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ISOELECTRIC FOCUSING IN GELS

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CONTENTS

I. INTRODUCTION

The development of the technique of isoelectric focusing (IEF) represents a major advance in the field of high-resolution separations of proteins and other amphoteric macromolecules. IEF is an equilibrium method in which amphoteric molecules are segregated according to their isoelectric points (p*I*) in pH gradients. The pH gradients are formed by electrolysis of amphoteric buffer substances known as carrier ampholytes. When introduced into this system, other amphoteric molecules such as proteins will migrate to pH zones that correspond to their respective p*I*s where their net charge is zero. By counteracting back-diffusion with an appropriate electrical field, the separated molecules can be concentrated into extremely sharp bands. The technique has now been refined to a level that permits the resolution of molecules whose p*I*s differ by as little as 0.005 pH unit or less. This degree of resolution cannot normally be obtained by conventional electrophoretic or chromatographic procedures. In these latter procedures, specially adjusted conditions have to be devised for particular separations, while in contrast, IEF, by virtue of being an equilibrium method, has a "built-in" resolution which usually allows one to separate in only one or two experiments all components with measurably different p*I* values. Further, because it is an equilibrium method, the system is self-correcting and therefore considerably less demanding in terms of experimental technique. IEF is particularly suitable for differentiating closely related molecules and provides a valuable criterion of homogeneity.

This review deals primarily with analytical and preparative procedures for IEF in anticonvective media. Particular emphasis is given to techniques for IEF in polyacrylamide gel, as this medium provides the closest approach to the high resolving potential of IEF and also offers considerable experimental flexibility. After a brief description of the development of gel electrofocusing, instrumental and practical aspects are discussed. These sections are followed by a review of some applications of the technique in biomedical research in order to illustrate its many advantages and a few of its limitations.

A. Background

The practical development of IEF can be traced to the pioneering work in 1912 of Ikeda and Suzuki[1], who found that a mixture of amino acids would assume an order during electrolysis that followed the ascending p*I* values between the anode and the cathode. This ordering resulted in the formation of a pH gradient between the electrodes. Williams and Waterman[2] refined the technique by designing a multichambered apparatus in order to reduce convective disturbances. The application of this system was limited, however, because of the variability of the field strength between the electrodes.

The technique of IEF received a major impetus in 1954–56 through the work of Kolin[3,4], who devised a series of pH gradients using diffusion of buffers in sucrose density gradients under an electric field. In these "artificial" pH gradients, Kolin was able to obtain "isoelectric line spectra" in a few minutes, a rapidity that is still unmatched in the field of electrophoresis. Unfortunately, these gradients were unstable because of the rapid migration of the buffering electrolytes during electrolysis[5] and the recovery of separated components proved difficult.

The concept of IEF was further extended in the 1960s by Svensson (now called Rilbe). In a series of theoretical articles entitled "Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients"[6-8], Svensson laid the foundation of IEF in its present form. Svensson advanced the idea of developing a "natural" pH gradient from amphoteric molecules with high conductances and closely spaced p*I*s. Under an electric field, these ampholytes would distribute according to their p*I* values to form a pH gradient in which the pH increased monotonically in the direction of the current. Svensson's concepts were soon achieved in practice with the synthesis by Vesterberg[9] of carrier ampholytes with many of the properties prescribed by Svensson. With these ampholytes, it became possible to develop smooth and stable pH gradients between pH 3 and 10, a range which encompasses the p*I* values of most proteins and many other amphoteric macromolecules.

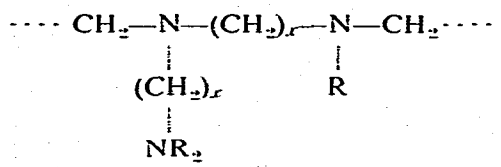
These ampholytes have now been available for about 5 years and have proved useful for both preparative and routine analytical procedures. Initially, IEF with Vesterberg's carrier ampholytes was conducted in sucrose density gradients as an anticonvective medium and was used primarily for preparative purposes (see reviews by Haglund[10] and Vesterberg[11,12]). However, the system was soon adapted for small-scale analytical procedures. In addition to reductions in the scale of sucrose density gradient techniques, other media such as paper, cellulose acetate and gels were investigated. Among the most promising adaptations was the use of a gel as an anticonvective medium. IEF in gels or gel electrofocusing (GEF) overcame many of the problems associated with convective mixing, diffusion and isoelectric precipitation in liquid media. In addition, GEF offered considerable savings in time and materials and could readily be adapted both for routine small-scale analyses of multiple samples and for preparative procedures.

Systems for GEF are now as convenient and adaptable as gel electrophoresis. Because of its remarkable resolution and sensitivity, GEF may soon become the method of choice for charge separations of proteins, particularly for analytical purposes. Early developments in GEF have been reviewed by Catsimpoolas[13,14], Williamson[15] and Wrigley[16]. Since last reviewed, more than 1000 papers dealing with this technique and its applications have been published (see "Acta Ampholinae", issued by LKB, Stockholm, Sweden). Two international conferences have been held, the proceedings of the first (New York, 1972) being published as a volume of the *Annals of the New York Academy of Science*[17]. Proceedings of a meeting in Glasgow (1973) will soon be published[18].

B. Properties of Ampholines

Some of the properties required of useful ampholytes, as envisaged by Svensson[6,7], can be summarized as follows: (1) good buffering capacity at the isoelectric point; (2) good conductivity at the isoelectric point; (3) low molecular weight; (4) good solubility in water at the isoelectric point and hydrophilic character; (5) low light absorption above 260 nm; (6) chemical properties different from the proteins to be separated. Substances with many of these properties have been developed by Vesterberg[9,19], who synthesized a series of isomers and homologues of polyamino polycarboxylic acids by the reaction of different proportions of acrylic acid with a variety of polyethylene polyamines. By this means, a large number of carrier ampho-

lytes with closely spaced pK and pI values in the pH range 3–10 were obtained. The general formula for these substances is



where $x = 2$ or 3 and R = H or $—(CH_2)_x—COOH$.

Ampholytes prepared in this way are available from LKB, Stockholm, Sweden, under the trade name "Ampholine". The wide-range pH 3–10 Ampholines have been fractionated and are available in several narrow ranges of 0.5 to 3 pH units. It should be noted that some pH ranges, notably those at each end of the overall range, may have been supplemented with amino acids such as glutamic acid and lysine in order to extend the pH range of Vesterberg's ampholytes. Whether other amino acids are present is not known. Although such additions might improve the quality of pH gradients, they might seriously complicate amino acid analyses of separated proteins.

These carrier ampholytes have been used successfully for many purposes, but they do not meet all of Svensson's criteria. Thus, even though their chemical structure is completely different from that of proteins, they nevertheless react with ninhydrin, biuret and Folin's reagent[20]. They also chelate with metal ions (especially divalent ions such as Mg^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}) and form insoluble complexes with commonly used protein stains[14].

Ampholines in different pH ranges also differ in their physico-chemical properties. Fig. 1 shows the conductance, pH gradient and UV absorbance profiles of three Ampholine pH ranges. In general, good conductance is obtained in most pH ranges, with the exception of the pH range 5–8, where the conductance is uneven. This pH range is, therefore, more sensitive to convective mixing caused by Joule's heating. As expected, the buffering capacity of the ampholytes follows their conductivity profile[10].

The size distribution of most Ampholines is in the range 300–1000 daltons, with a median of 600 daltons[10]. Studies with ^{14}C-labelled Ampholines, however, indicate that about 1% may be as large as 1000–5000 daltons[21]. Gasparic and Rosengreen[21] synthesized ampholytes of lower molecular weight, with a mean distribution of about 300 daltons, only 0.2% being above 1000 daltons. These are also available from LKB and should prove useful for fractionating oligopeptides and other substances of low molecular weight.

Most pH ranges of the Ampholines have a low absorbance between 280 and 430 nm, although each exhibits characteristic spectra with peak absorbances at 285, 310, 340, 350, 365 and 430 nm (ref. 22). Most ranges also have characteristic UV spectra (Fig. 2), owing to differences in UV absorbance of component ampholytes. The pH range 3–5 shows a particularly strong UV spectrum between 280 and 360 nm, and is also distinctly yellow in colour compared with other pH ranges. The absorbance of most chromophores varies with pH (ref. 22). Such differences in UV absorbance, when taken together with the uneven distribution of ampholytes along the pH gradient, can create considerable problems in quantitating focused proteins by UV densitometry[23].

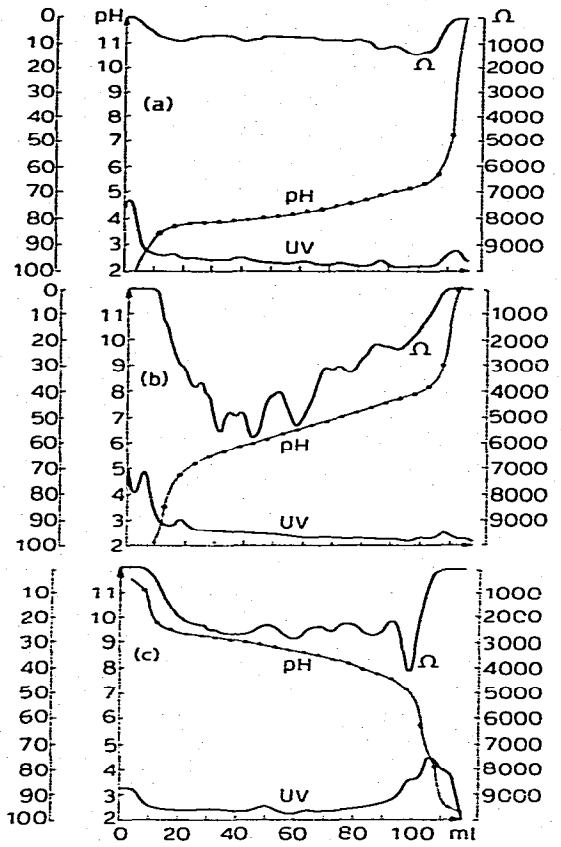


Fig. 1. Conductance, pH gradient and UV absorbance profiles of three Ampholine pH ranges. The conductance course of the pH gradients pH 3–6 (a) and 7–10 (c) are very even. The pH gradient 5–8 (b) contains low-conductivity ampholytes around pH 7 and shows a more uneven conductance course. This gradient is consequently more sensitive to thermal convection caused by Joule's heating in the low-conductance region. Generally, the conductance course is more even when shorter pH regions are used. (By permission of Pergamon Press. See ref. 20.)

Together with a characteristic UV spectrum, most Ampholine ranges also exhibit a typical fluorescence emission spectrum. Fig. 3 shows the pH dependence of the spectrum obtained by excitation at 310 nm of a 4% Ampholine solution of pH range 3–5. There is a striking correlation between the UV/visible and the fluorescence spectra. As the intensity of the 310-nm peak decreases with increasing pH, quenching in the fluorescence emission spectrum increases markedly[22]. These spectra indicate the presence of heterocyclic nitrogen structures, which are probably part of the population of amphoteric molecules within each pH range as their removal by charcoal substantially alters the pH gradient formed during IEF. The fact that the UV and fluorescent chromophores are particularly strong in the pH range 3–5 suggests that their formation is favoured at low pH.

Until recently, the only source of suitable carrier ampholytes was the expensive commercial variety produced by LKB. However, Vinogradov *et al.*[21] described a

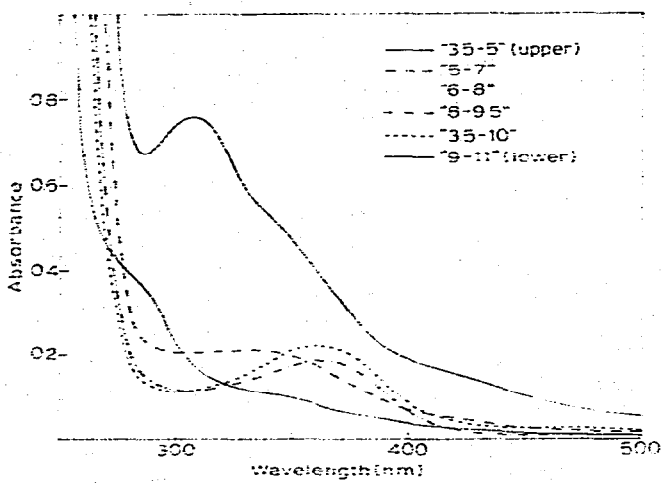


Fig. 2. UV/visible spectra of different Ampholine pH ranges. ——— (upper solid curve), pH 3.5–5 range (actual pH 4.31); –·–·–·–, pH 5–7 range (actual pH, 5.92); ·····, pH 6–8 range (actual pH, 6.89); – · – · – · –, pH 8–9.5 range (actual pH, 8.96); ———, pH 3.5–10 range (actual pH, 7.05); ———, (lower solid curve), pH 8–11 range (actual pH, 9.58). All spectra taken with a Jasco UV ORD 5 spectropolarimeter using a 4% Ampholine solution and 3-cm light path cuvette. (By permission of Academic Press. See ref. 22.)

synthesis of carrier ampholytes that seems practical for most laboratories. Acrylic acid (Aldrich, Milwaukee, Wisc., U.S.A.) and pentaethylenehexamine (PEHA) (Union Carbide, New York, N.Y., U.S.A.) are distilled under vacuum, then acrylic acid is added dropwise under nitrogen to a stirred solution of 0.15 mole of PEHA in

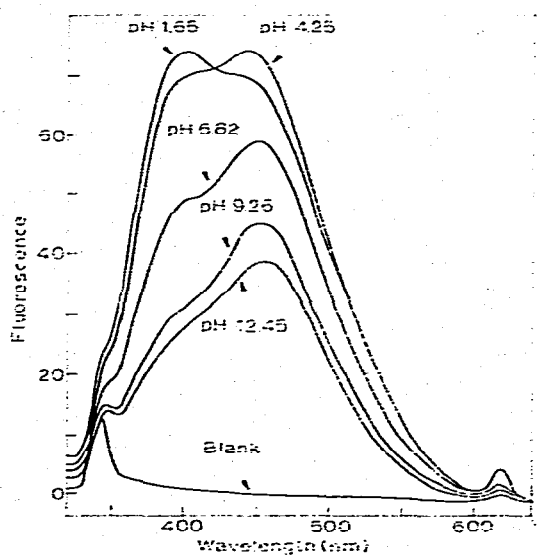


Fig. 3. Fluorescence emission spectrum of a 4% Ampholine solution, pH 3–5 range. Excitation wavelength, 310 nm (7 nm band width); emission band width, 8.9 nm; recorder sensitivity, 5; chart speed, low; scan speed, medium; filter, 290 nm. The spectra were recorded with a Perkin-Elmer MPF-2A spectrofluorimeter fitted with a Hitachi recorder. (By permission of Academic Press. See ref. 22.)

35 ml of water during 60 min so as to provide the desired nitrogen:carboxyl ratio. The reaction mixture is adjusted to 70° and stirred overnight then, after cooling to room temperature, distilled water is added to make a 40% (w/v) solution. The most satisfactory ampholytes are obtained with nitrogen:carboxyl ratios of about 2:1. These ampholytes have satisfactory buffering and conductance properties in the pH range 4–8 and give good resolution in gel electrofocusing[24]. Their cost is much less than that of the commercial variety.

C. Detection of Ampholines

The number of different amphoteric species within each pH range of the Ampholines is not known exactly. For some time, it was thought[25] that there might be several hundreds in the range between pH 3 and 10, but direct analysis indicated that considerably fewer were present. Focused Ampholines can be detected by several methods. Those displayed in the pH range 3–10 by the Ampholine–glucose caramel technique of Felgenhauer and Pak[26] are shown in Fig. 4. Rilbe[27] demonstrated striations caused by strong refractive index gradients of focused Ampholines in a quartz cell (Fig. 5). Focused Ampholines can also be observed by precipitation with heavy metals in acidic solutions, *e.g.*, 2% potassium hexacyanoferrate(II) in 5% trichloroacetic acid. This complex can be dissolved by the addition of 5% hydrochloric acid (P. G. Righetti and J. W. Drysdale, unpublished work). It would appear from such results that there may be only about 50 ampholyte species between pH 3 and 10 and perhaps fewer than 20 in most the narrow ranges of two pH units. It is also apparent that the various ampholytes are present in vastly different amounts and are not uniformly distributed throughout the pH range. Vinogradov *et al.*[24] distinguished approximately 30 different species with nine major components in their ampholyte preparations in the pH range 4–8.

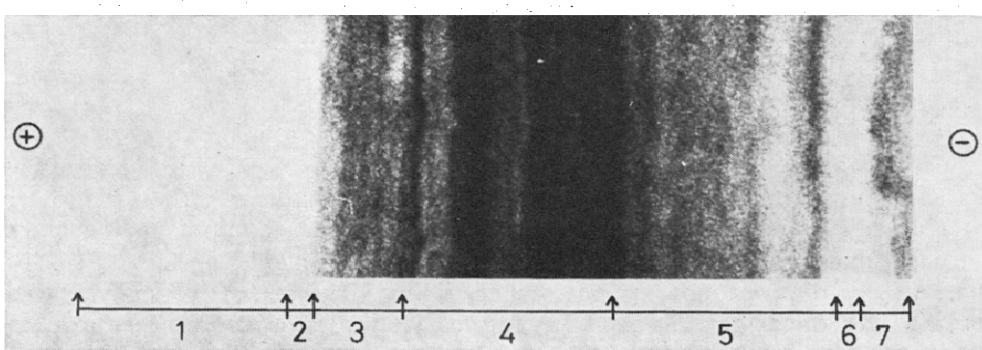


Fig. 4. Band pattern of Ampholine (pH 3–10) after Sephadex thin-layer focusing. For detection, a sheet of filter paper soaked in 5% glucose was rolled over the thin layer. After heating for 8 min at 110°, the Ampholine pattern has developed and can be evaluated by its fluorescence at 350 nm or in daylight. The following zones can be distinguished: (1) broad, faintly stained zone below pH 3.6; (2) an almost unstained narrow zone; (3) an acidic Ampholine region between pH 3.6 and 5, with several bands of medium intensities; (4) a region between pH 5 and 8 with numerous narrow bands of very different intensities; the four most prominent bands are about 1 pH unit apart; (5) an alkaline region above pH 8.0 with broad bands not well demarcated from each other; (6) a second almost unstained narrow zone; (7) an electrode zone above pH 10, in which glucose caramelization proceeds in the presence of NaOH. (By permission of the New York Academy of Sciences. See ref. 24.)

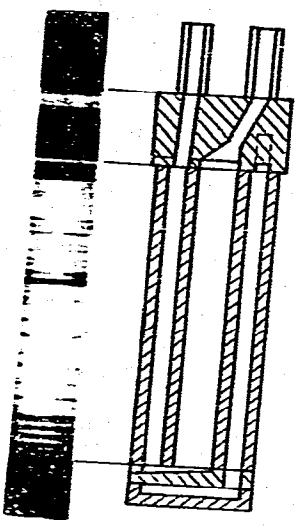


Fig. 5. Photograph of cell after isoelectric focusing of carrier ampholytes. The striations are due to strong refractive index gradients caused by focused ampholytes. (By permission of the New York Academy of Sciences. See ref. 25.)

2. INSTRUMENTAL AND METHODS

This section deals largely with the instrumentation required for IEF in gels: the information required for IEF in sucrose density gradients has been given in previous reviews[10-12]. Three items are required for gel electrofocusing —an electrofocusing cell, a high-voltage power supply and a means of cooling the gels efficiently. These are discussed below, together with methods for their application in analytical and preparative procedures. Detailed information on published methods is not given here.

A. Analytical procedures

The technique of GEF was originally described in a number of papers published independently and almost simultaneously in 1968 (refs. 28–34). Initially, many workers used apparatus designed primarily for gel electrophoresis although others, especially those using thin-layer techniques, built their own equipment[28,32]. Their results clearly indicated the considerable potential of the technique, particularly in its remarkable resolution and experimental flexibility. Unfortunately, many of the systems suffered from a marked instability in the pH gradients. This problem can be attributed mainly to the use of inappropriate apparatus. Most of the apparatus for electrophoresis in gel cylinders requires large volumes of electrolyte in order to minimize changes in the pH of the electrolyte during electrophoresis and, if used for gel electrofocusing, considerable convective mixing can occur in these large volumes of electrolyte, which disturbs the pH gradient formed between the ampholytes in the gel and the electrode and can be a major contributing factor to pH gradient instability.

During the transition from sucrose gradients to gels, few systematic studies were made in order to optimize the conditions of gel composition and electrolysis to achieve equilibrium focusing. Further, in the understandable desire to obtain rapid

analyses, insufficient attention was given to the problem of cooling, which can be a critical factor in view of the wide variation in the conductivity and distribution of focused ampholytes. As a result, local heating may occur, with its associated problems of protein denaturation and convective mixing in the gel, which is another factor contributing to pH gradient instability.

(a) Gel cylinders

Fawcett[35] designed a very simple apparatus to overcome heating problems by thermostating gel tubes in a bath of an electrolyte solution (see Fig. 6). As the coolant was also the catholyte, the cold finger and stirrer were plastic-shielded. In order to minimize the volume of electrolyte used, Fawcett devised the ingenious solution of inserting a platinum electrode wire directly into a layer of Ampholine on top of each gel tube.

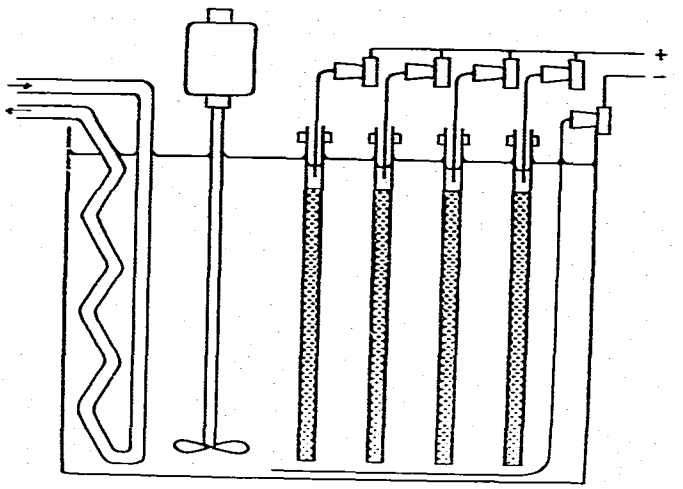


Fig. 6. Apparatus for gel electrofocusing according to Fawcett[35], where the tubes are thermostated by inserting them into a bath containing one of the electrodes and the corresponding electrode solution. At the anode, the platinum wire is inserted directly into a layer of Ampholine on the top of each gel tube. (By permission of Pergamon Press. See ref. 35.)

Righetti and Drysdale[23,36] developed an apparatus specifically for IEF in gel cylinders in which stable and reproducible pH gradients can be obtained (Fig. 7). The salient features of this apparatus include efficient cooling of gels by circulating fluid and small electrolyte compartments. The gel tubes are held in a water-tight compartment through which coolant at -5° to $+4^{\circ}$ is passed. Circular platinum electrodes are positioned close to the extremities of the tubes so as to minimize convection disturbance of the pH gradient between gel and electrode. The apparatus holds twelve gels of 10 × 0.3 cm I.D. Usually, the gels are cast in plastic tubes from which they are readily extruded. Quartz tubes, which can be scanned directly in the UV region, may be used interchangeably. A commercial adaptation of this apparatus is now available from Medical Research Apparatus, Boston, Mass., U.S.A.

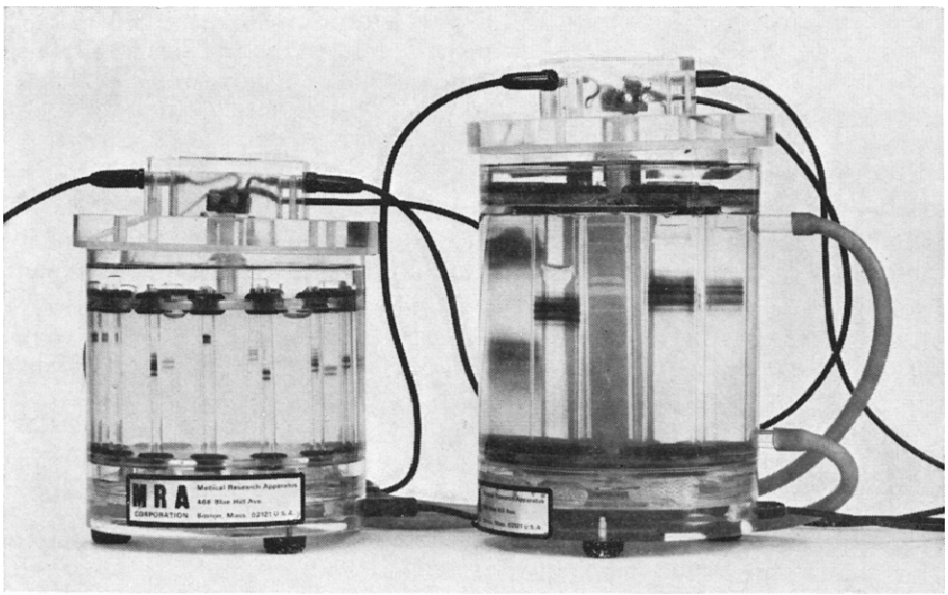


Fig. 7. Analytical and preparative apparatus for isoelectric focusing in gel cylinders showing fractionations of haemoglobin variants. The preparative apparatus also contains a fractionation of 50 mg of human placenta lactogen. The multiple forms of this hormone are evident when viewed against the dark background. (By permission from the New York Academy of Sciences. See ref. 36.)

(b) Gel slabs

Delincée and Radola[37] developed an efficient cooling system for IEF in a thin layer of granulated gel (Sephadex, Sepharose or granulated polyacrylamide gel) (Fig. 8). The gel was supported on a glass plate (20 × 20 cm or 40 × 20 cm) and cooled by contact with a metal block maintained at 4°. Two filter-paper strips soaked in 0.2 *M* sulphuric acid and 0.4 *M* ethylenediamine were laid directly on top of the

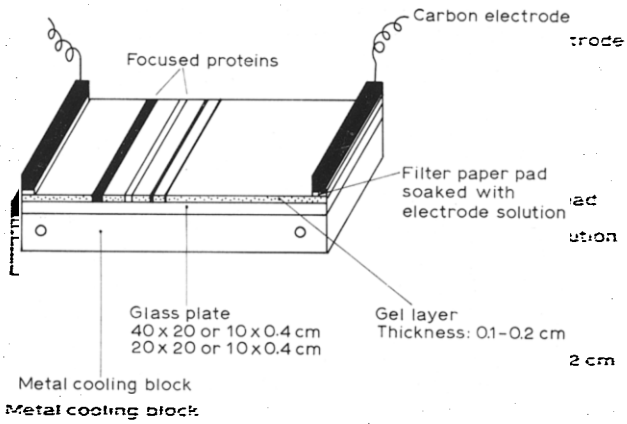


Fig. 8. Apparatus for small-scale isoelectric focusing in a layer of granulated gels, according to Radola. Notice the flat carbon electrodes, which are laid directly at the two gel extremities on top of a filter-paper pad soaked with electrode solution. (By permission of Butterworths Scientific Publishers.)

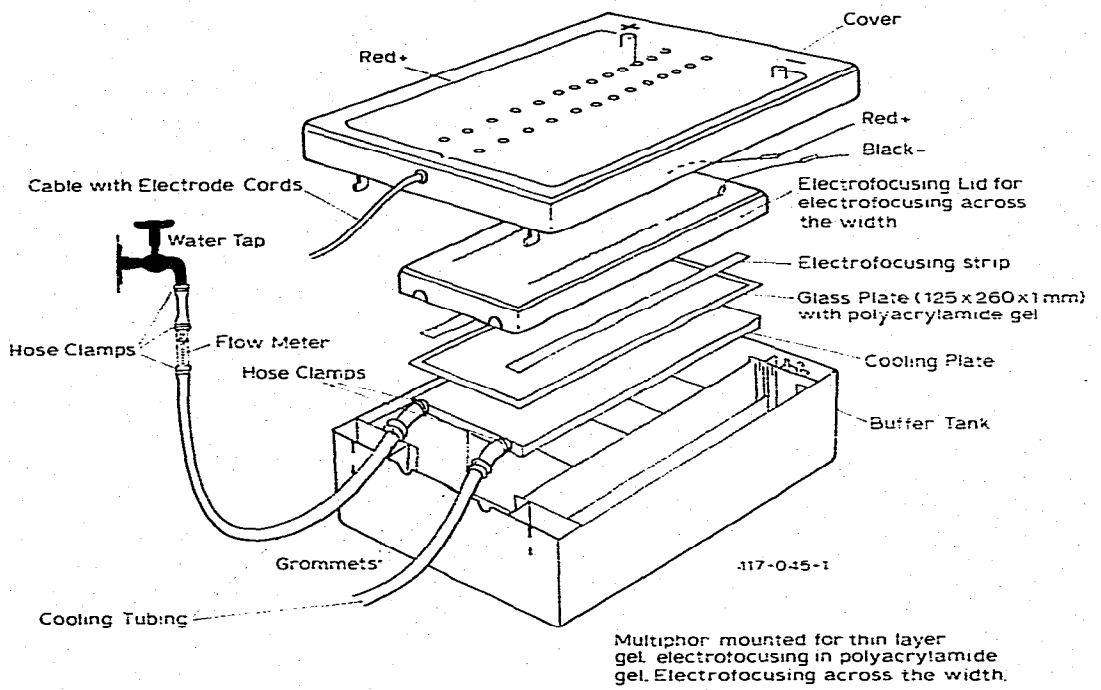


Fig. 9. Exploded view of the LKB 2117 Multiphor apparatus for thin-layer isoelectric focusing in polyacrylamide gels. By changing the cover lid, IEF can be performed either across the width (10 cm) or across the length (25 cm). The sample is applied soaked on rectangular pieces of filter paper (1 × 0.5 cm). (Courtesey of LKB Produkter AB.)

ends of the gel so as to provide a support and electrical contact for two flat carbon electrodes.

Another apparatus for thin-layer IEF in gel slabs has now been produced by LKB. This apparatus exploits the original idea of Awdeh *et al.*[28] as further modified by Vesterberg[38]. In this system, 60 ml of gel mixture are used to polymerize a flat bed of dimensions, 25 cm × 10 cm and 2 mm thick. Fig. 9 gives an exploded view of this apparatus. The gel solution is polymerized between two glass plates. Samples are usually applied to the gel surface in a small piece of filter paper, and as many as 20 samples can be run simultaneously. Dilute samples or samples of unknown concentration can be applied from a long strip of paper cut in the form of an acute-angle triangle. Focused zones appear as long, thin lines of linearly increasing concentration, allowing a rapid estimate to be made of the proper amount to be loaded. A minor disadvantage, as Smyth and Wadström[39] pointed out, is that many proteins are not readily eluted from cellulose filter papers, and other adsorbents, such as cellulose acetate or desiccated polyacrylamide, may be more suitable. Alternative systems for slab gel electrofocusing in which samples are loaded directly into pockets in the gel are available from Hoeffer (San Francisco, Calif., U.S.A.) (vertical unit) and Medical Research Apparatus (horizontal unit).

The choice between gel cylinders or slabs depends upon the particular require-

ments. Slab gels allow excellent comparisons of similar samples in parallel tracks. In addition, samples can be applied as anodic, cathodic or near isoelectric species without the risk of exposure to extremes of pH at the electrodes, which gives an easy check for possible artifacts and for the attainment of equilibrium focusing conditions. Gel rods, on the other hand, allow greater experimental flexibility. For example, different pH gradients can be run simultaneously, and multiple analytical procedures can be used for replicate samples. Focusing in gel cylinders also permits the analysis of more dilute samples, generally requires less Ampholines per sample analysis and offers the possibility of using scanning devices and other techniques devised for analysis after electrophoresis in gel rods.

B. Preparative procedures

IEF in gels appears to be particularly suitable for preparative fractionations as the resolution achieved during the run is not lost during sample recovery procedures, which has been a major problem in preparative electrofocusing in sucrose density gradients. Isoelectric precipitation is also less troublesome, as demonstrated in Fig. 7, which shows a fractionation of human placental lactogen at levels sufficient to detect the major components. This pattern is identical with that obtained with minimal sample inputs. The larger gels shown in Fig. 7 permit the fractionation of up to 200 mg of protein. The preparative apparatus has essentially the same overall dimensions as the small-scale analytical model. The interchangeable core accommodates gels of 20- and 50-ml capacity and also contains an "indicator" gel of the same length but with a volume of only 2 ml. This indicator gel enables one to determine the position of components in the larger gels which remain under voltage during the analysis. As in the analytical systems, multiple samples can be fractionated simultaneously in different pH gradients. Separations are usually achieved in about 12–24 h and patterns generally remain stable for at least 72 h. Because heat transfer is less efficient in these large gels, particular attention must be given to cooling procedures.

Although such gels usually allow higher sample inputs and superior resolution compared with all-liquid systems of comparable capacity, they are considerably less convenient in terms of recovery of separated proteins. Once the bands have been detected, either from an indicator gel or a paper imprint[40], the gel segment has usually to be cut, squashed and eluted serially with buffer. A more efficient and convenient system has recently been devised by Suzuki *et al.*[41] in which the separated proteins are eluted electrophoretically from gel segments, with excellent recovery.

As far as sample recovery is concerned, preparative focusing in Sephadex beds may be a more attractive alternative. By scaling up his thin-layer granulated technique, Radola[40] separated gram amounts of proteins in Sephadex gels of dimensions 40 × 20 × 1 cm. Fig. 10 shows the seperation of a mixture of 500 mg each of ovalbumin, horse myoglobin, ribonuclease and cytochrome *c* in a 700-ml gel of Sephadex G-75 Superfine, containing 1% of pH 3–10 Ampholine and 0.05% of lysine and arginine. After focusing and protein detection, the sample zone is simply scooped up with a spatula and the protein can then be separated from ampholytes by gel filtration.

Another highly promising approach to preparative systems is the continuous-flow IEF technique in gel layers described by Fawcett[42]. Fig. 11 illustrates the principle of continuous-flow IEF compared with continuous-flow electrophoresis. In both methods, an electric field is applied at right-angles to the direction of flow of electro-

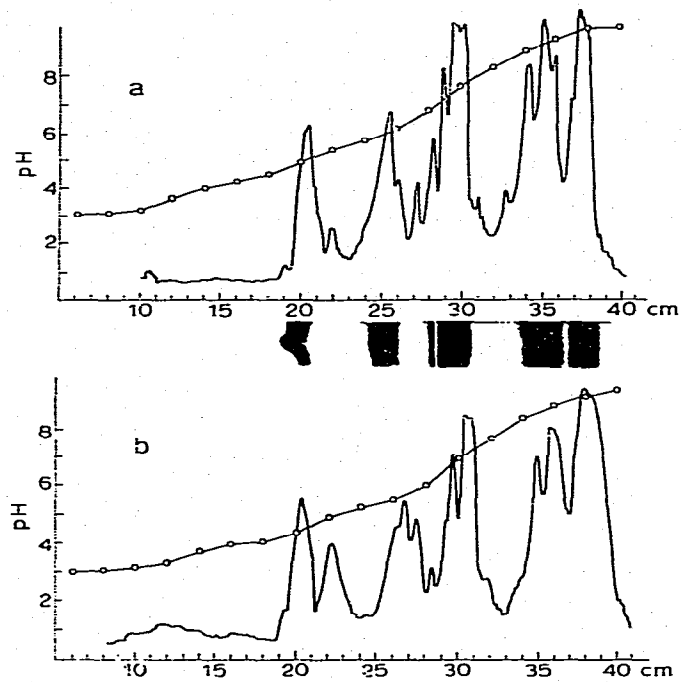


Fig. 10. Preparative isoelectric focusing of 2 g of protein in a 40 × 20 × 1 cm trough in pH 3–10 ampholytes. Gel stabilization, Sephadex G-75 Superfine; thickness of the gel layer, 1 cm. Proteins: ovalbumin, horse myoglobin, ribonuclease and cytochrome *c* (500 mg of each). Focusing: (a) 400 V for 20 h followed by 800 V for 2 h; (b) 400 V for an additional 10 h and 800 V for 2 h. Densitogram of a print stained with light green SF, ○—○, pH gradient. (By permission of the New York Academy of Sciences. See ref. 40).

lytes. In the latter, the protein mixture is continuously injected within a narrow zone and components of different electrophoretic mobilities migrate as bands that radiate from the origin. In continuous-flow IEF, the amphoteric components migrate to their p*I*s, where they have zero electrophoretic mobility. They will then move only in the direction of the liquid flow (illustrated in Fig. 11 as two black lines becoming perpen-

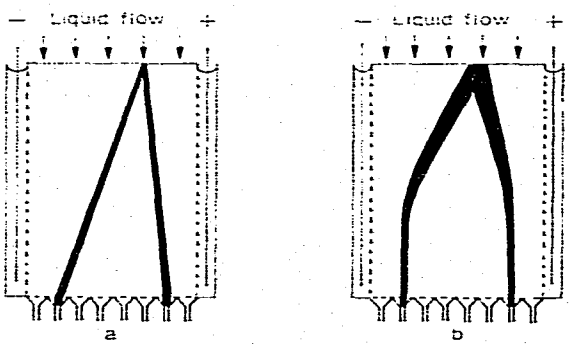


Fig. 11. Diagram illustrating the principle of (a) continuous-flow electrophoresis and (b) continuous-flow isoelectric focusing. (By permission of the New York Academy of Sciences. See ref. 42.)

dicular to the electric field). In contrast to continuous-flow electrophoresis, the width of the injected zone is unimportant. Indeed, the sample solution can be applied over the whole width of the apparatus.

A major advantage of continuous-flow IEF is that separated zones are removed from the apparatus under voltage, thereby eliminating the diffusion effects commonly encountered in all-liquid systems during sample recovery in the absence of an applied potential differential[43]. Another important feature is that the apparatus can be run for extended periods with continuous sample inflow and recovery, thus allowing purification of several grams of material. Fig. 12 shows this apparatus in operation. The vertical separation chamber is filled with Sephadex G-100 equilibrated with 1% of Ampholine of pH 3–10. Two streams of haemoglobin can be seen to merge toward the middle of the cell, whence a single line moves downwards, perpendicular to the electric field.

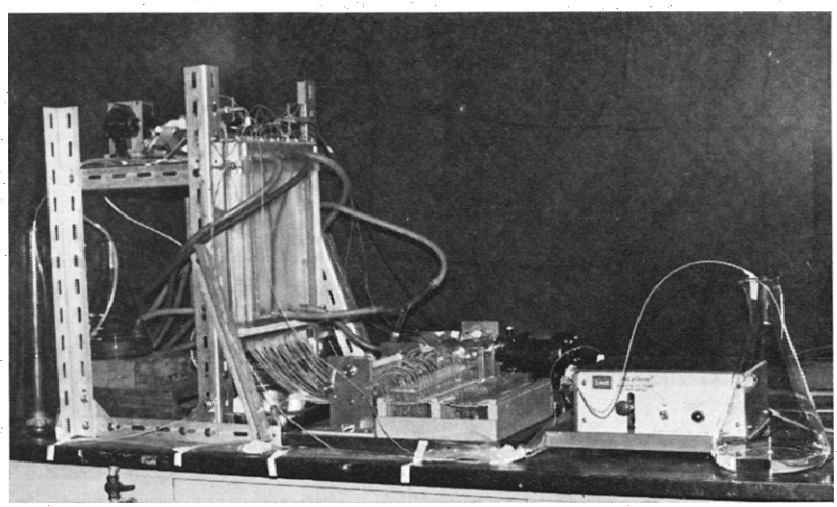


Fig. 12. Photograph of continuous-flow isoelectric focusing in Sephadex-stabilized media. View of complete apparatus with pump units and collecting tubes. (By permission of the New York Academy of Sciences. See ref. 42.)

C. Power supply

In the past few years, considerable attention has been paid to improvements in the design of apparatus, supporting media and the composition of media and electrolytes for IEF. Until recently, however, surprisingly little attention has been given to the source and nature of the electric potential used. It is generally agreed that high resolution and faster separations are produced by high potential differences. However, high voltages can generate a considerable amount of heat in the cell, causing band distortion, convective mixing and often denaturation or inactivation of labile substances. As Joule heating is also dependent on the current carried by the cell (Joules = volts × amps), several investigators have turned to pulse power supplies, such as the Ortec Model 4100 (Ortec, Oak Ridge, Tenn., U.S.A.) and the Pulsephor (Grainer, Milan, Italy), to generate high voltages at pre-determined frequencies with lower

currents. Although such pulse power supplies effectively reduce Joule heating, they do not regulate the actual power delivered to the cell. This regulation, however, is a critical factor in electrofocusing experiments, particularly during the early stages when considerable changes in conductivity occur. This usually necessitates careful monitoring and manual adjustments of frequency and the height and/or width of the voltage pulse. Such continued monitoring is not only inconvenient but also makes experimental reproducibility difficult. Several systems are now being developed in order to overcome these problems in power regulation. Schaffer and Johnson[44] modified an unregulated d.c. power supply by incorporating a regular circuit to control the average power delivered to the cell (see Fig. 13). Söderholm and Wadström[45] built a regulated power supply capable of delivering 3000 V, with which the time required for equilibrium focusing in thin-layer gels can be reduced to about 1 h. Medical Research Apparatus has recently produced a regulated pulse power supply that continuously monitors and regulates the average power delivered to the cell by automatic control of pulse frequency. Such instruments should allow electrofocusing to be conducted with the same facility as electrophoresis.

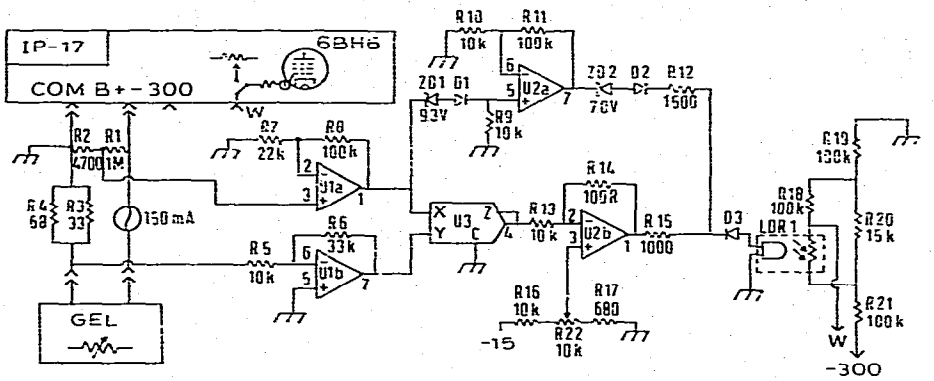


Fig. 13. Constant-power regulator circuit. U1 and U2, Motorola MC1458G (dual "741" type op amp); U3, Burr-Brown 4094 15C (multiplier); D1–D3, 1N457A; ZD1 and ZD2, Zener diodes; R1–R21, 1/2 W; R22, 10k potentiometer; LDR 1, Raytheon CK1122. (By permission of Academic Press. See ref. 44.)

D. Types of gel media

Because of its low electroendosmotic flow, optical clarity and satisfactory mechanical properties, polyacrylamide gel has been used extensively as a support medium for electrofocusing and is presently the medium of choice for most small-scale analytical procedures. However, the same attractive anticonvective properties that give such excellent resolution also represent a major hazard —molecular sieving— which must not be overlooked. It is well known that polyacrylamide gels severely restrict the mobility of many proteins. Although molecular sieving is often advantageous in electrophoretic procedures, it can have disastrous consequences in gel electrofocusing, particularly when attempting to focus large proteins in systems that do not develop stable pH gradients. Because IEF is an equilibrium method, reproducible and meaningful results can be obtained only if the experimental conditions allow all proteins

to reach their equilibrium positions in the time available for the experiment. All too often investigators have had to compromise by curtailing electrolysis periods in order to counteract instability in the pH gradient, which has resulted in a failure to achieve equilibrium focusing conditions and much confusion in the literature.

Several factors in addition to apparatus design, gel temperature and electrolysis conditions can affect the results obtained by gel electrofocusing, one of the most important being the composition of the gels, particularly in the concentration of ampholyte and acrylamide[23,36]. We have found that an ampholyte concentration of 2% (w/v) gives optimal results. Although lower levels (1%) can be used in order to obtain more rapid focusing, the banding patterns and pH gradient are often unstable, which may be related to a lack of adequate conductivity in parts of the gel at low ampholyte levels.

For routine analysis, we generally use 10 × 0.3 cm I.D. gels containing 4% of acrylamide, 0.16% of N,N'-methylenebisacrylamide (both recrystallized)[16] and 2% (w/v) of ampholytes. Occasionally, glycerol is added to a level of 5% (v/v). The gels are cooled by circulating antifreeze solutions at 0 to −5 . Most proteins with molecular weights up to 500,000 will focus in less than 6 h in these gels when subjected to an average power of 0.1 W/gel. For larger proteins, it is advisable to use lower gel concentrations. Florini *et al.*[17] used very dilute gels containing only 2.6% of acrylamide for the analysis of myosin preparations. For proteins with molecular weights of less than 100,000 or for oligopeptides or oligonucleotides, more robust gels with 5 or 6% of acrylamide may be preferred[36]. Faster banding can be obtained when higher initial voltage levels are used, but often at the expense of gradient stability. This can be avoided by using lower temperatures or by using a pulse power supply to deliver a high voltage at low current levels. It is usually simple to establish that equilibrium focusing is achieved in the system by showing that similar banding patterns and p*I*s are obtained from samples applied to the top of the gel or distributed throughout the gel by adding the protein to the gel solution before polymerization.

Agarose gels, owing to their very large pore size and satisfactory mechanical strength, would seem to be an ideal support for IEF. Catsimpoolas[48] and Riley and Coleman[33] were among the first workers to use this medium and obtained separations in 1% agarose. However, owing to high electroosmotic flows[49], these gels did not give stable pH gradients and equilibrium patterns were difficult to establish. Låås[50] has recently attempted to remove charged groups by alkaline hydrolysis of sulphates and reduction of carboxyl groups with lithium aluminium hydride in dioxane. The product is a virtually charge-free agar and may prove to be a convenient alternative to dilute acrylamide gels for focusing proteins of high molecular weight. Paper and cellulose acetate, which minimize molecular sieving and subsequent sample detection, also suffer from excessive electroendosmosis and are unsuitable as support media in IEF.

Radola[51] overcame many of the problems associated with molecular sieving by performing IEF in granular gels, particularly Sephadex. The Superfine grades of Sephadex G-75 and G-200 were the most suitable. Normal grades and Sephadex G-25 and G-50 were unsatisfactory, the latter giving irregular patterns and diffuse zones with proteins of low molecular weight. Fawcett[42] used Sephadex G-100 beds and graded particles of polyacrylamide gel as stabilizing media for continuous-flow IEF. Bio-Gel P-60 (−400 mesh) is preferable to Sephadex when staining for carbo-

hydrates by the periodic acid–Schiff reaction[52]. As there is no sieving effect for macromolecules above the exclusion limit of these gels, high-molecular-weight substances, such as virus particles, can be focused without steric hindrance. The risks of artifacts caused by polymerization catalysts in polyacrylamide gels are also avoided in granular gels. Free radicals detectable by electron spin resonance seem to persist in polyacrylamide gels, even after a pre-run[53].

3. SAMPLE DETECTION

A. Staining and destaining

Considerable problems were encountered in the early stages of GEF in detecting separated proteins because many of the stains commonly used for detecting proteins also formed insoluble complexes with Ampholines in acidic media[33]. This problem could be circumvented by first precipitating the proteins in trichloroacetic acid and leaching out the Ampholines before staining and destaining in the usual manner. With the exception of Radola's paper print technique[51] for detecting proteins in Sephadex beds, this process was, however, inconvenient and time consuming.

Many of the problems associated with Ampholine–dye binding have now been overcome. Most procedures appear to discriminate between complexes of ampholytes and protein with dye on the basis of their differential solubility in alcoholic solutions and their stability at different temperatures[54,55] and pH (ref. 56). In general, the Ampholine–dye complex is less stable or more soluble at higher levels of alcohol, temperature and pH than the protein–dye complexes. Awdeh[57] developed a direct staining procedure for proteins with bromophenol blue in 45% ethanol–10% acetic acid. Riley and Coleman[33] stained proteins with a solution of 0.1% of fast green in 25% ethanol–10% acetic acid. Both methods are rapid and give little interference from ampholytes, but unfortunately neither is very sensitive. Several attempts have been made to develop direct staining procedures with Coomassie brilliant blue, a group of stains that are widely used in gel electrophoretic analysis. Spencer and King[58] devised a satisfactory method in which proteins absorbed Coomassie brilliant blue from a 0.01% solution in 5% trichloroacetic acid, 5% sulphosalicyclic acid and 25% methanol. This method gives little background staining but is less sensitive than when particulate Coomassie blue in trichloroacetic acid solutions is used for staining. The intensity of the stained bands may, however, be enhanced by increasing the level of Coomassie blue to 0.05%, but at the expense of higher backgrounds. We have found a method that combines high sensitivity and low backgrounds and may have wide applicability. Focused gels are immersed with shaking for at least 4 h at room temperature in a solution of 0.05% of Coomassie blue and 0.1% of copper(II) sulphate in acetic acid–ethanol–water (10:25:65), then for a further 4 h in the same solution, but containing only 0.01% of dye. Final destaining is effected in acetic acid–ethanol–water (10:10:80).

Frater[59] described a series of dyes with high colour indices (C.I.) that can be used for direct staining in the absence of alcohol. These dyes include Kiton Rhodamine B (C.I. 45100), Disulphine Blue UN (C.I. 42045), Fast Acid Blue B (C.I. 44035) and Coomassie Violet R (C.I. 42650). Most of these dyes contain two sulphate residues and one or two charged nitrogen atoms in addition to bulky, unsubstituted aromatic rings that favour strong hydrophobic binding to proteins. For staining,

Frater used a mixture of 0.05% of Fast Acid Blue B and 0.05% of Coomassie Violet R in 5% acetic acid. Removal of excess of dye apparently takes only a few hours.

It can be deduced from the large variety of staining procedures presently used in gel electrofocusing that no single method is totally satisfactory. Future developments might include staining with fluorescent dyes, such as fluorescamine[60], which are extremely sensitive but also subject to rapid hydrolysis. 8-Anilino-1-sulphonate[61] and dansyl chloride[62] are being investigated at present.

An attractive alternative would be to devise procedures for detecting uncoloured proteins by pre-labelling them with suitable chromophores. Preliminary experiments with fluorescamine have been encouraging[63]. In this procedure, pre-labelled proteins are detected after focusing by exposure to UV light. Interestingly, the interaction with fluorescamine does not seem to alter the p*I* substantially or introduce much additional complexity to the banding patterns of many proteins. Such methods may be helpful in preparative procedures for recovering proteins.

B. Histochemical staining for enzymes

IEF in gels is particularly suitable for the histological detection of enzymes. Most of the methods developed for specific enzyme detection after gel electrophoresis can usually be adapted for IEF, provided that appropriate steps are taken to counteract adverse pH effects from ampholytes. Fig. 14 shows a histochemical stain for lactate dehydrogenase isozymes separated from tissue extracts by gel electrofocusing. In this case, reduced pyrimidine nucleotides formed during conversion of lactate to pyruvate form an insoluble coloured product by reduction of tetrazolium salts[64].

In addition to direct histochemical stains, some interesting "zymogram" techniques have been developed, in which a second substrate-impregnated gel is layered over the focused gel. Vesterberg and Eriksson[65] detected staphylokinase activity by

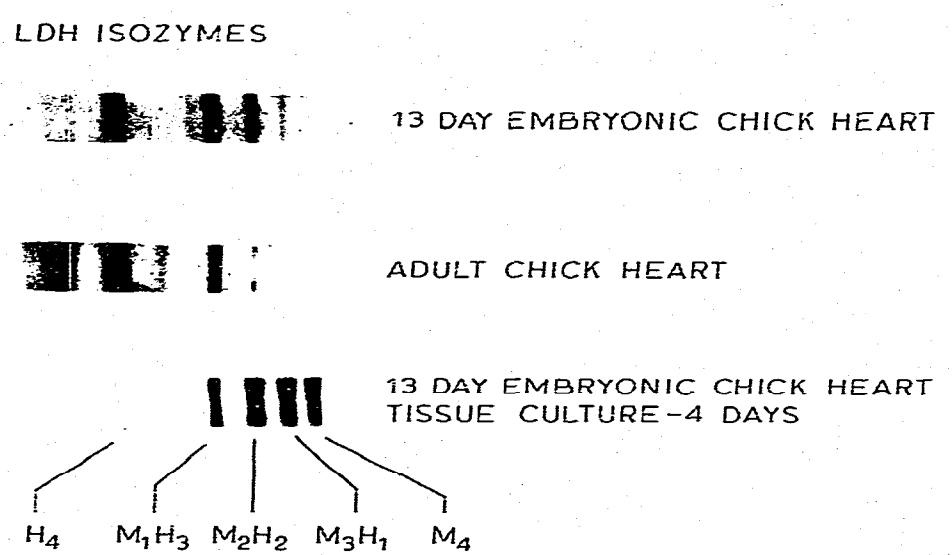


Fig. 14. Histochemical stain for lactate dehydrogenase (LDH) isozymes after gel electrofocusing in the pH 3–10 range. (By permission of the New York Academy of Sciences. See ref. 36.)

overlaying focused gels with a layer of fibrinoclot containing plasminogen as an impurity. The focused staphylokinase converted the plasminogen into plasmin so that the fibrinolytic activity which corresponds to staphylokinase activity appeared as a clear spot on the opaque fibrin plate. Wadström[66] used an overlay of an agar plate containing 1 % of casein in order to detect extracellular staphylococcal proteases after gel electrofocusing. Vesterberg[67] detected pepsin activity in gels by its ability to digest albumin in an overlying agar plate. After staining with Coomassie blue, the zones of enzymatic activity appeared as white, unstained zones against the stained background (Fig. 15). Similar *in situ* procedures can be used to detect enzyme activity by autoradiography after forming an insoluble radioactive product.

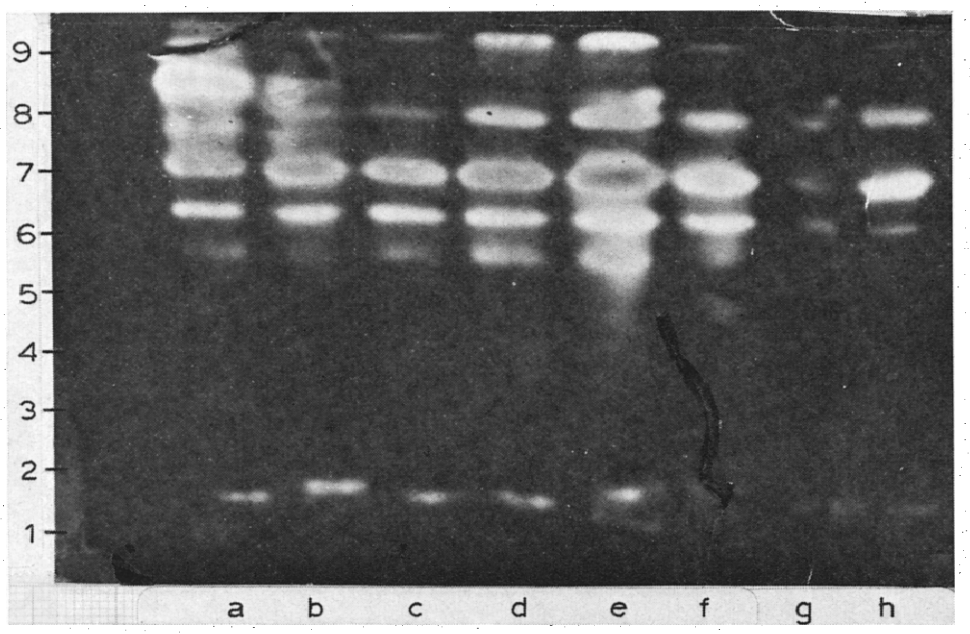


Fig. 15. Zymogram detection of proteolysis after isoelectric focusing. The sample of gastric juice (10 μl) was applied to pieces of filter paper measuring 1.0 × 0.3 cm and placed close to the cathode. The zones with enzymatic activity can be seen as white unstained zones against the stained background, which represents undigested albumin. Distance from the cathode (cm) is shown on the left. (By permission of Munksgaard. See ref. 67.)

C. Gel fractionation

When no suitable histochemical or autoradiographic method is applicable, enzyme activities can be detected in eluates from serial gel sections. A convenient and highly reproducible method of gel fractionation is illustrated in Fig. 16. Gels are cast in plastic syringe tubes that fit into the small-scale analytical apparatus (Fig. 7). After focusing, a No. 20 snub-nosed syringe needle is fitted on to the hub of the tube, and the gel is extruded through the needle by advancing a rubber-tipped plunger activated with a Hamilton syringe fractionating device. Discrete segments of homogenized gel of 10 μl are obtained at each stroke of the plunger. Many enzymes can be assayed directly in this gel homogenate. Bagshaw *et al.*[68] used this method to assay DNA-dependent RNA polymerases with both native and denatured DNA templates. De-

Fig. 16. Fractionating device for gel rods. The gel tubes have a round collar at one end and a standard syringe taper tip at the other. After focusing, a syringe needle is attached to the tapered tip and the gel is extruded in uniform fractions through the needle with the aid of a Hamilton repeating dispenser. Note that no band distortion or compression occurs. (By permission of the New York Academy of Sciences. See ref. 68.)

spite the large size of the focused enzyme (molecular weight 600,000) and the added template (*ca.* $2 \cdot 10^6$), all of the reactants were apparently mutually accessible in view of the high activities obtained. The transfer of fractions for the assay of insoluble radioactive products is greatly facilitated by using diallyltartaramide as the gel cross-linker because the gel segments can be solubilized in 2% periodic acid before counting.

D. Scanning devices

IEF in gel cylinders offers many advantages in sample detection and quantitation. The dimensions of the gels are often suitable for scanning densitometry either directly after focusing or after appropriate staining. In our system, gels can be scanned in a 10-cm cuvette in a Gilford Model 240 recording spectrophotometer fitted with a linear transport device. Quartz or glass gel tubes fit directly into the cuvette holder so that the focused gels can be scanned directly in the tubes. Fawcett[35] adapted a Unicam SP 800 spectrophotometer for scanning gels at a constant wavelength and for obtaining spectra of individual components. In this way, information about the identity of registered zones can be collected.

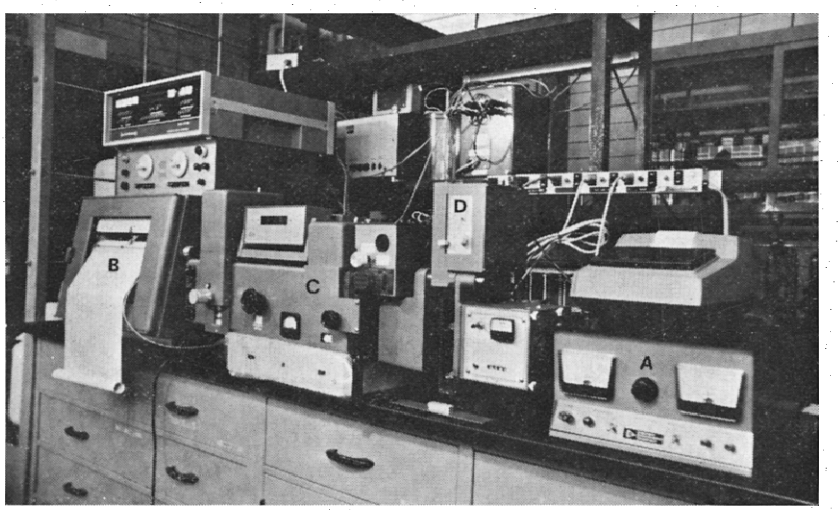


Fig. 17. Photograph of the equipment used for performing scanning isoelectric focusing. A, power supply; B, recorder; C, monochromator, dual lamp housing and photometer (note that this equipment is resting on its side); D, linear transport system, positioned vertically. (By permission of the New York Academy of Sciences. See ref. 43.)

Catsimpoolas[43] devised an interesting approach, called "analytical scanning isoelectric focusing" for the analysis of focused patterns. He performed IEF in a quartz cell held vertically in the chamber of a modified Gilford linear transport device (Fig. 17). The focusing column can be monitored continuously and the developing patterns observed. This system gives more reliable analyses of banding patterns in sucrose density gradients as it permits the detection of closely spaced peaks that might not be apparent from the analysis of fractions eluted in the absence of high voltage. In the conventional system, the patterns obtained on elution often do not reflect the true resolution obtained in the column because of diffusion in the absence of current and sample mixing during elution. *In situ* scanning also indicates the end-point in

IEF, which is important because a progressive flattening of the pH gradient often occurs after equilibrium is reached with a resulting loss in resolution (plateau phenomenon)[69]. Catsimpoolas extended his technique to form the basis of a new kinetic method, called "transient state isoelectric focusing" (see p. 314).

Densitometric evaluation in thin layers has been carried out by Radola[51] with a Schoeffel SD 3000 spectrodensitometer (Schoeffel Instruments, Westwood, N. J., U.S.A.). The Zeiss MPQ II chromatographic spectrophotometer allows densitometry by transmittance, reflection and fluorescence emission and is compatible with gel cylinders, polyacrylamide thin layers, granulated flat beds, paper and cellulose acetate up to dimensions of 20 × 20 cm and can also be used to obtain the spectra of single bands.

E. pH measurements

The p*I* of a protein determined by IEF also represents its isoionic point in the absence of complex-forming ions[11]. By definition, the isoionic point is a measure of the intrinsic acidity of a pure protein, as it is defined as that pH which does not change on addition of a small amount of pure protein[70]. This definition is also applicable to a protein analyzed by IEF, as the pH of the isoelectric zone does not alter on addition of more protein. It should be remembered that p*I* values estimated by IEF are temperature dependent and usually decrease with increasing temperature[71]. The pH should, therefore, be measured at a constant specified temperature, preferably the focusing temperature. In addition, pH measurements are often affected by the presence of glycerol or sucrose, which are commonly used in IEF. As the p*I* measurement is a valuable parameter for characterizing proteins, it would be most desirable to standardize the conditions used for measuring p*I* values observed by IEF. The importance of routinely estimating pH gradients in each experiment cannot be over-emphasized. No two preparations of Ampholines of the same nominal pH range give identical pH gradients, and in fact the same preparation will give slightly different gradients in gels of different composition or with different electrolysis conditions. This variability can be corrected by correlating banding patterns with the actual pH gradient developed in each experiment[23]. In general, estimates of p*I* values determined by electrophoresis are lower than p*Is* obtained by IEF[8,71], largely because of interactions of proteins and buffer ions during electrophoresis[72]. Usually, the lower is the ionic strength of the electrophoresis buffer, the higher are the apparent p*I* values. Estimates of p*Is* from electrophoretic data extrapolated towards zero ionic strength give results similar to those obtained by IEF.

The simplest way of measuring the pH gradient in gel cylinders is to cut the gel lengthwise into equal segments, elute the ampholytes into a small volume of distilled water and read the pH of the gel eluates with a combination microelectrode[23,28,34]. When using this method, the buffering capacity of the ampholytes should not be exceeded as a result of excessive dilution. As a general rule, the volume of the eluting water should be less than seven times the volume of the gel section. When measuring gradients in alkaline pH ranges, absorption of atmospheric carbon dioxide can be minimized by using boiled water or by flushing eluates with nitrogen. Finally, it is advisable to have a small amount of salt, *e.g.*, 10 m*M* sodium chloride, present so as to ensure adequate conductivity.

The pH gradient in gel slabs can be measured directly from the gel surface

with a flat-membrane electrode. Preferably, the electrode should have a cross-section of not more than 6 mm (for example, type LOT 403-30-M8, Ingold, Zurich, Switzerland[35]). Beeley *et al.*[73] reported a method for pH gradient determinations in unsectioned gel cylinders or slabs by using an antimony microelectrode (1 mm in diameter) in conjunction with a calomel reference electrode. These electrodes are manufactured by Activion Glass (Kinglassie, Fife, Great Britain) according to the design of Kleinberg[74]. Measurements are made by pressing the reference electrode at any position along the gel and scanning at regular intervals with the antimony electrode. In contrast to a glass electrode, where pH readings are obtained directly from the pH scale, the antimony electrode gives measurements of electromotive force (EMF) on the millivolt scale. These values are then converted into units of pH by means of an appropriate calibration graph. The antimony electrode appears to give more accurate measurements at the acidic and basic ends of the gel and is very stable. J. A. Beeley (personal communication) reported the frequent use of this electrode over a period of 2 years with no changes in the original calibration graph.

The determination of the p*I* values of proteins of known chemical structure in the presence of denaturants, such as 6 *M* urea, can also be valuable in conformational studies[75]. However, it should be noted that urea appreciably decreases the activity coefficient of hydrogen ions, to give apparently higher p*K* and p*I* values of the carrier ampholytes. Correction factors should, therefore, be used for p*I* determinations of proteins in urea[75].

F. Plateau phenomenon

The plateau phenomenon[69] or cathodic drift[36] occurs in many separations in most gel media. This instability of pH gradients is indicated by a progressive flattening of the pH gradient at the centre of the gel and results in the migration of basic components towards the cathode. Its origin is still not completely clear. Chrambach *et al.*[69] concluded that it does not depend qualitatively on gel concentration, Ampholine p*I* range, Ampholine concentration, the presence of protein, the presence or absence of urea, temperature or the geometric arrangement of electrodes. The process can be retarded by increasing the viscosity of the medium by incorporating 12.5% of sucrose[69] or 10% of glycerol[36] into the gels.

Radola[51] reduced the cathodic drift in Sephadex gels by treating them with propylene oxide in order to remove carboxyl groups. This suggests that a major cause of the plateau phenomenon is electroendosmosis. Chrambach *et al.*[69] argued that electroendosmosis *per se* can be responsible to only a limited extent for the plateau phenomenon, as the latter is symmetrically two-directional whereas the former is one-directional. However, we have found the major effect to be a cathodic shift in which proteins and Ampholines slowly migrate into the cathodic chamber, where they can be detected by the ninhydrin reaction. By the same criterion, little, if any, Ampholine is found in the anodic compartment. During IEF in cellulose acetate, which has a high electroendosmosis, the cathodic drift is accompanied by a net transport of water from the anode to the cathode. The consequences of the cathodic shift are evident from the results depicted in Fig. 18 from experiments performed with the Multiphor thin-layer apparatus of LKB[76]. The haemoglobin sample (applied as a cathodic, anodic and near isoelectric species) reached equilibrium in 1.5 h, as shown by the convergence of the three samples. Thereafter, the focused bands began to drift slowly toward the

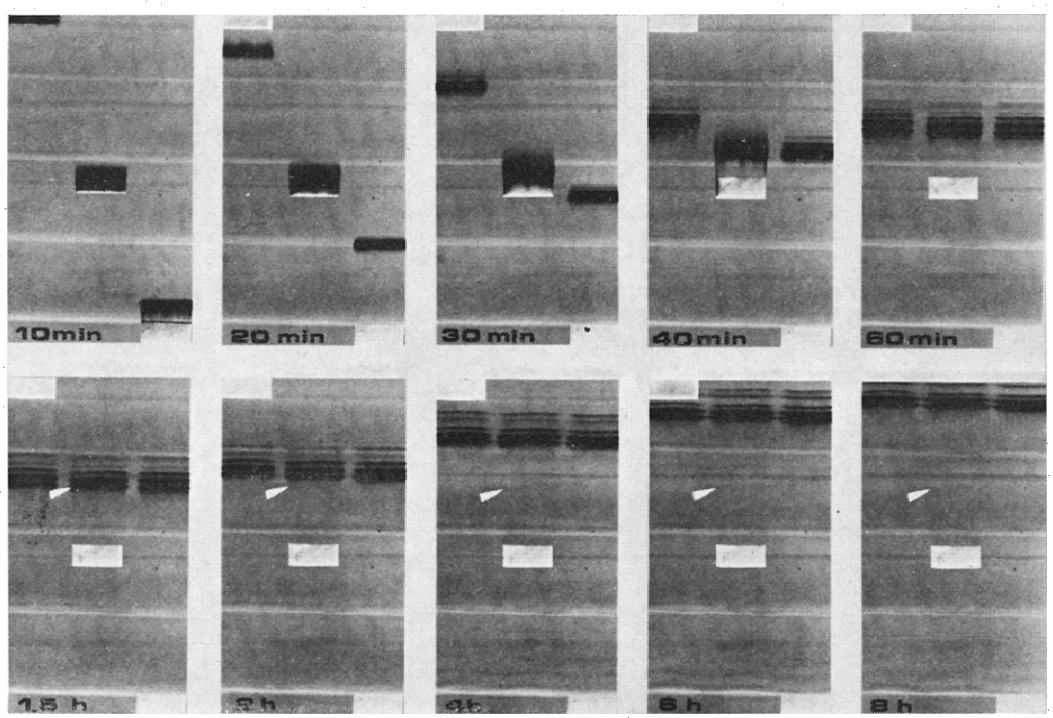


Fig. 18. Cathodic shift. Three haemoglobin samples (applied as cathodic, anodic and near isoelectric species) in the LKB 2117 Multiphor thin-layer equipment reach equilibrium in 1.5 h. The equilibrium position is marked with a white triangle. Thereafter, the focused bands begin to drift towards the cathode. After 8 h, the bands have almost migrated from the gel. (Courtesy of H. Davies; by permission of Butterworths Scientific Publishers. See ref. 76.)

cathode. After 8 h, the bands had almost migrated from the gel.

Most evidence indicates a multifactorial basis for the cathodic shift with many independent contributing variables. In addition to electroendosmotic effects, much of the problem may stem from the design of the apparatus. It is particularly prevalent in apparatus with large electrolyte chambers where appreciable convective mixing occurs. Local heating effects due to discontinuities in conductance between focused ampholytes may also aggravate the problem. Haglund[10] pointed out that this latter problem may also arise when pH ranges are used that do not include ampholytes buffering near pH 7. In this case, a zone of pure water will develop between the gel ampholytes and the electrolytes, which can be prevented by adding a small amount (*e.g.*, 1%) of pH 6–8 Ampholine. When the cathodic shift is appreciable, it is important to determine the minimum focusing time required for equilibrium conditions before banding patterns disintegrate. From this point of view, the analytical scanning technique of Catsimpoolas[13] appears to be very valuable.

4. APPLICATIONS

This section deals with various applications of GEF in biomedical research and gives examples of the discovery of heterogeneity in many apparently homogeneous

proteins. Where possible, attempts are made to define the structural basis for some of this heterogeneity.

A. Enzymes

By most criteria, IEF is a fairly mild procedure for protein fractionation. Although separations occur in salt-free media, the ampholytes serve to maintain proteins in solution and, because of their polyvalent nature, may afford greater stabilization than inorganic salts. Such a phenomenon has been shown for α-haemolysin, protease and a hexosaminidase from *Staphylococcus aureus*[77] and with DNA-dependent RNA polymerase[78]. In some instances, enzyme stabilization may be due to the formation of complexes between the carrier ampholytes and inhibitory heavy metals such as Cu^{2+}, Hg^{2+} and Pb^{2+}.

On the other hand, loss of activity may occur on prolonged exposure of an enzyme to unfavourable pH ranges at its p*I* or to chelation of necessary metal cofactors. Such losses in enzymatic activity can often be largely restored by incubation with the appropriate cofactor[79]. Additional problems may arise from the oxidation of cysteine and methionine residues to cysteic acid and methionine sulphoxides[80], but can be avoided by performing IEF in the presence of antioxidants such as thiodiglycol or ascorbic acid. The latter seems more effective and also prevents possible modification of tyrosine and arginine residues. These antioxidants can be incorporated directly into sucrose density gradients or added at the cathode during gel electrofocusing. Similar precautions have been used by Park[81] and Bunn[82] to analyze mixtures of oxy- and deoxyhaemoglobins. Many sulphydryl-dependent enzymes may also lose activity on electrofocusing unless they are kept in the reduced form. For their stabilization, the antioxidant should ideally carry no net charge in the p*I* range of the enzyme. 2-Mercaptoethanol, 2,3-dimercaptopropanol and dithiothreitol are effective in concentrations of about 10^{-3} *M* (ref. 83).

B. Glycoproteins

Glycoproteins from a variety of sources have been purified by IEF in sucrose density gradients or in gels[56,84-93]. Indeed, one of the first and most striking demonstrations of the high resolving power of gel electrofocusing came from the studies of a glycoprotein, L-amino acid oxidase, by Hayes and Wellner[56] in 1969. This glycoprotein contains galactose, mannose, N-acetylglucosamine, fucose and sialic acid. Disc electrophoresis of crystalline samples of this enzyme isolated from *Crotalus adamanteus* revealed three components, each of which appeared homogeneous when re-run. However, when analyzed by gel electrofocusing, these three electrophoretic components were resolved into 18 enzymatically active forms, isoelectric between pH 5.2 and 8.4 (Fig. 19). This heterogeneity does not seem to be due to partial loss or different redox states of the FAD cofactor or to metal chelation. Several of the isoelectric species appear to differ in amino acid composition. This heterogeneity may reflect genetic variation in the pooled sample, as venom from individual snakes may contain different combinations of the isozymic forms (Fig. 20). However, in addition to differences in primary structure, much of the heterogeneity may be attributed to variations in carbohydrate in several of the forms[94]. Such non-uniform distribution of carbohydrates may turn out to be a major cause of heterogeneity in other glycoproteins.

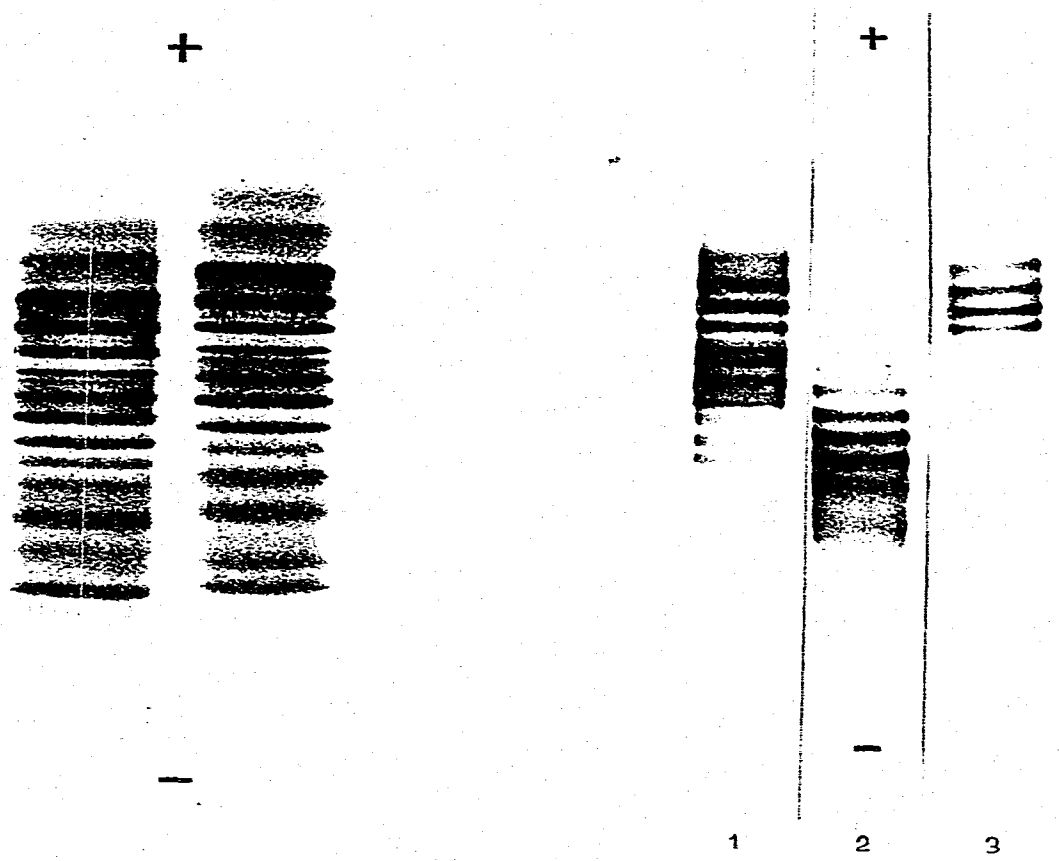


Fig. 19. Separation of L-amino acid oxidase isoenzymes by isoelectric focusing in gel. Crystalline enzyme from pooled venom was focused in 10-cm gels containing 2% of pH 3–10 carrier ampholites. The gels were stained for protein with Coomassie blue. Top: anode. (By permission of the New York Academy of Sciences. See ref. 94.)

Fig. 20. Isoelectric focusing of L-amino acid oxidase from single snakes. Venom samples from three snakes were submitted to isoelectric focusing and stained for enzymatic activity. The venom used in gel 1 was shown by electrophoresis to contain isoenzymes A, B and C; the venom in gel 2 had only C; the venom in gel 3 had only A. (By permission of the New York Academy of Sciences. See ref. 94.)

C. Immunoglobulins

The enormous heterogeneity of immunoglobulins is, as Williamson and colleagues[95,96] have stated and elegantly demonstrated, a problem worthy of the resolving power of IEF. Two types of protein heterogeneity arise in these proteins: (a) multiple species produced biosynthetically as the products of distinct structural genes, these immunoglobulins being closely related in sequence (alleles at one or more loci); and (b) microheterogeneity generated by post-synthetic modifications of a "biosynthetically homogeneous" protein. The heterogeneity of myeloma proteins is a good example of the latter case. A myeloma protein is the immunoglobulin product of a single

neoplastic plasma cell clone. The individual plasmacytoma synthesizes a single molecular species of immunoglobulin, which appears homogeneous on gel electrofocusing. However, after secretion into the serum, this single protein gives rise to a characteristic microheterogeneous isoelectric spectrum. Much of this heterogeneity seems to be due to post-synthetic loss of amide groups from glutamine and asparagine residues[95,96] although part may also arise from differences in carbohydrate content.

In the case of genetically determined heterogeneity in immunoglobulins, the purification to homogeneity of a single antibody can be a formidable task. For instance, in the case of inbred CBA/H mice, injected with 3-nitro-4-hydroxy-5-iodophenyl acetate (NIP) coupled to bovine γ-globulin, it has been estimated that the minimum number of anti-NIP molecules likely to be synthesized is about 8000. Most of these antibodies will focus over a range of only 2 pH units. As the present resolving power of gel electrofocusing is of the order of 0.005 pH unit, the maximum theoretical number of distinguishable bands would be about 400 (ref. 96). Thus, even at this high resolution, homogeneity of a single band is not assured. An example of this overwhelming complexity has been described by Hoffman *et al.*[97]. Fig. 21 shows the isoelectric spectra of anti-X_p antibody 2883 and of fractions thereof after separation by preparative IEF. Most of these fractions appear to band as discrete, single entities when analyzed by gel electrofocusing. However, after mild reduction and alkylation, a heterogeneous population of light and heavy chains is generated from each apparently homogeneous fraction[97] (Fig. 22). Purification of a single antibody, therefore, represents a very difficult task. However, by using antibodies to a restricted immune response, it may be possible to isolate individual species that meet several criteria of homogeneity.

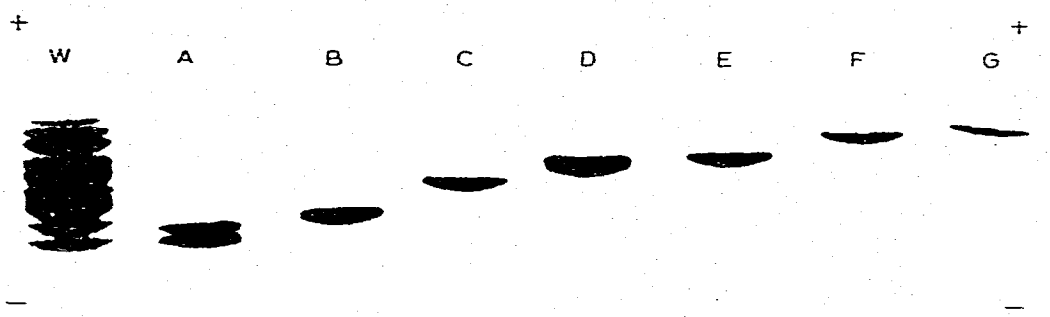


Fig. 21. Isoelectric spectra of anti-Xp antibody 2883 and fractions A to G taken from a sucrose density gradient electrofocusing separation. The cathode is at the bottom. Analytical electrofocusing was performed on disc gels. Staining of proteins was carried out with Coomassie blue. W: unfractionated antibody. (By permission of Williams and Wilkins. See ref. 97.)

Keck *et al.*[98] have developed an interesting method for detecting separated antibodies. They first precipitated the focused immunoglobulins in 18% sodium sulphate solution, then cross-linked the protein chains with glutaraldehyde. This is a well-known method used in X-ray crystallography in order to prevent crystal fractures[99]. The aldehyde forms a continuous copolymer, trapping and immobilizing the protein within the gel matrix, but without hampering the binding activity of the anti-

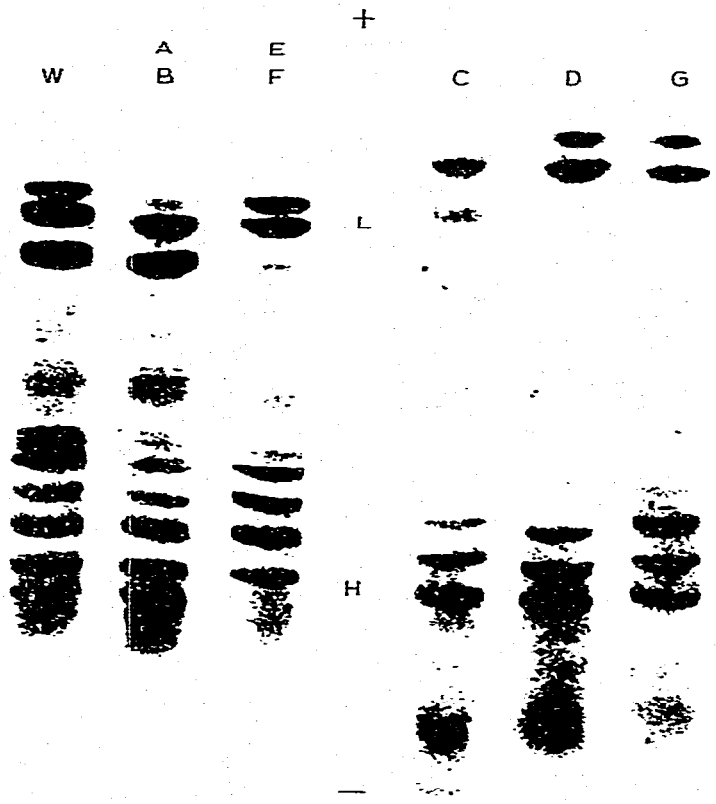
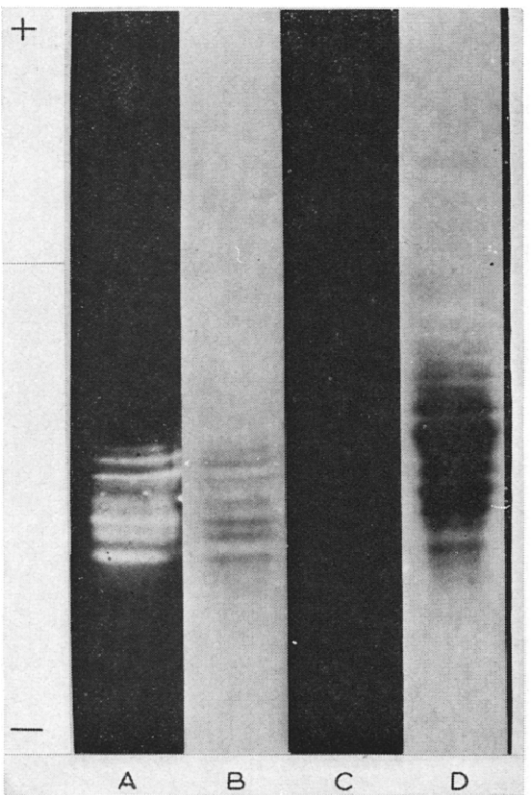


Fig. 22. Isoelectric spectra of mildly reduced and alkylated antibody 2883 and fractions A to G. From left to right: W, unfractionated antibody; A and B, pooled fraction; E and F, pooled fraction; fraction C; fraction D; fraction G. Light chains (L) focus at lower p*I* values than heavy chains (H). (By permission of Williams and Wilkins. See ref. 97.)

bodies. The antibody bands are then located by treating the gels with ^{125}I-labelled specific antigens. After washing out unbound radioactivity, the immune complex is detected by autoradiography. Fig. 23 shows an interesting example of this technique. The method can be extended to the use of fluorescent-labelled proteins and radio-labelled polysaccharide antigens as locators.

D. Lipoproteins

GEF has proved useful for analyzing and characterizing both native lipoproteins and their constituent polypeptide chains. The analysis of lipoproteins requires the presence of non-ionic detergents such as Tween 80, Emasol, Brij-39, Triton X-100 (ref. 100) or tetramethylurea[101] in order to maintain their solubility. Ionic detergents cannot be used as they do not allow equilibrium focusing. Kostner *et al.*[100] pre-stained human serum lipoproteins with Sudan black and obtained about eight distinct bands after focusing in gels containing 33% of ethylene glycol. The relationship of these various forms to those obtained by other methods was not determined. Multiple forms of lipoproteins presumably could arise through differences in bound lipids,



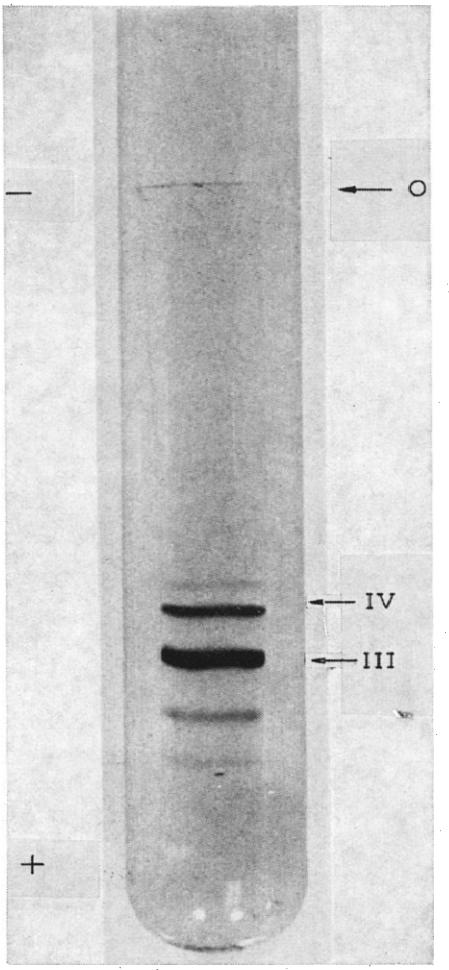


Fig. 23. Detection of electrofocused rabbit anti-4,1-aNS antibodies by treatment with ^{125}I-labelled 4,1-aNS ovalbumin. A and B, specifically purified rabbit anti-4,1-aNS antibody; C and D, normal rabbit IgG (DEAE-purified). A and C are radioautographs and B and D are patterns following staining with bromophenol blue. (By permission of Verlag Chemie. See ref. 98.)

Fig. 24. Gel isoelectric focusing of apo-HDL$_2$ in 6 *M* urea. (By permission of the New York Academy of Sciences. See ref. 103.)

the apoproteins, or in associated carbohydrate. In addition, although pre-staining greatly facilitates the detection of separated components, it might introduce an artificial heterogeneity from variations in dye binding.

Scanu and colleagues[102,103] also used IEF for analyzing human serum lipoproteins and their component polypeptides. A typical separation of apo-HDL$_2$ by GEF is shown in Fig. 24. The pattern given by GEF is considerably more complicated than that given by sodium dodecyl sulphate (SDS) gel electrophoresis, indicating heterogeneity in polypeptide chains of similar size. Gidez and Murnane[101] also found rat serum apolipoproteins to be more heterogeneous on GEF than is indicated by

SDS gel electrophoresis. This point is depicted in Fig. 25, which compares electrophoretic and electrofocusing analyses of unfractionated rat apo-HDL and sub-fractions separated by gel filtration. The unfractionated material resolved into about seven fractions on SDS gel electrophoresis but over twenty on GEF. The sub-fractions obtained by gel filtration permit a partial correlation of components displayed by both procedures. The reasons for the additional complexity seen in these experiments have not yet been established. However, it is significant that the complexity revealed in a single electrofocusing analysis of human apolipoproteins is of the same order as might be expected from combined techniques of gel filtration and ion-exchange chromatography[103].

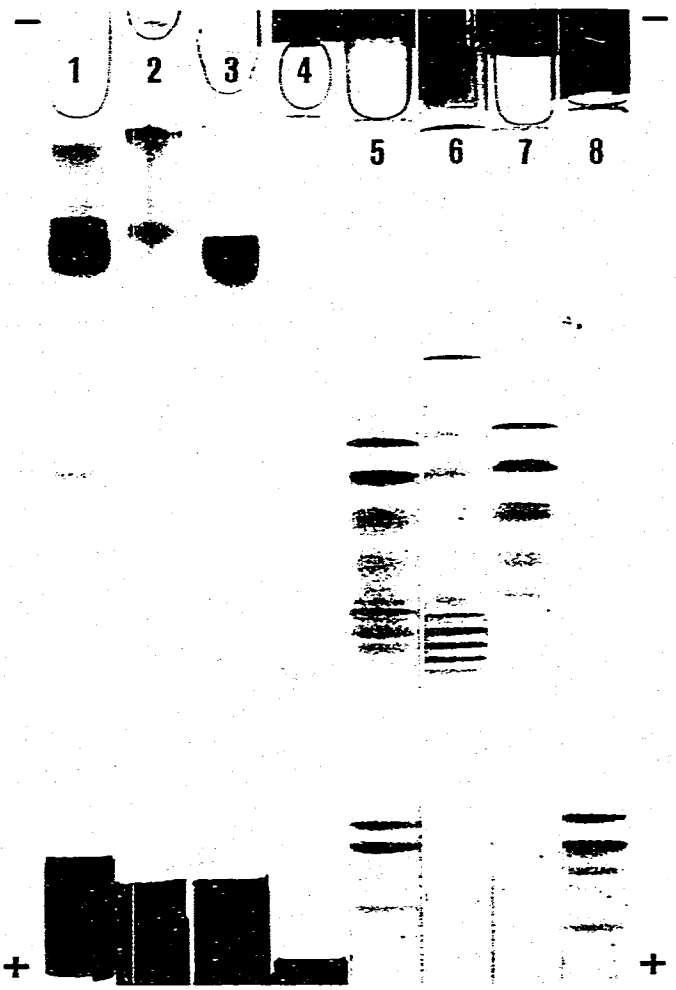


Fig. 25. Comparison of fractionation of rat apolipoproteins by SDS gel electrophoresis and by gel electrofocusing. Left to right: 1, apo-HDL unfractionated; 2–4, sub-fractions of apo-HDL separated by gel filtration (all analyzed by SDS gel electrophoresis); 5–8, gel electrofocusing patterns of 1–4. (L. I. Gidez and S. Murnane, in preparation[101].)

The present situation can be summarized as follows: (a) for lipoproteins, IEF is more useful as an analytical procedure because of the tendency of lipoproteins to precipitate at their p*I*s; (b) IEF may have wider applications for both analytical and preparative purposes with apolipoproteins; (c) highly reproducible separations of the apolipoproteins are obtained by focusing in gels containing 6–8 *M* urea in order to prevent aggregation, but at the risk of generating an artifactual heterogeneity by carbamylation of the polypeptide chain.

E. Membranes

At present, gel filtration and gel electrophoresis, both in the presence of SDS, have been the methods of choice for fractionating and characterizing membrane components. Recent experiments, however, indicate that IEF may provide a useful alternative. Jamieson and Groh[101] separated human erythrocyte and lymphocyte populations by IEF in sucrose density gradients. Similar procedures were used to isolate and characterize plasma membranes from human blood platelets[105]. Bonsall and Hunt[106,107] employed IEF to study interactions of human red blood cell (RBC) membranes with sodium trinitrobenzenesulphonate and surfactants. Several attempts have also been made to analyze disaggregated membrane components by IEF. Merz *et al.*[108] solubilized RBC ghosts in 8 *M* urea, 20 m*M* EDTA and 0.2% 2-mercaptoethanol, and fractionated the extract in 2.5% acrylamide gels, containing 12.5% of sucrose, 1% of pH 3–10 Ampholine and 8 *M* urea. Fig. 26 shows the results of thisseparation. Approximately 40 components, isoelectric between pH 5.90 and 8.25, were obtained by this method. A similar number of components are given by SDS electrophoresis in polyacrylamide gels[109]. It should therefore be possible to characterize these components with a two-dimensional technique (charge in the first dimension and size in the second, and so to assign both a p*I* and a molecular weight to each polypeptide).

F. Peptides

Although IEF would seem a useful method for separating both peptides and proteins, it is presently of limited applicability because of difficulties encountered in distinguishing and separating peptides from ampholytes of similar size and p*I*. Peptides with appreciable UV absorbance may, of course, be readily detected over the background noise of the ampholytes[43]. However, their subsequent separation from ampholytes by gel filtration or ion-exchange chromatography may prove difficult. Pre-labelling with chromophores such as dinitrophenol was used by Kopwillem *et al.*[110] to analyze a series of peptides of human growth hormone synthesized by the Merrifield technique. Fluorescamine may be an attractive alternative for pre-labelling peptides if the reaction proceeds to completion and only one product results. Occasionally, at high inputs, focused peptides may be detected as opalescent precipitates at their p*I* values.

Righetti and Coronelli[111] have developed a method for analyzing water-insoluble substances such as antibiotics by incorporating dimethyl sulphoxide (DMSO) into the gel. After focusing, the DMSO is removed by washing the gel in water where the focused antibiotics precipitate. The relative distribution of antibiotics can be obtained by scanning the gel at 600 nm. The antibiotics are subsequently eluted from the gel.

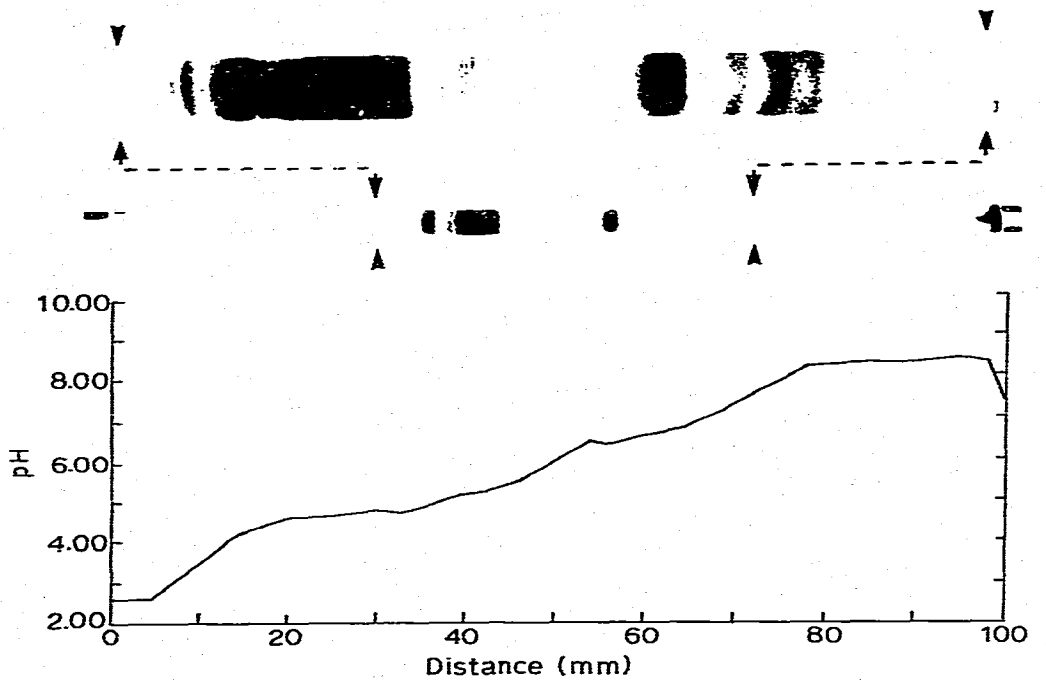


Fig. 26. Isoelectric focusing of human erythrocyte membrane components in 2.5% acrylamide gels containing 8 *M* urea, 12.5% of sucrose and 1% of Ampholine (pH 3–10). Sample load: 1 mg. Electrofocusing time: 24 h. (By permission of Academic Press. See ref. 108.)

G. Metalloproteins

In many respects, isoelectric focusing has proved to be of variable value in the analysis of metalloproteins. On the one hand, it has permitted the resolution of various classes of molecules that bind different levels of metal or in which the metal exists in different oxidation states. On the other hand, it can create an artifactual heterogeneity by metal chelation. The behaviour of transferrin is a good example of both possibilities. Wenn and Williams[112] and Van Eyk *et al.*[113] demonstrated three molecular species of transferrin, corresponding to molecules containing zero, one or two atoms or iron. Although all three species occur naturally, the relative amounts of each as observed by IEF may differ considerably from those in the original mixture. Medellin[114] found that both iron-containing forms were eventually converted into the apoprotein by chelation of iron by ampholytes on prolonged electrofocusing. As might be expected, the extent of this reaction is dependent on the relative amounts of ampholyte and metalloprotein present. Using small amounts of transferrin labelled with ^{125}I and ^{59}Fe, Medellin found no iron remaining on the transferrin after electrofocusing for 64 h. In contrast, all three forms of the protein were detectable at high transferrin inputs, together with two additional unstable forms. As one of the two latter forms was apparently a half-saturated species, it is possible that the two unstable forms represent different conformers, depending on which site is occupied by iron. Aisen *et al.*[115] have also distinguished two transferrin molecules by IEF, each containing only one atom of iron. A similar phenomenon seems to occur with the protein

metallothionein, which contains one Cd^{2+} and one Zn^{2+} moiety per molecule and which may be separated by electrofocusing into two forms, one cortaining only Cd^{2+} and the other both Cd^{2+} and Zn^{2+} (ref. 116). The extent of metal chelation seems to be largely dependent on the p*I* range of the protein, and seems more extensive in the case of proteins that focus below physiological pH ranges where binding of the metal ion to the protein is reduced.

Of course, not all metalloproteins suffer from these problems. A particularly interesting example is the iron-storage protein ferritin, which exists in cells in combination with vastly different amounts of iron, up to 2500 atoms per molecule. The iron is present as an iron(III) oxyhydroxide micelle inside a spherical multimeric protein shell, but can readily be removed after reduction. Most tissue ferritins appear homogeneous on electrophoresis, but are resolved into multiple isoferritins by IEF. However, despite the large and variable iron contents of these isoferritins, the multiple forms are not due to differences in iron content, either pre-existing or resulting from chelation by ampholytes[117–119]. Recent evidence suggests that much of the heterogeneity represents hybrid molecules fashioned from multiple sub-unit types[120].

In addition to the possibility of introducing an artifactual heterogeneity through metal chelation, there is also the possibility of generating multiple forms with different redox states of the metal cofactor. Vesterberg[121] and Satterlee and Snyder[122] fractionated met-myoglobin into nine components, six of which contained iron(III), while the other three contained iron(II). These latter forms seem to arise from interaction with ampholytes and riboflavin or persulphate[123]. On the other hand, this does not seem to happen with all haemoproteins, for example, GEF may be used to quantitate oxidized forms of haemoglobin, such as occur in methaemoglobinaemia, in the presence of Fe^{2+} oxyhaemoglobin. Such studies have also demonstrated interesting intermediates in the formation of methaemoglobin. In preparations that contain partially oxidized haemoglobins, one might predict the existence of three species corresponding to oxyhaemoglobin ($\alpha_2^{2+}\beta_2^{2+}$), methaemoglobin ($\alpha_2^{3+}\beta_2^{3+}$) and perhaps intermediate half-oxidized forms in which the haem on either the α- or β-chain is oxidized[124]. Four species are evident on GEF, of which the middle two have been tentatively identified as $\alpha_2^{3+}\beta^{2+}$ and $\alpha_2\beta^{3+}$ (Fig. 27)[125].

H. Clinical applications

IEF is finding increasing application in routine clinical procedures, which can be largely attributed to the use of gels that allow simultaneous fractionation and rapid evaluation of multiple samples at low cost. The technique is likely to be of considerable value in the analysis of genetic variants. GEF has proved useful for detecting haemoglobinopathies as it permits the separation and quantitation of several haemoglobins that can not readily be achieved by electrophoretic procedures. Fig. 28 shows typical profiles given by some haemoglobin variants. Of particular interest to the diagnosis of sickle cell disease is the good separation of haemoglobins A, S and F. By focusing in glass tubes, the relative amounts of the various forms can be quickly assessed by densitometric scanning[126]. Although GEF will separate many known variants, it is interesting that it does not allow a clear distinction between haemoglobins A_2, C or E. These haemoglobins have very similar p*I*s, despite large differences in their primary structures[121]. On the other hand, as most haemoglobins are isoelectric near pH 7, a large number of expected variants with neutral amino acid substitutions may be

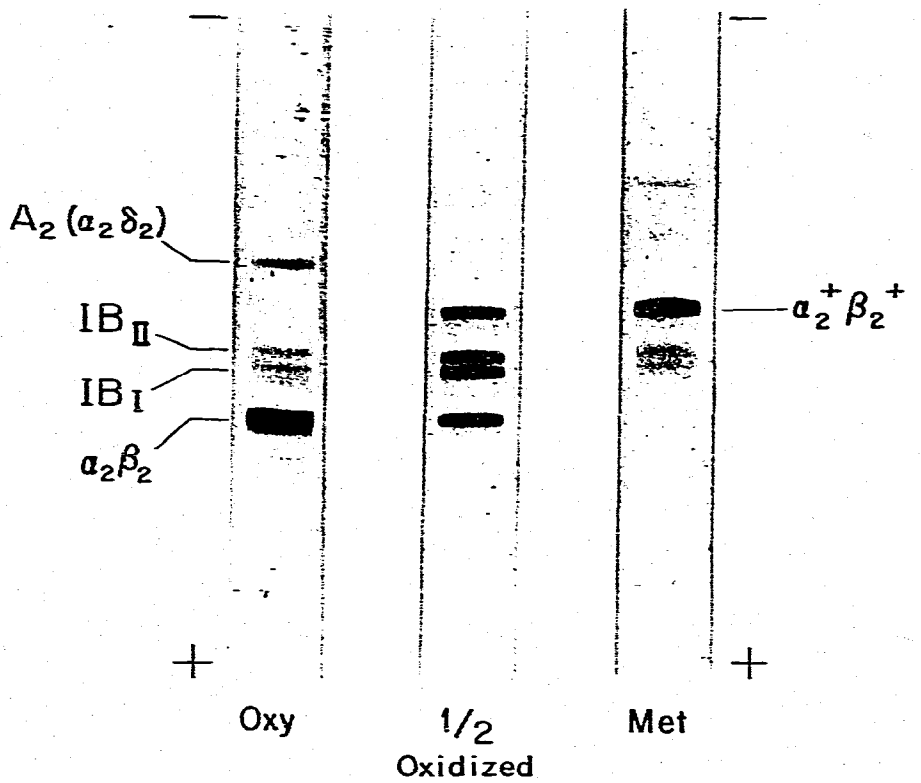


Fig. 27. Gel electrofocusing patterns demonstrating partially oxidized haemoglobins IB_I and IB_{II}, separated from oxyhaemoglobin and methaemoglobin (pH 6–8). (By permission of ASP Biological and Medical Press. See ref. 125.)

detectable by GEF, if such substitutions alter the molecular configuration of the protein. By focusing in a thin gel slab at high pulsed voltage, Righetti and Bianchi Bosisio Righetti[127] screened as many as 100 haemoglobin samples a day.

GEF will probably also be useful for analyzing tissue extracts and body fluids. Beeley[128] fractionated human salivary proteins from parotid, submandibular and mixed salivas. The proteins, which are isoelectric between pH 5 and 8, give gland-specific patterns. Rotbol[129] analyzed human urinary proteins from normal individuals and from patients with chronic pyelonephritis. Marked changes, both qualitative and quantitative, were detected in the regions of albumins and γ-globulins in the diseased state.

Using a two-dimensional technique involving IEF followed by gel electrophoresis, Dale and Latner[130] analyzed sera from patients suffering from IgG and IgA type myelomatosis and from cirrhosis. In type IgG myelomatosis, they found a marked increase within the lower IgG arc, a diminution of the remaining IgG area and a virtual absence of IgA. In myelomatosis type IgA, there was a distinct increase in the IgA area and a virtual absence of IgG. In cirrhosis, both the IgA and IgG areas were more pronounced than those in normal sera. Latner[131] applied this method to

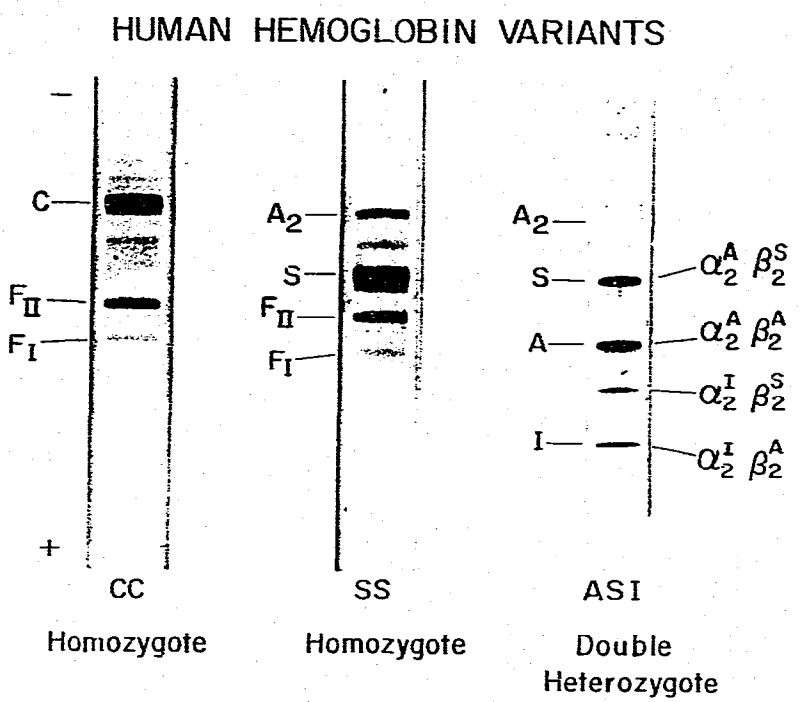


Fig. 28. Gel electrofocusing patterns of human haemoglobin variants (pH 6–8). (By permission of ASP Biological and Medical Press. See ref. 124.)

the analysis of tissue fluids from a number of human diseases and obtained characteristic protein maps for several diseases.

GEF has been useful for isolating and characterizing human α-fetoprotein. This foetal globulin is present in the serum of many patients with primary hepatoma and in children with embryonal carcinoma of the gonads. Its presence in serum is used in the serodiagnosis of these malignancies. Alpert *et al.*[132] separated two major molecular forms of α-fetoprotein by GEF, with p*I*s of 4.85 and 5.2. Both forms are present in most hepatoma and foetal sera, but their biological relationships are unknown. The two forms are converted into an apparently homogeneous form of higher p*I* after neuraminidase treatment, which suggests that they differ in sialic acid content.

Unexpectedly, GEF has not yet been routinely applied to serum protein analyses, a frequently used diagnostic procedure. Perhaps the major reason has been difficulties in staining proteins with high sensitivity. This should no longer be a problem with recently developed procedures, particularly when thin gel slabs are used, with which staining and destaining can be achieved in a few hours. Considerable work will, however, be required in order to characterize normal protein patterns so as to identify components of particular interest among the many additional components revealed by GEF.

1. Isoelectric focusing as a probe of interacting protein systems

GEF promises to be of value in studying interacting protein systems. It can

provide information on the interactions between protein molecules or sub-units and between proteins and small molecules, including conformational changes that may accompany these interactions. Ultimately, these findings may offer a better insight into the molecular basis of cooperative interactions in allosteric proteins and in larger biological systems.

Owing to its availability in a pure form and its intense colour, haemoglobin is a particularly favourable model for such studies. Park[81] and Bunn[82] investigated sub-unit exchange between several human haemoglobins and the formation of mixed tetramers between human and canine haemoglobins. These intermediate forms could be detected only by using deoxyhaemoglobins, focusing under strictly anaerobic conditions and at low temperature. Fig. 29 demonstrates an example of mixed tetramer formation. Hybrids from oxyhaemoglobin are demonstrable but dissociate rapidly during electrofocusing[126]. Park also used GEF to study the dimerization plane in deoxyhaemoglobin and in oxyhaemoglobin, and its pH dependence. These studies showed that both liganded and unliganded haemoglobins cleave along the same plane at pH 7 and 10.6. Such observations on sub-unit exchange may provide valuable information about sub-unit organization in oligomeric proteins.

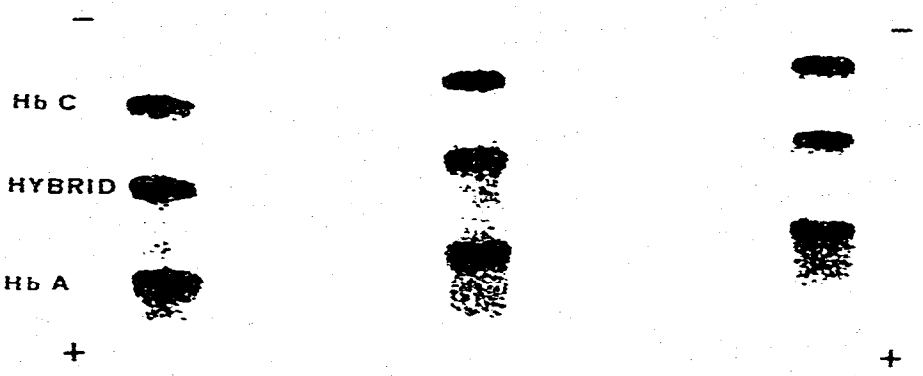


Fig. 29. Demonstration of mixed tetramers in haemoglobin. Each gel contains a mixture of CO-HbC and CO-HbA, both purified by starch block chromatography. The bands have just reached their isoelectric point. The gels (2 mm in diameter) were pre-focused for 2 h at 1.2 mA per tube. The samples were focused at 800 V. (By permission of the New York Academy of Sciences. See ref. 81.)

Haemoglobin is also useful for studying interactions between macromolecules and small molecules. Thus Park[81] and Bunn[82] were able to demonstrate the conformational change associated with the Bohr effect by separating deoxyhaemoglobin and oxyhaemoglobin by gel electrofocusing from a mixture of partially oxygenated molecules. At equilibrium, deoxyhaemoglobin is detected as a purple-violet band (p*I* 7.15) above the red oxyhaemoglobin (p*I* 6.95). A unique advantage of the focusing system occurs in those studies which combine equilibrium and kinetic processes. For example, after a protein is focused, ligands, chemical reactants or interacting proteins can be passed through the gel and various kinetic processes can be studied. In this way, Park[81] was able to study the binding of ATP to haemoglobin and to calculate the stoichiometry of the complex. Similar approaches may be helpful in studying the molecular basis of cooperative interactions in allosteric proteins.

Another example of macromolecule–ligand interaction is evident from the shift in p*I* values of concanavalin A on binding carbohydrates. This substance possesses hemagglutinating and mitogenic activities, regulates the growth of transformed fibroblasts and binds to cell surfaces. Thus, the study of its interactions may shed light on its mode of action on cells. Crystalline preparations of concanavalin A show a range of seven or eight components, isolectric between pH 5.9 and 8.0. When preincubated with increasing amounts of D-mannose or α-methyl-D-glucoside, there is a progressive shift of the lower p*I* bands toward the band at pH 8.0. At high concentrations of these ligands, the conversion is almost complete (Fig. 30). On the other hand, D-galactose, which binds only poorly to concanavalin A, has much less effect. As the former ligands are uncharged molecules, these pronounced p*I* shifts may result from conformational changes in concanavalin A such as have been demonstrated by measuring circular dichroic spectra[133].

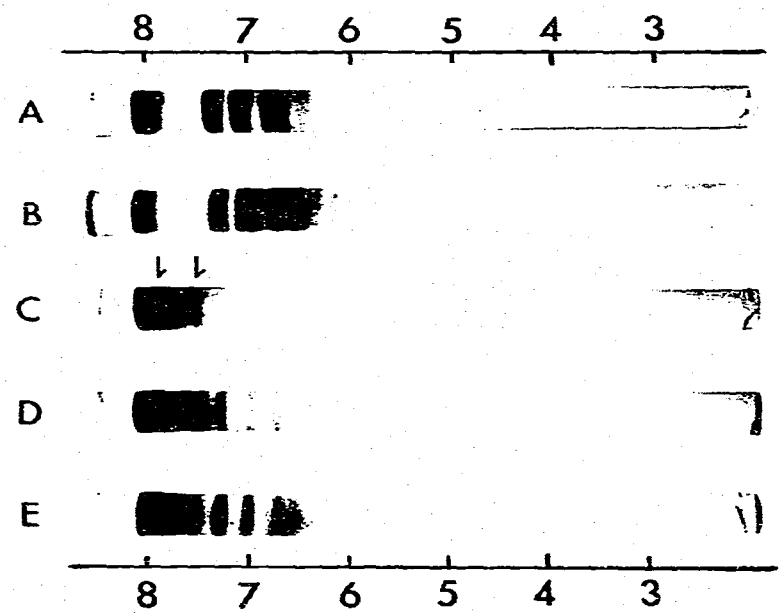


Fig. 30. Isoelectric focusing of concanavalin A pre-incubated with sugars. Concanavalin A (1.2 mg ml) was pre-incubated with sugars (0.06 *M*) for 30 min at 37 . A, with D-galactose; B, with no sugar (control); C, with D-mannose; D, with α-methyl-D-glucoside; E, with D-glucose. Proteins were stained with Coomassie blue. Numbers indicate pH gradients along the gel. Arrows indicate protein bands produced by pre-incubating concanavalin A with specific sugars. (By permission of Academic Press. See ref. 133.)

IEF may also offer an alternative means of studying enzyme–substrate and enzyme–coenzyme complexes. This possibility was suggested from studies of an apparently pure preparation of folate reductase, which unexpectedly resolved into three enzymically active components on GEF (Fig. 31). These multiple forms were not artifacts. As analyzed originally, the enzyme preparation contained both substrate and cofactor, added as stabilizing agents, and these substances were found to be the

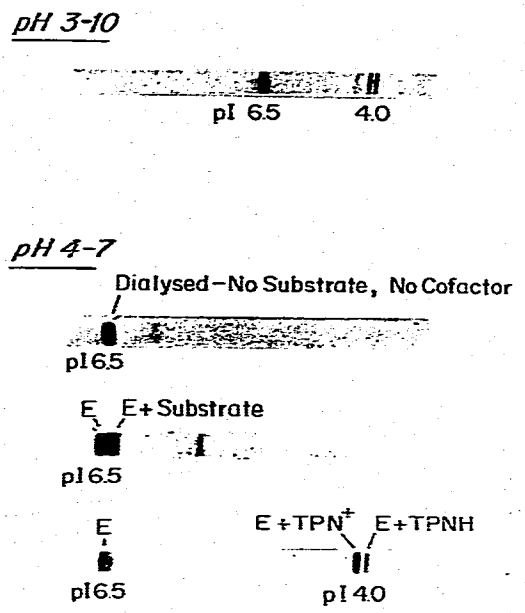


Fig. 31. Isoelectric fractionation of enzyme, enzyme–substrate and enzyme–coenzyme complexes. (By permission of the New York Academy of Sciences. See ref. 36.)

cause of the apparent enzyme heterogeneity. When the substrate and cofactor were removed, only a single component, with a p*I* of 6.5, was obtained. Upon addition of half-saturating amounts of substrate, another band of similar p*I* was generated. Upon addition of coenzyme, two additional bands with p*I*s close to pH 4 were obtained. Spectral analysis indicated that one corresponded to E + TPN$^+$ and the other to E + TPNH[36].

IEF has also been found to be particularly useful in monitoring chemical modifications that affect the net charge of proteins and in determining the homogeneity of the product. Bobb and Hofstee[134] and Bobb[135] studied the progressive carbamylation (reduction of positive charges) and maleylation (replacement of positive with negative charges) of chymotrypsinogen A. By following the successive decrease in p*I* with increasing additions of carbamyl or maleyl groups, they were able to construct an "isoionic titration" curve, relating the p*I* value to the actual number of added substituents. Fig. 32 shows the pattern obtained by progressive maleylation of chymotrypsinogen A: it can be seen that the p*I*s decrease from the three native forms (p*I* 8.8–9.6) to p*I* 2.4 for the maximum maleylation number. However, the product at each step of maleylation is highly heterogeneous.

5. TWO-DIMENSIONAL PROCEDURES

A. Immunoelectrofocusing

By using the high resolving power of gel electrofocusing rather than gel electrophoresis, one might expect to obtain additional information from immunoelectrophoretic analyses. The feasibility of immunoelectrofocusing is evident from the early

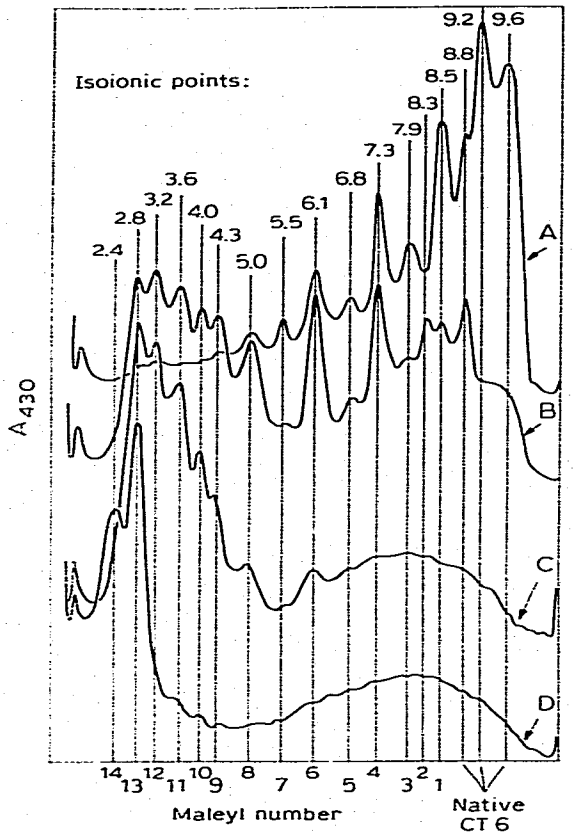


Fig. 32. Conversion of chymotrypsinogen A into maleylated derivatives as shown by gel isoelectric focusing in the pH 3–10 region. The gels were stained with bromophenol blue and scanned at 430 nm. The isoionic points shown at the top of each curve were estimated from the position of the peak (determined on duplicate gels scanned at 280 nm) with respect to the pH gradient. In C and D, the bulge in the curve in the alkaline region is a staining artifact. These preparations had an average of 3.1 (A), 6.8 (B), 9.4 (C) and 13.7 (D) amino groups per molecule blocked by maleylation. (By permission of the New York Academy of Sciences. See ref. 135.)

work on agarose gels by Riley and Coleman[133] and Catsimpoolas[136] but is unlikely to become a standard procedure until agarose preparations of low electro-osmotic flow become available. Until then, polyacrylamide gel of high porosity, *e.g.*, 3.5%, would seem a more appropriate medium. Immuno-IEF has been described in which the initial protein separation is achieved by IEF in polyacrylamide gel, followed by immunodiffusion or immunoelectrophoresis into agar gels in the second dimension[137] (see Fig. 33). After focusing, the gel rod is embedded between parallel trenches in an agar gel. The trenches are filled with antiserum, which forms precipitating arcs with proteins diffusing from the gel rod. Alternatively, the focused gel may be embedded in an antibody-impregnated agarose gel, then subjected to electrophoresis at right-angles to the IEF run. Antigen–antibody complexes form conical-shaped precipitating zones in which the migration distance of the leading boundary edge gives a measure of antigen concentration (Laurell's rocket technique[138]). This technique not only

quantitates antigens but can also give qualitative information about possible immunological relationships.

A third variation of the immuno-IEF technique is to incubate the focused gel directly in an appropriately buffered solution containing a specific antiserum[139]. In this instance, it is particularly advisable to use gels of high porosity, *e.g.*, 4%, so as to allow rapid penetration of the large antibody molecules. After incubation for 24 h at room temperature, non-precipitated proteins and excess of serum are removed by exhaustive washing. The remaining immunoprecipitates can be detected directly or after staining.

In addition to immunological techniques with whole gels, antigens can be located in squashed gel segments[140] or in macerated gel fractions (Fig. 16) by double diffusion, radial immunodiffusion or immunoelectrophoresis (Fig. 33).

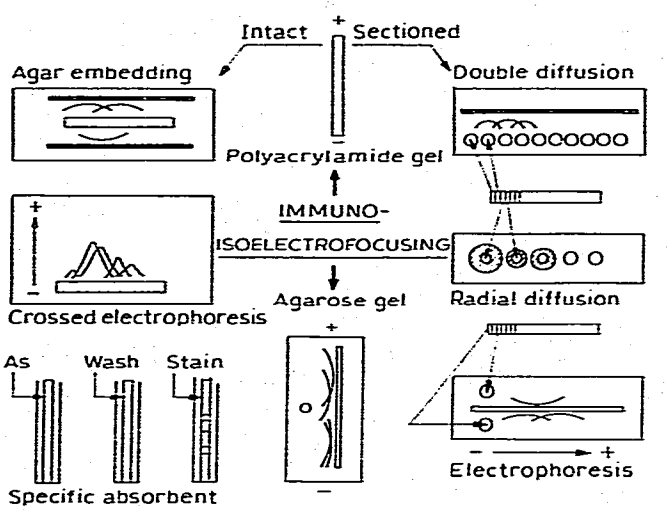


Fig. 33. Various forms of immunoisoelectric focusing analysis in gels. (By permission of the New York Academy of Sciences. See ref. 137.)

B. Isoelectric focusing–gel electrophoresis

Dale and Latner[130] and Latner[131] demonstrated the potential of this technique in detecting qualitative and/or quantitative changes in protein maps from normal and pathological sera, which were not apparent by either gel electrophoresis or gel IEF alone. Such "protein maps" or "protein prints" are valuable for genetic studies in assigning phenotypes to the chromosomes that control their synthesis. Wrigley and Shepherd[141] mapped wheat grain proteins from several wheat varieties. An extract of gliadin proteins, when analyzed by SDS gel electrophoresis or by gel IEF alone, was resolved into about 20 components. However, when analyzed by a two-dimensional technique (gel IEF followed by electrophoresis), twice as many components were revealed. This indicates that many apparently single zones, separated by either method alone, may in fact be heterogeneous. Fig. 34 shows an example of this two-dimensional separation: above is the pattern from gel IEF and on the left the pattern from starch gel electrophoresis.

C. Isoelectric focusing–electrophoresis in gel gradients

This technique is a variation of the two-dimensional procedure of Margolis and Kenrick[142] in which proteins are separated in the first dimension according to charge by electrophoresis in 2–3% acrylamide gels, then in the second dimension according to size by electrophoresis in a gel of decreasing pore size, towards a "pore limit". Kenrick and Margolis[143] modified this technique by performing gel IEF for charge separations in the first dimension. This gel is then embedded into the top of a

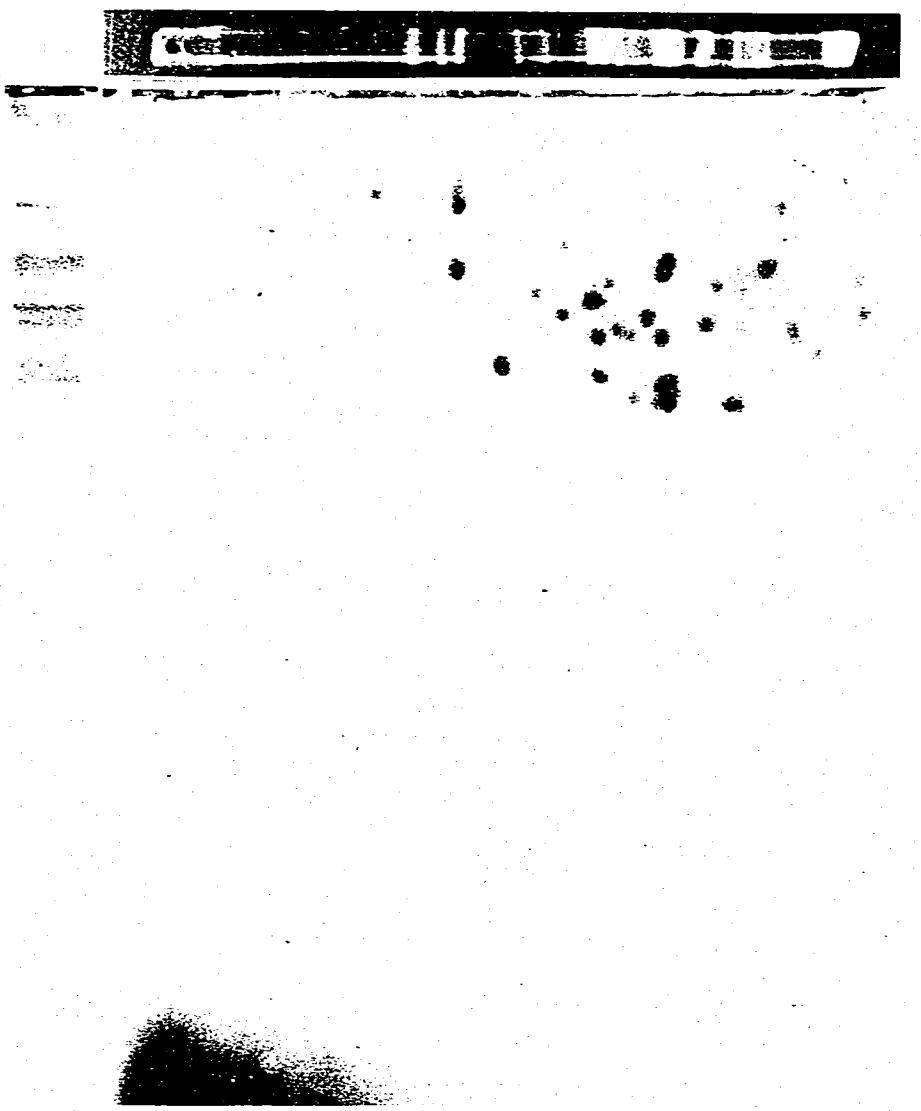


Fig. 34. Protein map of wheat grain protein. Above is the pattern for gel isoelectric focusing alone (pH 5 (left) to pH 9, 120-mm gel). On the left is the pattern for starch gel electrophoresis alone (origin at top). Note that the two-dimensional technique resolves nearly twice the number of components separated by either method alone. (By permission of Academic Press. See ref. 143.)

concave 4.5–26% gradient gel slab and the focused proteins are separated in the second dimension according to size by electrophoresis. This technique generally gives more information than IEF–gel electrophoresis and disc electrophoresis–gradient gel electrophoresis. In addition to the increased resolution, one obtains estimates of both the p*I* and the molecular weight of the separated components. Moreover, if the shape of the gel gradient is known, it is also possible to calculate the Stokes radius of the macromolecule being analyzed. The validity of gradient gel electrophoresis as an equilibrium technique has been recently challenged[144]. However, if the system is run for a sufficiently long period, it is at least an "approach to equilibrium" method and seems to give good estimates of molecular weight[145]. Fig. 35 shows some results obtained by this two-dimensional technique.

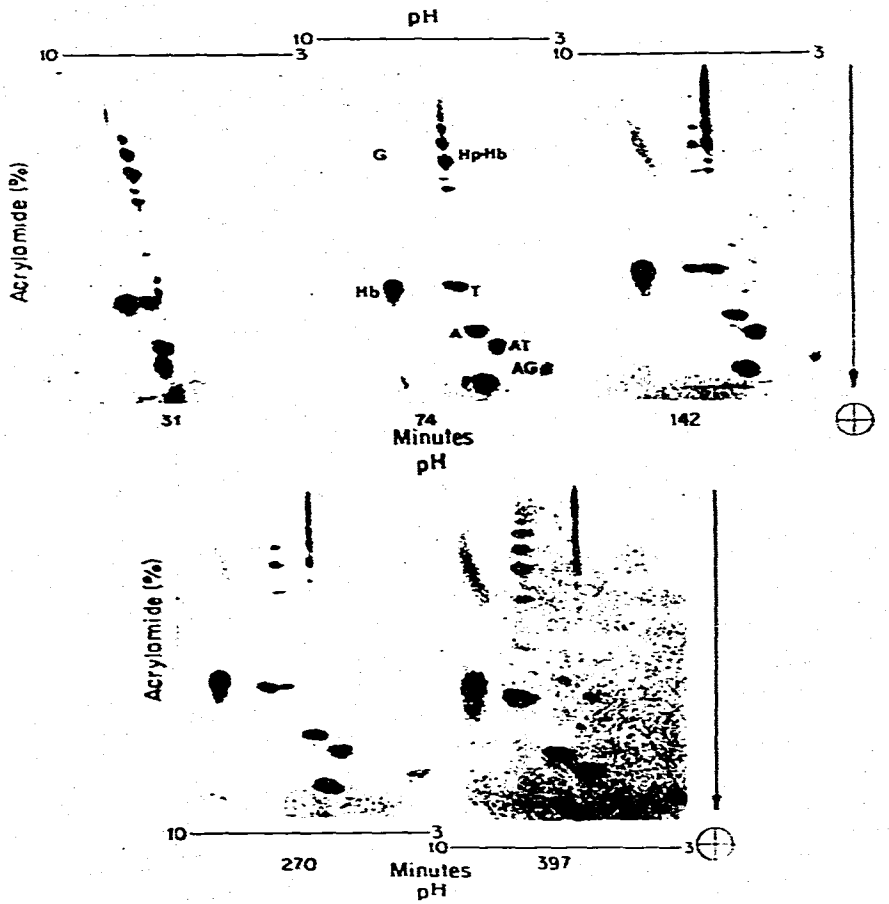


Fig. 35. Two-dimensional separation of a mixture of serum proteins in a pH 3–10 gradient for 31, 74, 142, 270 and 397 min, respectively, in the first stage and 4.5–26% acrylamide concave gradient gel for 20 h in the second stage. Hp–Hb, haptoglobin–haemoglobin complex; Hb, free haemoglobin; T, transferrin; A, albumin; AT, α_1-antitrypsin; P, prealbumin; G, immunoglobulin (IgG); AG, α_1-acid glycoprotein. (By permission of Academic Press. See ref. 143.)

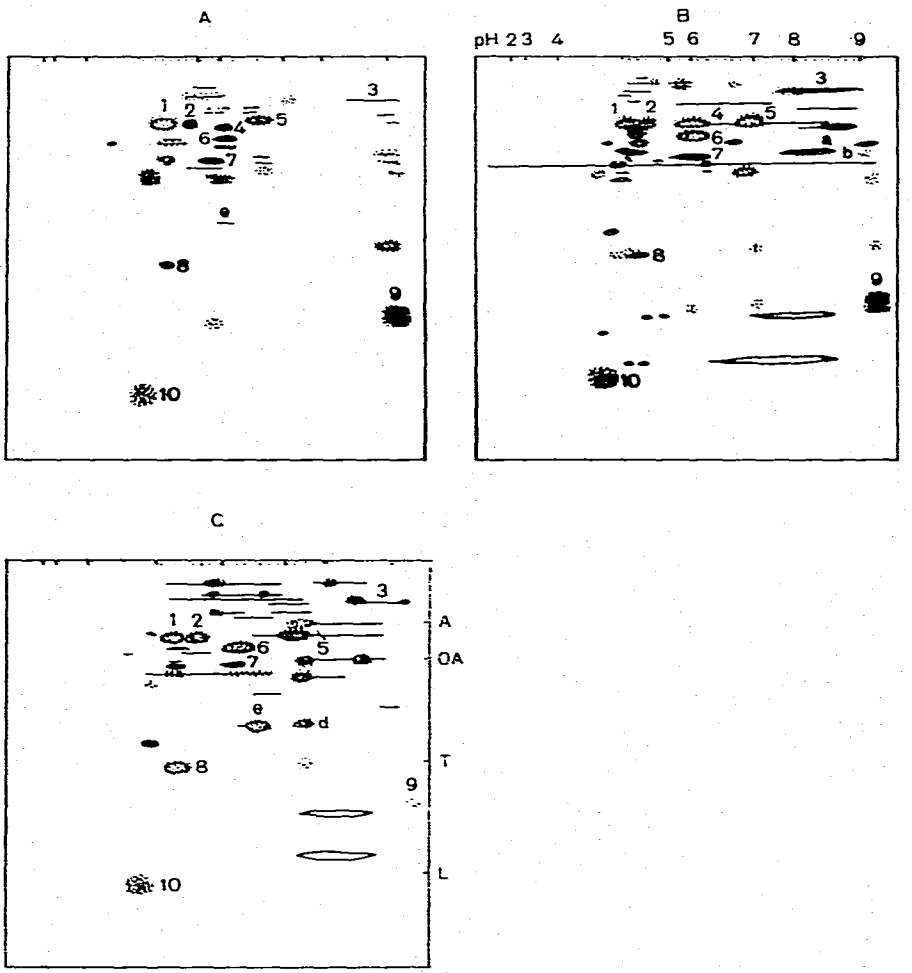


Fig. 36. Two-dimensional gel electrophoresis of non-histone protein fractions prepared by chromatography of mouse chromatin on hydroxyapatite. The horizontal axis represents isoelectric focusing in the first dimension, the range of the pH gradient being indicated as shown in B. The vertical axis shows the separation in the second dimension using electrophoresis in SDS, the position of proteins of known molecular weight being indicated in C for albumin (A), ovalbumin (OA), trypsin (T) and lysozyme (L). Proteins stained with Coomassie blue are shown as lines or filled areas. The position of ^{32}P-labelled proteins is shown by grains superimposed on lines or filled areas; or by grains alone where no protein was detectable by staining. (A) Liver; (B) kidney; (C) brain. (By permission of Springer Verlag. See ref. 148).

D. Isoelectric focusing–SDS gel electrophoresis

This technique may prove a useful alternative to IEF–gradient gel electrophoresis for obtaining additional information about quaternary structure and polypeptide molecular weights[146]. After IEF, the focused proteins are subjected to SDS gel electrophoresis in the second dimension. Barret and Gould[147] and MacGillivray and Rickwood[148] applied this technique to characterize non-histone proteins in chro-

matin. After reduction and alkylation, the proteins were fractionated by IEF in gel rods. The separated proteins were first denatured, then dissolved in 1% SDS for electrophoresis in the second dimension in a gel slab containing SDS. Fig. 36 illustrates the results obtained by this method for comparison of non-histone proteins from mouse chromatin. The accuracy of the method for these proteins was given as ± 3000 in molecular weight units and ± 0.2 pH units for p*I*. It should be noted, however, that not all multimeric proteins may dissociate completely by this method and that additional procedures might be necessary in order to ensure complete denaturation and degradation.

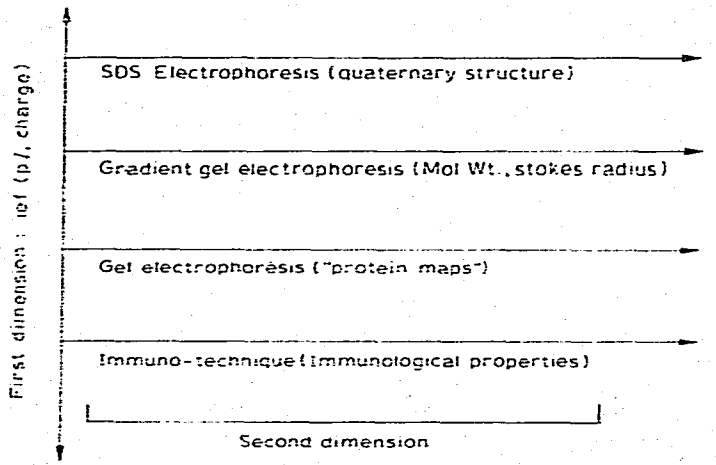


Fig. 37. Summary of various two-dimensional techniques.

Fig. 37 summarizes the various features of the four two-dimensional techniques described above. Judicious use of these methods will often define, in a few simple experiments, several physico-chemical parameters of the protein under study.

6. TRANSIENT STATE ISOELECTRIC FOCUSING (TRANSIF)

TRANSIF is a new kinetic method developed by Catsimpoolas[142, 151] from his technique of "analytical scanning IEF"[43], which gives quantitative information about parameters pertaining to experimental aspects of IEF and to the physical characterization of amphoteric molecules. TRANSIF data are obtained by coupling a scanning IEF assembly to an on-line digital data acquisition and processing system which continuously monitors and records changes in peak position, peak area, segmental pH gradient and p*I*. In contrast to IEF, which is a steady-state method, TRANSIF is essentially a kinetic method characterized by three different stages: initial focusing (IF), defocusing (DF) and refocusing (RF). The IF stage involves the electrophoretic migration of the species towards the p*I* and the attainment of steady-state focusing. The DF process is performed in the absence of current, where zone spreading is assumed to proceed by diffusion. Finally, in the RF stage, the electric field is re-applied and the distribution re-approaches the steady state.

TRANSIF has been very useful in determining the minimal focusing time for a protein zone, and has also provided quantitative data on the instability of the pH gradients with time, the so-called "plateau phenomenon"[69] or "cathodic drift"[36]. In agreement with our previous report[23], Catsimpoolas also found that the plateau phenomenon results from a progressive migration of the more basic ampholytes towards the negative electrode. The anodic zones remain essentially unaltered for periods of up to 10 h (ref. 149).

The DF stage in TRANSIF has been used in order to assess the diffusion coefficient in proteins. In following the rate of zone spreading in the absence of an electric field, the measurement of variance (σ^2) of the diffusing zone as a function of time and gel concentration yields a linear relationship, the slope of which corresponds to the apparent diffusion coefficient (D) of the protein. However, measurements of diffusion constants given by this method give substantially higher values than the free diffusion coefficient (D_0) calculated from ultracentrifugation data. It appears, therefore, that the apparent D measured by DF in polyacrylamide gels may not represent a true diffusion coefficient but a "dispersion coefficient"[152]. However, Catsimpoolas predicted that it should be possible to overcome this problem so as to obtain a true diffusion coefficient by his method. Moreover, by using the retardation coefficient measured at various gel strengths, information may also be obtained about the effective molecular radius and molecular weight of the protein.

7. DISCUSSION

The examples of applications of gel electrofocusing described above demonstrate the remarkable resolution that can be obtained with this technique and its advantages over other procedures for separating proteins on the basis of their surface charge. Although these features are widely recognized, the true potential of GEF is not always realized in practice. Perhaps the greatest problems have arisen from difficulties in developing sufficiently stable pH gradients to overcome molecular sieving effects. It must always be remembered that meaningful and reproducible results can be obtained only if the system allows all amphoteric species to reach their pI in the time available for the experiment. Gradient instability has often necessitated curtailing electrolysis periods to the extent that true isoelectric fractionations of large proteins seem unlikely. These problems have now been largely resolved by GEF systems that involve the use of thin rods of polyacrylamide gel. In our analytical system, pH gradients are established after 4 h and, if undisturbed, remain essentially constant for at least 24 h. As most proteins of molecular weight 500,000 or less will reach their equilibrium positions after 6 h, there is no need to compromise with electrolysis periods and experiments can be left safely to run overnight in order to ensure equilibrium focusing. More rapid separations may be obtained at lower gel temperatures and with higher potential differences. For example, Righetti and Bianchi Bosisio Righetti[127] and Bunn[126] reported equilibrium focusing of haemoglobins (molecular weight 68,000) in this system in about 1 h by using a pulse power supply to reduce heating effects at high potential differences.

For reasons that are still obscure, it is more difficult to obtain stable pH gradients in gel slabs, especially those run in a horizontal plane. Although we cannot account for this anomaly, it might be related to the formation of a very dense layer

between the top of gel rods and the catholyte after a short electrolysis period. This layer could be the most basic ampholyte species or an electrolysis product that prevents convective mixing in the vertical tubes. Although troublesome, instability of pH gradients in gel slabs need not prevent equilibrium focusing if the cathodic shift occurs after proteins have reached their p*I*s. However, instability of pH gradients may lead to considerable variability in banding patterns from day to day. The apparent discrepancies that often result can be reconciled only by characterizing the separated components by their p*I* values rather than their R_F values.

Several of the advantages of GEF are self-evident from the examples given above. Much of the finer resolution and superior resolution of GEF is attributable to its "built-in" resolution, whereby all molecules that differ in p*I* by as little as 0.01 pH unit will be separated. In addition, the focusing action allows the detection of minor components that may not be detected by other non-equilibrium methods. For example, during electrophoresis, protein bands generally become more diffuse with time and trailing edges may obscure minor components. In contrast, protein bands separated by GEF become sharper with time as their band density increases. Consequently, the gels can be overloaded with major fractions in order to facilitate the detection of components that may constitute as little as 0.2% of the total protein[121].

Since the advent of IEF, and particularly GEF, there have been many demonstrations of heterogeneity in proteins that appear homogeneous by other criteria. This has led many investigators to reevaluate the present criteria of homogeneity —or to take a strong position against IEF by rejecting the new-found heterogeneity as artifactual unless demonstrated by another method. The situation is reminiscent of the sceptics who blamed artifacts when new subcellular components were revealed by the electron microscope because they were not apparent under the light microscope. In this review, we have presented several examples of electrophoretically "pure" proteins that are resolved into multiple forms on GEF. In many of these cases, it has been possible to establish the basis of the new heterogeneity. As is apparent, there are many ways in which charge heterogeneity in proteins may arise, and not all need involve primary structures. Among other factors are post-synthetic modifications, ligand binding, different redox states in metalloenzymes and variations in prosthetic groups or non-protein components. Many of these differences may be considered trivial and an unnecessary complication. While this may sometimes be the case, it is surely preferable for most purposes to characterize systems as completely as possible and pursue those components of interest than to be completely unaware of sample heterogeneity and to miss many important observations.

As in all procedures, and particularly those in their formative stages, artifacts can occur with GEF. Many can be prevented by the use of improved techniques; others seem to be inherent in the nature of the procedure. Among avoidable artifacts are patterns given by incomplete focusing and chemical modifications of samples by gel constituents other than the ampholytes. The former can be checked by determining whether the apparent p*I* of separated proteins alters with time. Equilibrium focusing is easily demonstrated in slabs by obtaining coincidence of banding patterns from samples applied at different positions in the pH gradient. In gel cylinders, equilibrium focusing can be confirmed by demonstrating the same banding pattern from samples pre-mixed throughout the gel solution before polymerization or applied to the top of polymerized gels. Occasionally, sample modification with persulphate may

occur, but we have not yet found this to be a major hazard with many proteins.

Unavoidable artifacts fall into two classes: those which arise from interaction with ampholytes and those induced by subjecting molecules to unfavourable conditions of pH and ionic strength. Hayes and Wellner[56] examined the possibility of ampholyte binding as an explanation for the heterogeneity in L-amino acid oxidase (Figs. 19 and 20), but found little evidence for irreversible ampholyte binding. Frater[59] reported binding of basic ampholytes to some acidic proteins from wool. The multiple forms found on subjecting nucleic acids to GEF[153] may also represent ampholyte binding. If so, such binding seems to be specific, as different classes of RNA give unique banding patterns and reproducible p*I*s (Shafritz *et al.*[154]). Possible interaction with ampholytes can be investigated by exposing the sample to different amounts of ampholyte before or during focusing, or by applying samples at different positions or at different periods during pH gradient development. In this way, they will encounter different ampholyte species during their migration to their apparent p*I*. If the same pattern is obtained with these various procedures, binding of ampholytes would seem unlikely.

A more insidious artifact may arise from structural alterations in macromolecules that are exposed to unfavourable conditions of pH or ionic strength. The multiple forms of tRNA and their anomalous p*I* range may represent such a situation[153]. Our results with individual iso-accepting species of tRNA indicated that the molecules underwent reversible conformational transitions on GEF. It appeared that the molecules were partially denatured as the bases were protonated and hydrogen bonds broken, to generate a spectrum of partially denatured molecules. Although such conformers may be of interest for structural analyses, they create considerable problems in the interpretation of banding patterns. Fortunately, such structural modifications seem to be uncommon in protein separations.

On balance, it seems that these artifacts will prove to be the exception rather than the rule, and that the additional information to be gained from GEF far outweighs possible risks arising from artifacts.

8. FUTURE TRENDS

Although techniques for gel electrofocusing are being standardized, there are still several areas that need improvement. Perhaps the greatest need is for more carrier ampholyte systems, particularly in the pH 5–8 range, and the development of alternative and preferably cheaper sources. It would also be desirable to characterize new ampholyte preparations more fully than has been the case for presently available species. With a variety of preparations, it would then be a simple matter to determine whether unexpected or inexplicable heterogeneity represented binding with specific ampholytes.

Preparative aspects of gel electrofocusing also merit further attention. As gels offer several advantages over liquid media, systems such as those used by Radola[10] and Fawcett[12] seem to be the most promising. An attractive possibility is the building of an automated system for continuous sample application and recovery of separated fractions, perhaps with on-line flow cells for pH measurements and UV densitometry. In the analytical field, it might be useful to develop alternatives to polyacrylamide gel for faster and simpler operation. A charge-free cellulose-based support would be

ideal, as it would allow rapid analyses and could be conveniently stored for reference purposes.

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10. SUMMARY

This review deals primarily with analytical and preparative isoelectric focusing (IEF) in gel media. We have tried to cover new developments over the years 1972, 1973 and early 1974 since the previous years have already been reviewed.

An introductory chapter on the properties and detection of Ampholines is followed by a section on analytical and preparative apparatus for gel IEF, with particular emphasis on thin-layer techniques and on continuous-flow IEF.

A chapter on sample detection deals with techniques for staining and destaining and for histochemical enzyme detection. New developments in pH measurements, such as the flat-membrane electrode and the antimony microelectrode, are reported.

Among the various applications of IEF to the study of biological systems we report the analysis of glycoproteins, immunoglobulins, lipoproteins, membrane proteins, peptides and metalloproteins. Particular emphasis has been given to the use of IEF as a probe of interacting protein systems. Examples are given of studies on sub-unit exchange and ligand binding in haemoglobin.

IEF can be effectively used in combination with other techniques for two-dimensional procedures. An IEF run in the first dimension can be followed by an immunodiffusion or immunoelectrophoresis, or by gel electrophoresis, or by electrophoresis in a gel gradient, or by SDS gel electrophoresis. Combined use of these methods will often define in a few simple experiments, several physico-chemical parameters of the proteins under study.

The review ends with a chapter on transient state isoelectric focusing and with remarks on future trends and developments.

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