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REVIEW

Isoelectric Focusing of Proteins in Gel Media

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Summary

Separation of proteins according to their isoelectric point can be performed in a pH gradient formed by stationary electrolysis of carrier ampholytes. The pH gradient is stabilized by the use of polyacrylamide, agarose, and Sephadex gels. Separated proteins can be detected by fixation with trichloroacetic acid followed by nonspecific staining, by specific staining, or through immunodiffusion techniques. Isoelectric focusing of proteins in gel media can be carried out in gel columns or on thin-layer plates by using conventional electrophoresis apparatus. Electrofocusing can be followed by electrophoresis in gel media for more complete separation of components.

Multiple samples of microgram quantities can be analyzed simultaneously by simple and rapid procedures. These methods have both analytical and preparative applications in protein fractionation work.

INTRODUCTION

Isoelectric focusing of proteins involves electrophoretic migration in a stable pH gradient with subsequent concentration of the amphoteric molecules in zones exhibiting pH values corresponding to their isoelectric points. Since the isoelectric point represents a pH value at which the protein has zero net charge, focusing is achieved by lack of electrophoretic migration at the particular pH. When a mixture of proteins having different isoelectric points is subjected to electrofocusing, the individual components are separated and form an isoelectric spectrum.

The theory of electrofocusing of proteins in stable pH gradients was

developed by Svensson (*1*, *2*). Practical application of the method was made possible by the synthesis of aliphatic polyamino-polycarboxylic acids which satisfied the criteria of good carrier ampholytes (*3*, *4*) necessary for the formation of the natural pH gradient. The problem of stabilization of the protein zones after electrofocusing was overcome by the use of sucrose density gradients (*5*). Experimental conditions, theoretical aspects of the technique, and application to the separation of proteins have been briefly reviewed by Haglund (*6*) and Vesterberg (*7*).

Although the isoelectric focusing method in columns containing sucrose gradients is suitable for preparative purposes, the technique cannot be used for analytical separation of microgram quantities of proteins. This is necessary when working with biological materials which cannot be obtained in large amounts. The need for such micromethods led to the development of new techniques involving the electrofocusing of proteins in gel media. Some of the advantages that have been claimed for gel electrofocusing procedures include (a) use of inert media for stabilization of the pH gradient, (b) analysis of microgram quantities of proteins, (c) employment of simple apparatus, (d) conservation of expensive carrier ampholytes, (e) resistance to convective mixing, (f) simultaneous separation of several mixtures, (g) employment of specific stains, (h) considerable shortening of the running time, (i) combination with electrophoresis in a two-dimensional fashion for increased resolution, and (j) visualization of separated proteins by use of immunodiffusion techniques.

This article is a review of the available gel electrofocusing techniques from the viewpoint of the media, procedures, and type of apparatus used rather than the biological importance of the separation achieved. It is hoped that this will provide a guide to original literature for reference when experimental details are needed.

DISC ELECTROFOCUSING

Disc electrofocusing methods were developed independently and almost simultaneously by Dale and Latner (*8*), Wrigley (*9*), Riley and Coleman (*10*), Fawcett (*11*), and Catsimpoolas (*12*). The common features of these methods are the use of polyacrylamide gel as the stabilizing medium, the employment of columns similar to those used for disc electrophoresis, and the appearance of the stained bands in the form of discs. Because of the similarity in appearance of the electrofocused protein bands with those obtained by disc electro-

phoresis, Catsimpoolas and Leuthner (*13*) proposed the term "disc electrofocusing" to describe this particular technique.

Apparatus and Choice of Carrier Ampholytes

The choice of apparatus, gel formulations, dimension of columns, electrode solutions, current applied and time of run, mode of sample insertion, and other experimental details varied among the reported procedures. Commercially available disc electrophoresis apparatus was used by Wrigley (*9*), Riley and Coleman (*10*), and Catsimpoolas (*12*) whereas Dale and Latner (*8*) and Fawcett (*11*) designed their own. The diameter of the columns used was approximately the same, but the length varied from short (65 mm) (*9, 10, 12*), to long (120 mm) (*8, 9*), to very long (150 to 200 mm) columns (*11*). Better resolution may be achieved with the long columns if the mixture of proteins to be separated is very complex. The pH range of carrier ampholytes can vary, depending on the degree of resolution desired and the isoelectric points of the proteins to be electrofocused. Usually carrier ampholytes in the pH 3–10 range are used in a screening run to determine the desired pH region of focusing. Subsequent experiments can then be carried out with ampholytes covering a range as narrow as two pH units. However, the narrow range ampholytes tend to produce broader bands in comparison to bands formed in the pH 3–10 range.

Polymerization of the Acrylamide Gel

Polymerization of the acrylamide gel can be achieved either by chemical means or by exposure to light. Formulations for both photopolymerization (*8–12*) and chemical polymerization (*14*) of the gels have been described. The essential components for the preparation of the gels include acrylamide, carrier ampholytes, *N,N'*-methylene-bisacrylamide, *N,N,N',N'*-tetramethyl ethylenediamine, riboflavin (photopolymerization only), and ammonium persulfate (chemical polymerization only). Riley and Coleman (*10*) suggested that *N,N,N',N'*-tetramethyl ethylenediamine is not required for polymerization, its function apparently being served by components in the carrier ampholytes. Chemical polymerization should be avoided if sensitivity of the proteins to ammonium persulfate leading to artifact formation (*15–17*) is suspected. However, if the pH 7–10 carrier ampholyte is used, chemical polymerization is necessary to form a gel. Photopolymerization can be used with the acidic range ampholytes including the pH 5–8 and pH 3–10 regions (*14*).

Acrylamide Concentration

The concentration of the acrylamide should be sufficient to obtain a self-supporting gel offering little resistance to the electrophoretic migration of proteins. A 5% polyacrylamide concentration is adequate for both purposes. A distinction should be made here between disc electrophoresis (*18, 19*) and disc electrofocusing. In the disc electrophoresis procedure the concentration of acrylamide regulates the pore size of the gel. By using different concentrations of acrylamide, a "sieving effect" can be achieved with proteins exhibiting different molecular size. Therefore their relative electrophoretic mobility is a function of both the electrical charge and molecular size. In disc electrofocusing, the "sieving effect" should be avoided since focusing depends only on the electrical charge of the protein. Concentrations of acrylamide above 5% can be used if the size of the proteins to be separated is such that no resistance to their migration is expected. Peacock and Dingman (*20*) have described a procedure for obtaining good gel strength at very low acrylamide concentrations by incorporating agarose in the formulation. Preliminary experiments (*10*) have shown the feasibility of using such gels for the separation of high molecular weight proteins by disc electrofocusing.

Sample Loading and Electrofocusing

The protein mixture to be electrofocused should be as free as possible of salts and inorganic buffers since their presence may cause variations in the position of bands from one run to another. However, relatively small contamination with salts can be tolerated. If the sample is available in the dry form, it can usually be dissolved in a small amount of water, or in the portion of carrier ampholyte solution used to prepare the gels. Generally, two modes of introduction of the protein sample into the gel have been described. These involve either direct addition to the gel formulation followed by photopolymerization of the gel-sample mixture (*9, 10, 12, 14*), or loading of the sample (in sucrose) on top of the gel and underneath a protective layer of ampholyte (*8, 11, 14*). The latter procedure should be used if exposure of the sample to the conditions of polymerization must be avoided. However, the gel-sample polymerization procedure is simpler and more rapid. The electrofocusing patterns resulting from either one of the loading procedures are identical. Different concentrations of urea can be incorporated in the gel if desired.

Solutions of phosphoric and sulfuric acids have been used as anolytes whereas solutions of ethylenediamine, ethanolamine, and tetramethyl ethylene diamine were employed as catholytes. The upper electrode is usually connected to the anode and the lower electrode to the cathode. The power supply should be capable of providing constant voltage since the current drops progressively during electrofocusing. Constant current power supplies are not suitable for disc electrofocusing. Current of 2–5 mA per column for a duration of 1–14 hr has been used. In the author's experience, a current of 5 mA per column for 1 hr is usually sufficient for complete electrofocusing.

Staining and Measurement of pH Gradient

An important step prior to staining the protein bands separated by disc electrofocusing is the removal of the carrier ampholytes which form insoluble complexes with a number of the common protein stains. This is accomplished by placing the gels in a beaker containing approximately 250 ml of 12% trichloroacetic acid and stirring with a magnetic stirrer, usually overnight. The trichloroacetic acid solution is replaced 5 to 6 times and is followed by a final rinsing of the gels in fresh trichloroacetic acid solution. The proteins can then be stained and destained by procedures similar to those used for disc electrophoresis (*19*). Some of the stains that have been used in disc electrofocusing include Coomassie Brilliant Blue, Amido Black, Ponceau S, and Lissamine Green SF. Riley and Coleman (*10*) have discussed the use of Light Green SF and Fast Green FCF for direct staining of gels. However, these procedures give poor color yield. Glycoproteins can be stained directly with periodic acid-Schiff reagent without removal of ampholytes (*21*). Similarly, the enzyme lipoxidase can be stained directly (*22*). Specific stains for lactate dehydrogenase activity (*8*) and acid phosphatase activity (*23*) have been described. It is conceivable that a number of other specific enzyme stains which have been successfully used in disc electrophoresis may also be used in disc electrofocusing perhaps without prior removal of ampholytes. Densitometric technique, detection of radioactive bands, and immunochemical analysis, all of which have found applications in disc electrophoresis, may also be applied to disc electrofocusing.

Determination of the pH gradient developed during electrofocusing can be performed by sectioning the gel column with the Canalco lateral gel slicer, or any other similar device, and eluting each section

in a small beaker with 1 ml of water for 4 hr. Care should be exercised to avoid the presence of CO_2 in the solutions to be measured. The pH gradient can also be determined directly from the gel column by the use of microelectrodes. Colored proteins and dyes of known isoelectric point can be utilized as pH markers for direct comparison with the electrofocused protein bands.

Applications

The disc electrofocusing method has been successfully applied to analytical separation work involving a wide variety of proteins. Some typical examples can be found in the separation of human serum proteins (*8*), water-soluble flour and gluten proteins (*9*), ovalbumin and plakalbumin (*9*, *14*), bovine serum albumin (*14*), hemoglobins and myoglobins (*10*, *11*), pH 4.5 soluble soybean proteins (*12*), lysate of human erythrocytes (*10*), soybean hemaglutinins (*21*), soybean lipoxidase (*22*), L-amino acid oxidase (*24*), soybean trypsin inhibitors (*25*), and human serum lipoproteins (*26*). An example of the analytical information that can be obtained from a mixture of proteins subjected to disc electrofocusing is shown in Fig. 1. The number of bands, their relative staining intensity, and the approximate isoelectric point of the separated components can be determined by using only microgram quantities of sample and a relatively simple and rapid procedure.

THIN-LAYER ELECTROFOCUSING

Essentially two variations of the thin-layer electrofocusing technique have been described employing either polyacrylamide gel or Sephadex as a stabilizing medium. The use of thin-layer plates of polyacrylamide gel as the electrofocusing matrix was introduced independently and almost at the same time by Awdeh et al. (*27*) and by Leaback and Rutter (*23*). Radola (*28*) used thin layers of Sephadex G-75 as an inert carrier for stabilization of the pH gradient. High resolution in the separation of proteins has been achieved with both methods. One of the advantages offered by the thin-layer electrofocusing method is that several samples can be compared simultaneously on the same plate. The maximum number of samples that can be compared directly on the same gel by disc electrofocusing is only two, provided double compartment glass tubes are used. Thus, thin-layer electrofocusing may be the method of choice when comparing electrofocusing patterns of samples with subtle variations in the position and intensity of bands.

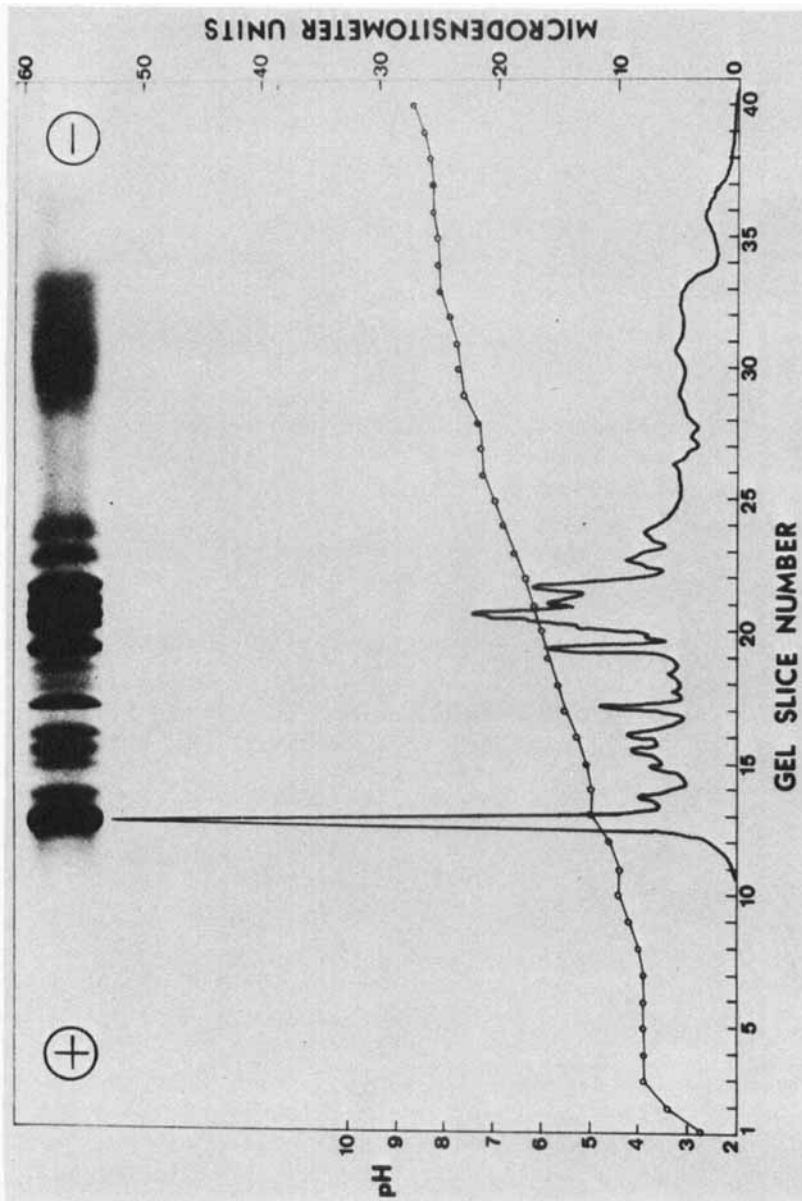

FIG. 1. Disc electrofocusing of soybean whey proteins in the pH range between 3 and 10. The densitometer tracing of the stained electrofocused bands was obtained with a Canaleo model F microdensitometer. Open circles represent the pH gradient along the polyacrylamide gel column after electrofocusing (*12*).

Polyacrylamide Gel

Many similarities exist between thin-layer polyacrylamide gel and disc electrofocusing. The mode of polymerization of the gel, the nature of electrolytes, the procedures for washing, staining, destaining, and pH measurement can be applied interchangeably. However, the apparatus used is constructed in an entirely different manner. Thin-layer electrofocusing is performed in a horizontal fashion whereas the disc electrofocusing technique requires a vertical arrangement.

Two types of apparatus have been devised for thin-layer electro-

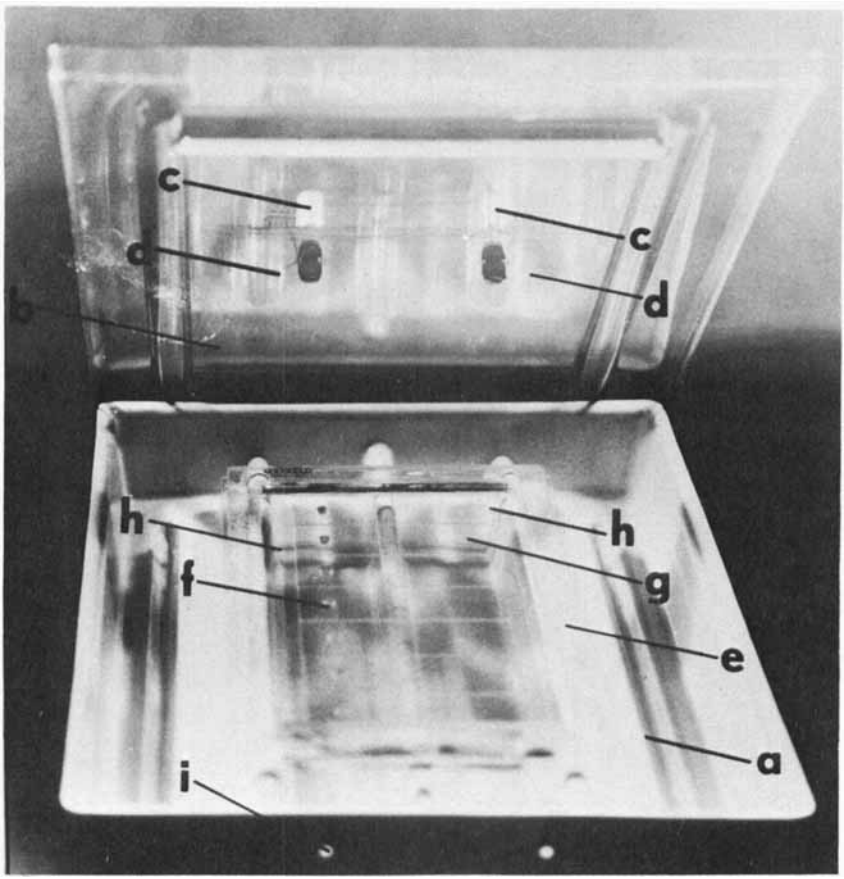

FIG. 2. Thin-layer polyacrylamide gel electrofocusing apparatus.

focusing. Leaback and Rutter (*23*) used a glass base to carry the gel slab and the electrode wells. A Perspex lid for the glass base has terminals connected to horizontal carbon or platinum electrodes which fit into the trenches. Awdeh et al. (*27*) formed the gel on a glass plate which was inverted and rested on two carbon electrodes spaced 20 cm apart. The author has devised an apparatus based on commercially available equipment (Fig. 2). The electrodes of a Gelman electrophoresis chamber were removed from position *i* and placed on top of the lid *b* at position *c* with the platinum wires *d* pointing downward. The holes *i* were closed with tape. Holes *f* were drilled at the bottom of a Shandon gel tray *e* for easy removal of microscope slides *g* which were accommodated in the tray. The tray *e* is rested on the shoulders of the electrophoresis chamber. Water is placed in the trough *a* to provide a moist atmosphere. During polymerization of the gel in the tray, glass rods are placed in position *h* to provide trenches for the electrolyte solutions. When the lid is closed, the platinum electrodes *d* make contact with the electrolyte at position *h*. A cooling plate can be placed underneath the gel tray if desired.

Thin-layer electrofocusing in polyacrylamide gels has been applied to the separation of immunoglobulins (*27*), hemoglobins and myoglobins (*23*, *27*), serum proteins (*23*), ribonuclease (*23*), erythrocyte phosphatase (*23*), and extracellular proteinase from Serratia marcescents (*29*). A pattern typical of the separations that can be achieved by this method is shown in Fig. 3. Rerunning of certain bands showed that the patterns observed were due to true molecular heterogeneity and not, for example, the complexing of a homogeneous protein with the carrier ampholytes (*23*).

Sephadex Gels

Thin-layer electrofocusing on Sephadex gels is permormed on plates 40×20 cm or 20×20 cm coated with a suspension of Sephadex G-75 (Pharmacia) in 1% carrier ampholyte solution (LKB) (*28*). The coated plates (0.75–1.0 mm thickness) are mounted horizontally on a metal cooling block. Contact with the electrode vessels, containing solutions of sulfuric acid and ethylene diamine, is made by Whatman 3MM paper strips shielded with a dialysis bag to prevent liquid flow from the electrode vessels to the gel. The protein sample is applied by means of cover slips as described for thin-layer gel filtration (*30*). Electrofocusing can be completed in 6–8 hr at 10–20 V/cm on the 20-

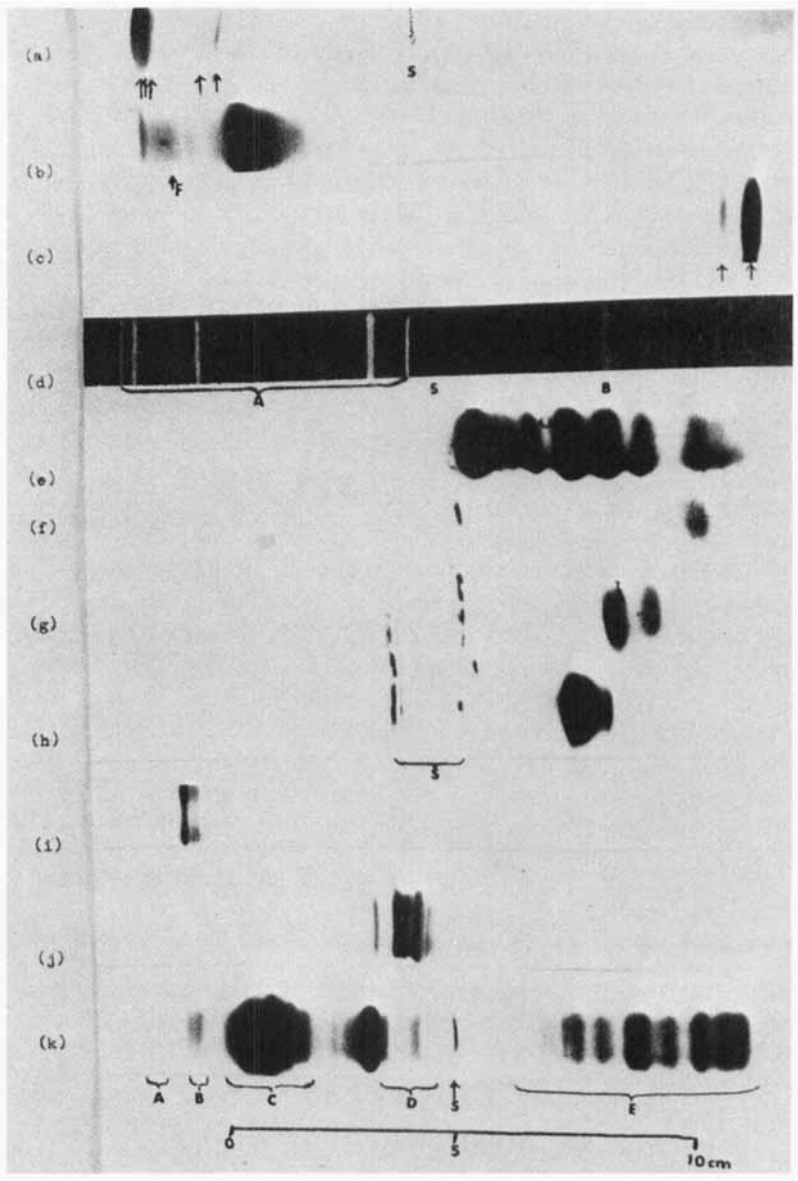

FIG. 3. Thin-layer polyacrylamide gel electrofocusing patterns. The portions of gels shown here correspond approximately to pH 3–10 (left to right) with (*a*) ovalbumin; (*b*) bovine serum albumin; (*c*) ribonuclease; (*d*) *drawing* of erythrocyte phosphatase activities (*A*) with

cm plates and in 18–24 hr at 5–10 V/cm on the 40-cm long plates. Protein bands are detected by taking a print from the surface of the gel with Whatmann 3MM paper, drying the paper, removing the ampholytes by extensive washing in sulfosalicylic acid or trichloroacetic acid, and staining with Coomassie Blue R25 or Lissamine Green. Densitometry can be performed in a Joyce-Loeble densitometer equipped with a thin-layer attachment. The pH gradient can be measured directly from the gel with microelectrodes. An example of the separation patterns obtained is shown in Fig. 4. So far, the method has been applied to the separation of myoglobins and hemoglobins, ovalbumin, bovine serum albumin, ferritin, and horseradish peroxidase (*28*).

ELECTROFOCUSING-ELECTROPHORESIS

This method involves the separation of a mixture of proteins by a combination of disc electrofocusing and electrophoresis. The proteins are first separated by disc electrofocusing and then are subjected to electrophoresis either on polyacrylamide gel slabs (*31–33*) or on agarose gels (*34*). A two-dimensional map of protein spots is obtained. The position of the spots is a function of both the isoelectric point and electrophoretic mobility of each protein in the particular electrophoresis buffer used. It should be recognized that this technique has great potentialities since the pH of the electrophoresis buffer can be varied, and one can take advantage of the "sieving effect" by varying the acrylamide concentrations of the gel.

Macko and Stegeman (*32*) used commercially available apparatus originally designed for disc electrophoresis and vertical flat gel electrophoresis. Dale and Latner (*31*) constructed an apparatus of their own design. Experimental details of the technique can be found in the above-mentioned works (*31*, *32*). In general, the factors involved in the separation of proteins by disc electrofocusing and polyacrylamide gel electrophoresis (*35*, *36*) are applicable to this method. Catsimpoolas (*34*) used agarose gel on microscope slides for the electrophoretic migration of the electrofocused proteins. This technique will be de-

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oxyhaemoglobin A indicated at position *B*; (*e*) human γ-globulins; (*f*) (*g*), and (*h*) fractions of (*e*); (*i*) human haptoglobins, (*j*) human transferrins; and (*k*) a "normal" human serum with prealbumin (*A*), haptoglobin (*B*), albumin (*C*), transferrin (*D*), and γ-globulin (*E*) zones. Sample slots are marked S (*23*).

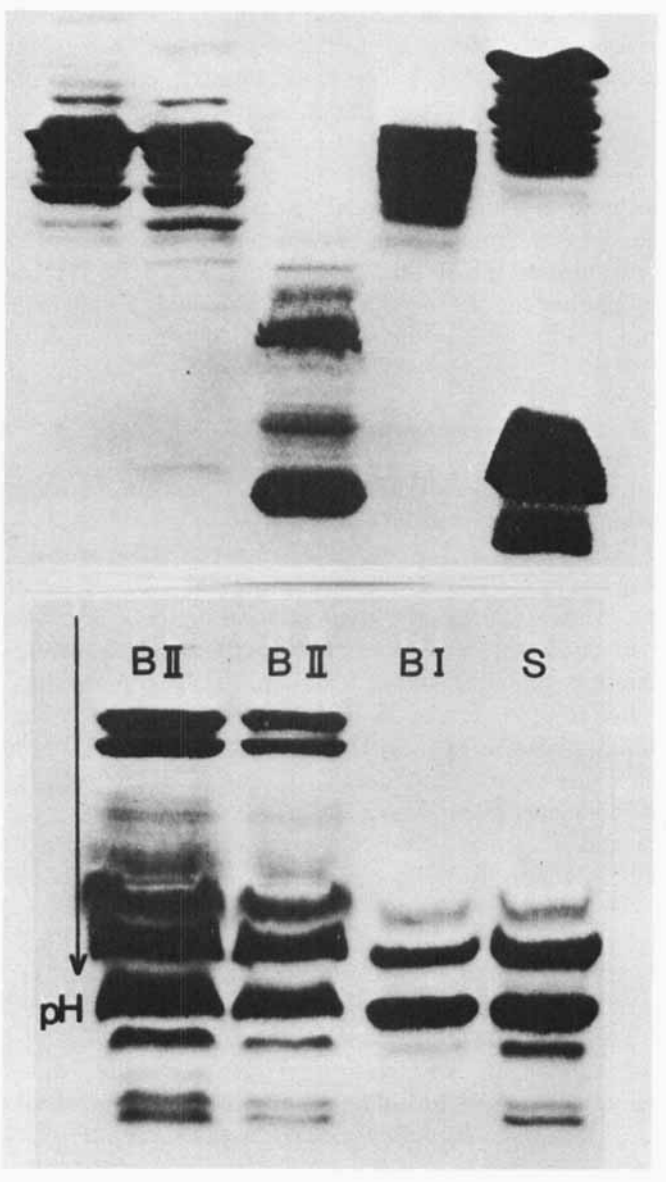

FIG. 4. (Upper): Thin-layer isoelectric focusing in a pH 3–10 ampholyte system, using Sephadex gel. From left to right: horse myoglobin (Koch and Light), horse myoglobin (Calbiochem), ovalbumin 1× crystallized (Serva), beef hemoglobin (Serva), and a mixture of (from top to bot-

scribed in more detail in the "Immunoelectrofocusing" section of this review.

Examples of the separation capabilities of the electrofocusing electrophoresis technique have been presented by Dale and Latner (*31*) using serum proteins, and Macko and Stegeman (*32*) in the analysis of potato tuber proteins (Fig. 5). Dale and Latner (*31*) were able to obtain characteristic "maps" of serum proteins showing changes in disease states. The importance of the potential clinical usefulness of this technique is apparent. As pointed out by Dale and Latner (*31*), one of the advantages of this method is that the biological fluid itself may be used to replace water in the ampholyte/acrylamide monomer recipe, thus eliminating the necessity of prior concentration.

PREPARATIVE GEL ELECTROFOCUSING

Although the various methods of isoelectric focusing of proteins in gel media have been used primarily for analytical purposes, preparative applications have been reported for thin-layer electrofocusing in polyacrylamide (*23*) and Sephadex gels (*28*). The preparative methods are simply scaled up procedures of the corresponding analytical techniques. The author has introduced a minor modification in the Prep-Disc apparatus manufactured by Canalco for possible application to preparative purposes. The flow slit disc is removed and the lower end of the upper column is covered with a square of Saran Wrap. The gel medium consists of 2 g acrylamide, 80 mg *N,N'*-methylenebisacrylamide, 20 ml of *N,N,N',N'*-tetramethylene diamine, 0.4 mg riboflavin, and 2 ml of the 40% stock ampholyte solution (LKB), made to 40 ml with water. The gel is photopolymerized in the column

tom) sperm whale myoglobin (Koch and Light), bovine serum albumin (Behringwerke), and horse spleen ferritin (Koch and Light). All proteins were used as 5% solutions, in mixture the concentration of each was 5%; 30 μl of the protein solutions were applied with a cover slip at the middle of the plate. Plate: 40 × 20 cm; layer, 0.75 mm. Focusing at 5–6 V/cm for 18 hr at 4–6°. Staining with Coomassie Blue (*28*). (Lower): Thin-layer isoelectric focusing of commercial horseradish peroxidase in a pH 3–10 ampholyte system. From left to right: B II (applied at two different amounts) and B I, peroxidase (Boehringer) with absorbance ratios of 0.6 and 2.8, respectively; S, peroxidase (Serva) absorbance ratio 2.6. About 100–200 μg of the preparations were applied. Plate: 20 × 20 cm, 0.75 mm layer. Focusing at 10 V/cm for 7 hr at 4–6°. Enzyme activity was detected by urea-peroxide and *o*-toluidine (*50*).

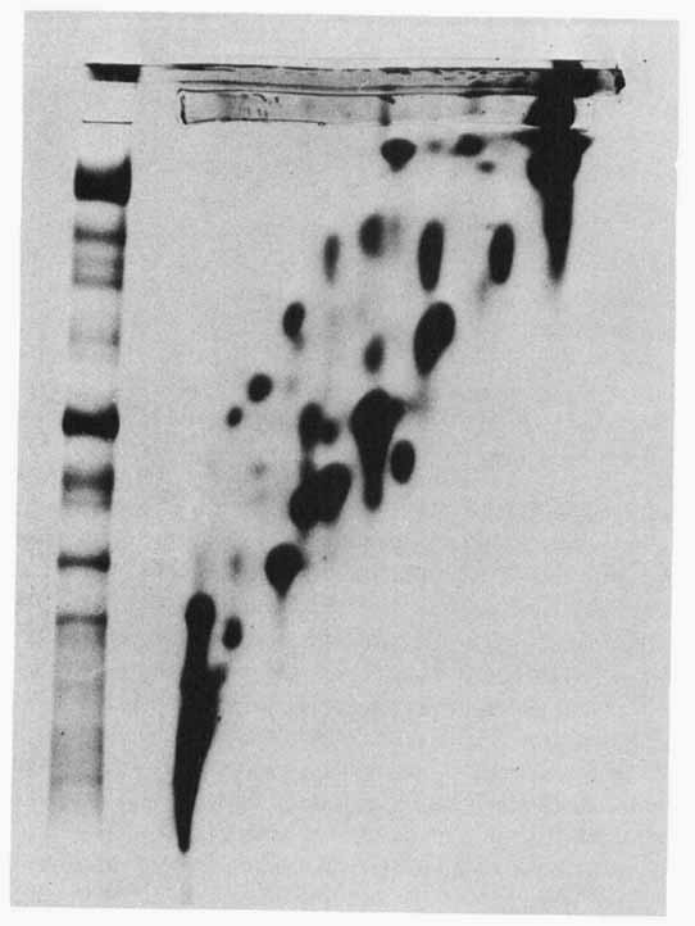

FIG. 5. Electrofocusing-electrophoresis pattern of proteins from potato tuber, variety Voran. The main part of the picture shows the "map" of protein spots obtained after polyacrylamide electrophoresis (pH 8.9. buffer 15.125 g Tris and 1.15 g boric acid per liter) of the electrofocused proteins (ampholine pH 5–7). The empty gel (barely visible on the picture), which has served as the starting line for the electrophoresis, is shown below the strip; it demonstrates that nearly all of the proteins entered the slab. On the left: the electrophoretic pattern run simultaneously (*32*).

filled to a height of approximately 18 cm. After polymerization the Saran Wrap is removed, and the column is placed in the top orifice of the basic apparatus where it automatically rests on the tapered shoulder. The protein sample in sucrose is loaded on top of the column

under a protective layer of ampholyte (*14*). The upper electrolyte reservoir is filled with 5% phosphoric acid solution, and the lower with 5% ethylenediamine. Electrofocusing is performed at 40 V for 24 hr using a constant voltage power supply. After electrofocusing, proteins are eluted by slicing the hollow gel cylinder at positions indicated by a marker longitudinal strip (3 mm wide) cut off the hollow gel and placed in 12% trichloroacetic acid solution. Excellent preparations have been obtained in this laboratory using this method.

IMMUNOELECTROFOCUSING

Immunochemical techniques, which the author has described by the general term "immunoelectrofocusing," were developed by Riley and Coleman (*10*) and Catsimpoolas (*34, 37–40*). In general, these methods involve electrofocusing of proteins in polyacrylamide or agarose gels followed by immunodiffusion or immunoelectrophoresis.

Polyacrylamide Gels

Three variations of the immunoelectrofocusing technique in polyacrylamide gels have been described. The disc immunoelectrofocusing method (*37*) involves isoelectric focusing of proteins in polyacrylamide gel columns followed by embedding of the intact column in pH 8.8 agar gel. Trenches cut parallel to the column are filled with appropriate antisera and immunodiffusion is allowed to proceed with consequent formation of immunoprecipitin arcs (Fig. 6). The technique is useful when an over-all immunodiffusion pattern of a mixture of antigenic components is desirable. All of the experimental conditions described for disc electrofocusing are applicable to this method. However, washing of the gel columns with trichloroacetic acid to remove the ampholytes prior to diffusion is not necessary. A buffered agar medium should be used for immunodiffusion in order to provide an environment suitable for antigen-antibody reactions.

In the "sectional immunoelectrofocusing" technique (*38*), the electrofocused gel column is sliced into approximately 40 sections using the Canalco lateral gel slicer. Each section is fragmented by extrusion through a microglass syringe and placed in sample wells punched in buffered agar gel plate. The antigenic components are eluted by addition of buffer in the wells and diffuse toward appropriate antisera placed in trenches parallel to the row of the sample wells. The arrangement of sample wells and antiserum trenches is shown in Fig. 7. Some of the advantages of this method are the appropriate esti-

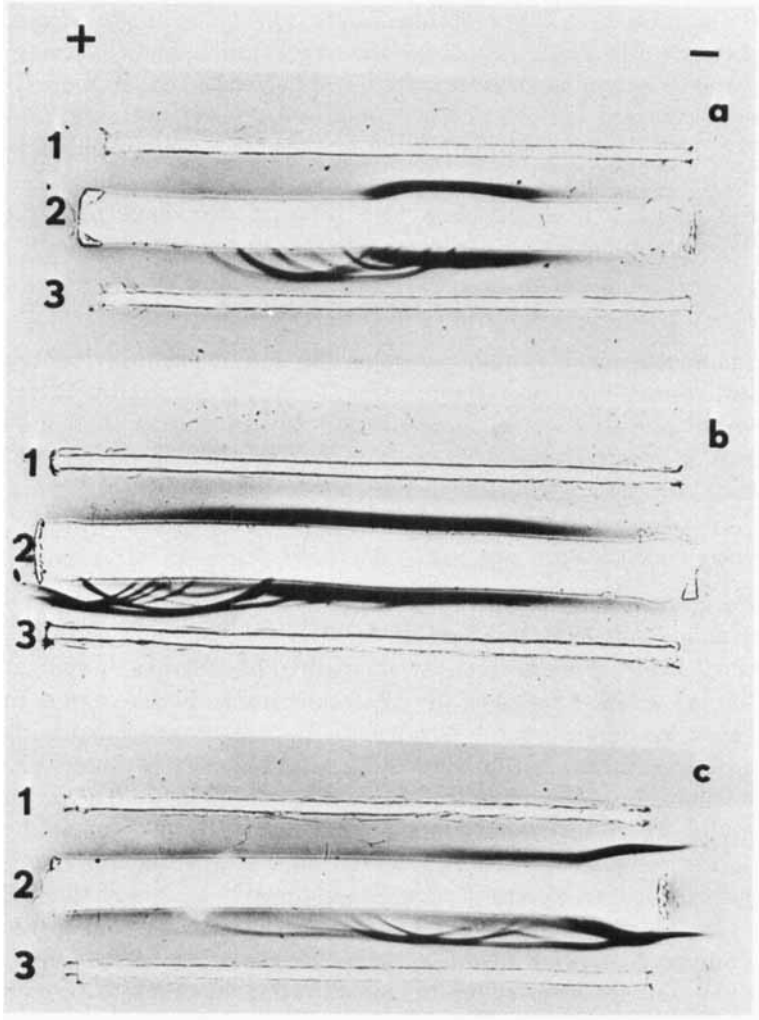

FIG. 6. Disc immunoelectrofocusing of rabbit serum proteins in the pH regions between pH 3 and 10 (*a*), pH 5 and 8 (*b*), and pH 3 and 6 (*c*). 1, Goat antiserum to rabbit γ-globulin; 2, electrofocusing column; 3, goat antiserum to rabbit serum (*37*).

mation of the isoelectric point of the sample placed in each well and the recognition of immunochemical identity or nonidentity reactions between samples in adjacent wells by loop or spur formation of the arcs (*41*).

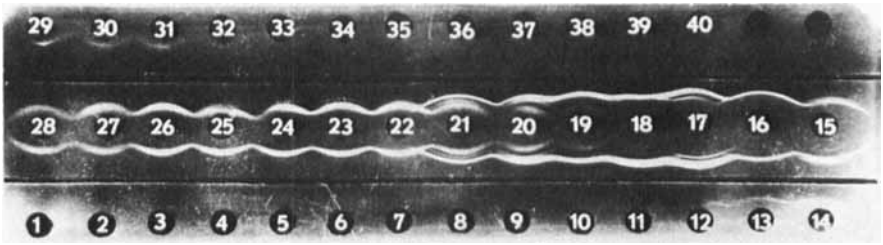

FIG. 7. Sectional immunoelectrofocusing of rabbit serum proteins electrofocused in the pH region between pH 3 and 10 and developed with goat antirabbit serum.

A useful extension of the "sectional immunoelectrofocusing" technique involves immunoelectrophoresis (*42, 43*) of the sections obtained from a disc electrofocusing gel column (*34*). The additional separation afforded by using immunoelectrophoresis after electrofocusing is of definite advantage in the examination of a complex mixture of antigenic proteins. As an example, serum proteins can be separated into simple groups of antigenic components by the combined electrofocusing-electrophoresis method which cannot be achieved by either technique alone (Fig. 8). This has also been demonstrated by Dale and Latner (*31*) in their combined two-dimensional electrofocusing-electrophoresis procedure.

Agarose Gels

Immunoelectrofocusing in agarose gels (*10, 39*) technically resembles immunoelectrophoresis (*42, 43*) in every respect except for the replacement of the electrophoresis buffer with carrier ampholytes and the corresponding electrode electrolytes such as phosphoric acid and ethylenediamine solutions. The principles of the separations afforded by the two methods are, however, different. An important aspect of the technique is the neutralization of the pH gradient developed in the agarose by brief immersion in buffers which will allow the antigen-antibody reaction to occur between pH 6.5 and 8.0 (*44*). Riley (*45*) has pointed out that the immunoelectrofocusing patterns of serum proteins described previously (*40*) may show more precipitin lines especially at the anodic and cathodic ends if buffers of higher concentration are used (e.g., 1.0 *M*, pH 7.0 phosphate buffer) for immunodiffusion. Immunoprecipitin lines of human serum developed with horse antihuman serum are shown in Fig. 9, as an example of the

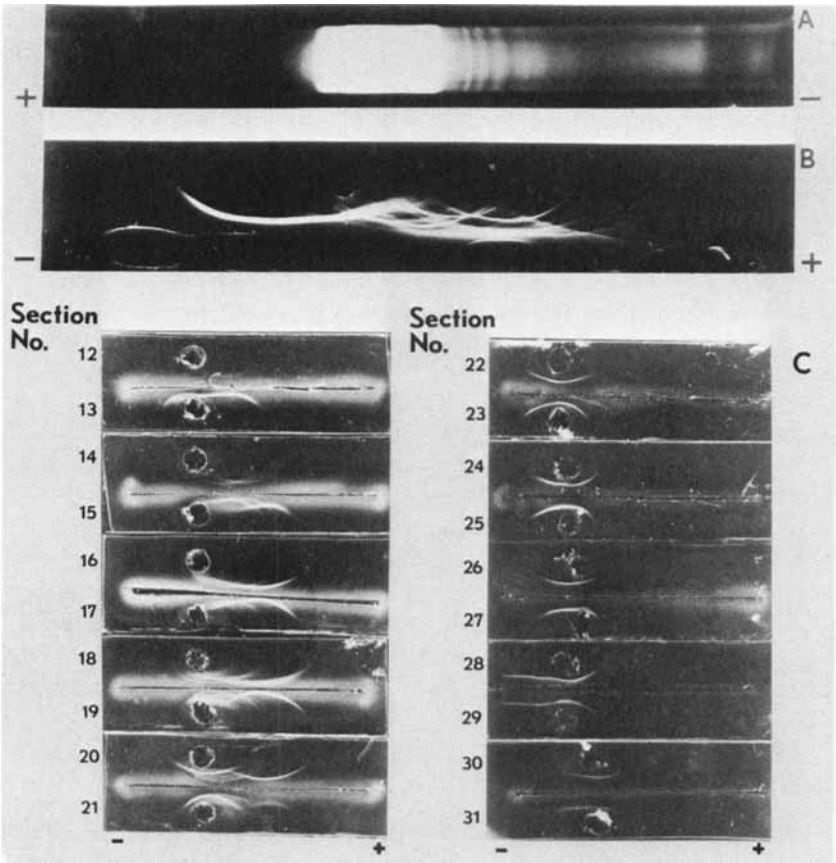

FIG. 8. Immunoelectrofocusing-electrophoresis patterns developed with goat antiserum to rabbit serum (*C*). The polyacrylamide gel column (*A*) shows the disc electrofocusing pattern (pH 3–10) of rabbit serum proteins. The immunoelectrophoresis pattern (*B*) was obtained from total rabbit serum proteins developed with goat antiserum to rabbit serum (*34*).

separations that can be achieved with this method. The agarose gel technique is more useful in pattern comparison rather than in the estimation of isoelectric points of separated components.

With the increased application of the immunelectrofocusing technique it is hoped that new methods and improvements in existing methods will be developed. Recently, Carrel et al. (*46*) introduced a new variation of the immunoelectrofocusing method which allowed

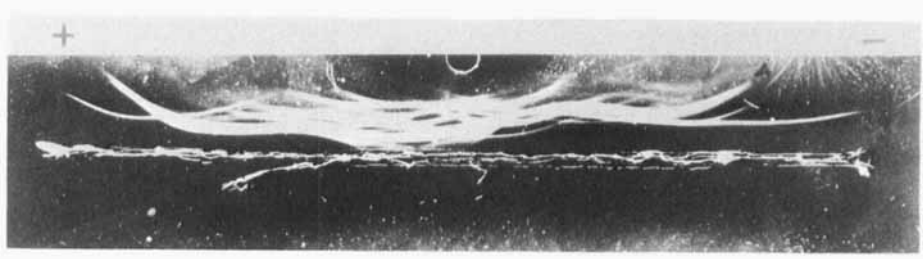

FIG. 9. Immunoelectrofocusing of human serum in pH 3 to 10 Ampholine in 1.5% agarose. Electrolysis was for 5 hr initially at 15 V and 1.2 mA. Visualization of precipitinogens was carried out by diffusion against horse antihuman serum (Hyland) following 4 min immersion of the slide in 1.0 *M* PO_4 buffer, pH 7.0 (*10*).

the immunoprecipitin bands to be formed directly on electrofocused polyacrylamide gel plates or in disc electrofocusing columns.

GENERAL CONSIDERATIONS

It seems reasonable to assume that the simplicity and high resolution of the gel electrofocusing method which offers definite advantages in the separation of proteins will eventually lead to its widespread use.

Important future developments may involve the elucidation of the nature of the heterogeneity observed with certain proteins which appear to be homogeneous by commonly accepted criteria. Some of the heterogeneity may be due to labile amide groups, conformational variations, subunit composition, and modification of the proteins during isolation or separation. These aspects of heterogeneity have been briefly discussed by Carlström and Vesterberg (*47*), Flatmark and Vesterberg (*48*), and Susor et al. (*49*). It is imperative that multiple molecular forms of a given protein, demonstratable by gel electrofocusing, should be further analyzed in order to rule out artifact formation.

The added dimension of isoelectric separation to existing analytical methods utilizing gel media may contribute significantly to our understanding of the intriguing complexities of protein structure.

ADDENDUM

A new method for staining proteins in the presence of carrier ampholytes has been described (*51*). The disc electrofocusing technique has been applied to the analysis of ferritin (*52*), antibodies of

restricted heterogeneity (*53*), carcinogenic hydrocarbon binding soluble protein from mouse skin (*54*), insulin (*55*), purified protein derivatives (PPD) (*56*), cathepsin D (*57*), purified antitype VIII pneumococcal polysaccharide antibodies (*58*), and human serum cholinesterase (*59*). Suggestions for specified conditions of pH placing of sample so as to avoid artifacts have been offered (*60*). Thin layer electrofocusing combined with radioautography has been described for the analysis of myeloma proteins (*61*), and in combination with immunodiffusion in the separation of *trypanosoma brucei* subgroup antigens (*62*). A two-dimensional technique combining disc electrofocusing and gradient gel electrophoresis was reported (*63*). An immunological method for identifying protein areas after electrofocusing–electrophoresis has been developed (*64*). The principle of Laurell's antigen–antibody crossed electrophoresis (*65*) has been utilized for identifying human urinary proteins after disc electrofocusing (*66*). It has been suggested that the frequently used terms "electrofocusing" and "immunoelectrofocusing" should be replaced by the more appropriate terms of "isoelectrofocusing" and "immuno-isoelectrofocusing" (*67*). The author of this review also suggests that the latter terms are adopted for easy subject indexing and uniform terminology.

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