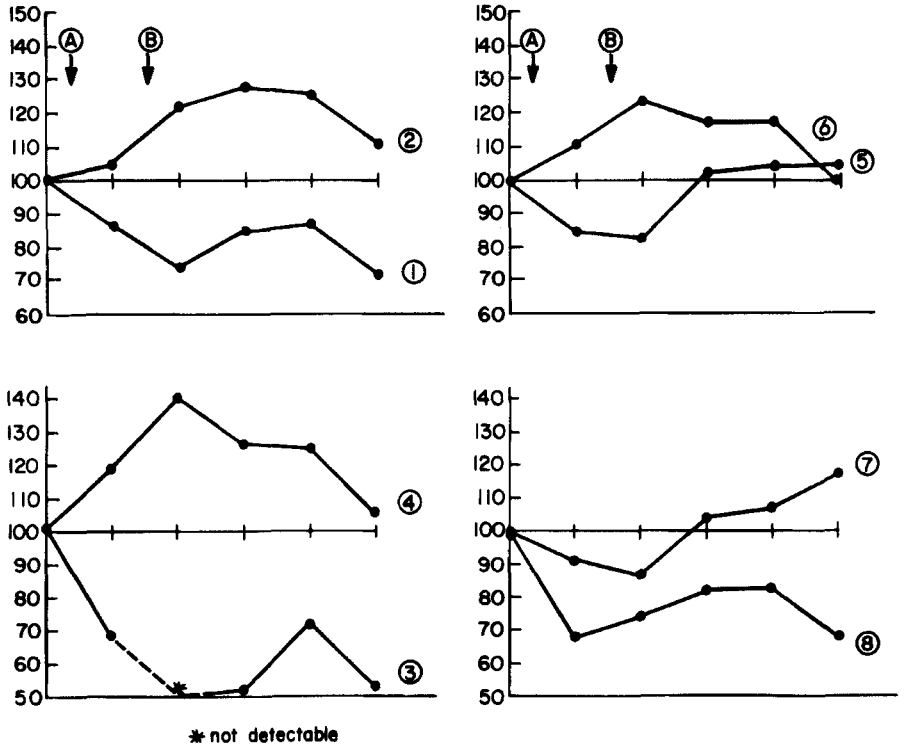


FIG. 10. Serial changes in various proteins in a single patient following major surgery and infection. All values are expressed as percent change from the presurgery level which is designated as 100%. The abscissa is an arbitrary time scale covering a 5-week period. *A*: Patient underwent hysterectomy for carcinoma of the uterus. *B*: Patient experienced the onset of fever subsequently found to be due to a kidney infection. Although 14 different proteins were measured simultaneously only 8 are shown in this graph. 1, albumin, 2, α_1-acid glycoprotein; 3, α_1-lipoprotein; 4, α_1-antitrypsin; 5, ceruloplasmin; 6, haptoglobin; 7, hemopexin; and 8, transferrin.

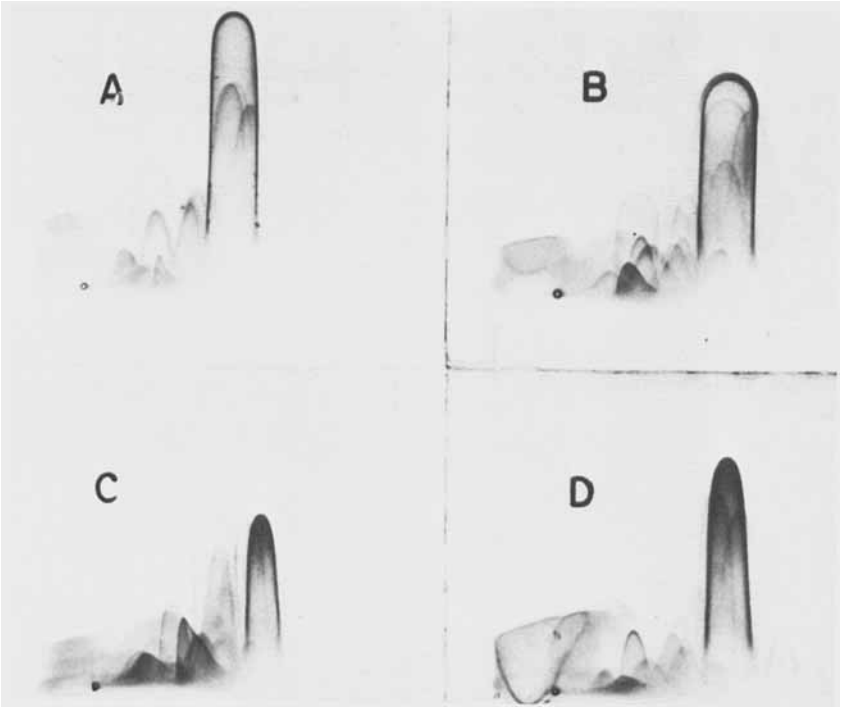


FIG. 11. Representative two-directional serum innumoelectrophoretic patterns in some diseases. *A*, Normal; *B*, acute viral hepatitis; *C*, nephrosis; and *D*, advanced alcoholic cirrhosis of the liver.

shown in Fig. 9 which is also taken from their work. The improvement in resolution is quite evident.

Sequential Changes in Disease

A number of recent reports have emphasized the usefulness and efficiency of this method for the measurement of sequential changes of serum proteins in a variety of diseases. Thus Clark and colleagues have quantitated 20 different proteins in normal adults (*26*) as well as in patients with multiple sclerosis (*27*), hemochromatosis (*28*), and certain other diseases (*29–31*) whereas Studd and colleagues compared protein changes simultaneously in parturient mother, fetus, and newborn (*23*), Abrams (*25*), on the other hand, measured the concentration of 11 dif-

ferent proteins in sera from normal children in a study of differences in protein concentration between the two sexes.

The application of two-directional immunoelectrophoresis in the study of sequential changes in specific protein fractions in serum following injury or inflammation has been particularly revealing (*24*), and Fig. 10 summarizes the data obtained in our laboratory in one patient undergoing major surgery and illustrates well the typical changes observed by other investigators as well. The sequential changes of only eight proteins are shown in this graph, and all values are expressed as a percentage change from the presurgery level. It becomes quite obvious that this technique could prove to be quite an elegant tool in the investigation of differences in response to the same stimulus among different individuals.

The full potential of the method can be applied to the reinvestigation of specific changes in many different protein fractions in a number of diseases previously studied with less sophisticated methods, and in Fig. 11 the protein changes in three patients with (a) acute viral hepatitis, (b) nephrotic syndrome, and (c) cirrhosis are compared with a normal control and illustrate quite nicely the improved resolution and specificity of this method over conventional methods in the study of certain proteins.

CONCLUDING COMMENTS

What is, then, the future potential of this method? At the moment it must still be considered a powerful analytical tool for research in a variety of problems in basic and clinical biochemistry of human proteins. The most costly item is the antiserum; however, modification of the system will allow further reduction in cost. It is, of course, an immunochemical method and as such is subject to all of the pitfalls of other immunochemical methods, the most important being the unavailability of high potency antisera producing multiple, sharply defined peaks. A large amount of data is generated, truly employing microquantities of sample, thus efficient instrumentation and methods will need to be developed to allow easy manipulation and meaningful interpretation of the data. Finally, it is quite conceivable that certain steps in the procedure can be partly or entirely mechanized so as to improve the efficiency of large-scale analyses. Thus, although we are at some distance away from the introduction of two-directional immunoelectrophoresis in the routine clinical laboratory, the gap is slowly closing.

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