
**Separation Science and Technology**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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**To cite this Article** Catsimpoolas, Nicholas(1973) 'Isoelectric Focusing and Isotachophoresis of Proteins', Separation Science and Technology, 8: 1, 71 — 121
**To link to this Article: DOI:** 10.1080/00372367308057990
**URL:** http://dx.doi.org/10.1080/00372367308057990

SEPARATION SCIENCE, 8(1), pp. 71–121, 1973

# 13TH EASTERN ANALYTICAL SYMPOSIUM ARTICLES

## Isoelectric Focusing and Isotachophoresis of Proteins

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### Abstract

Two relatively new electrophoretic methods for the separation and characterization of proteins and other ion species, isoelectric focusing in natural pH gradients and isotachophoresis, are reviewed.

## ISOELECTRIC FOCUSING

### Introduction

*Principle*

Isoelectric focusing is an electrophoretic method of concentrating molecules, exhibiting amphoteric properties, at a pH zone corresponding to their isoelectric point. This can be achieved with the application of an electrical field (dc potential) to an electrolyte system in which the pH increases progressively from the anode to the cathode. An amphoteric molecule, such as a protein, will be negatively charged at the alkaline

side of its isoelectric point, and positively charged at the acidic side. Thus the protein will migrate electrophoretically toward the positive or negative electrode until it reaches the pH of its isoelectric point at which it exhibits a zero net charge (Fig. 1). Concentration or focusing of the protein zone results at a particular pH as an equilibrium process between electrical mass transport and back diffusion.

Since proteins have isoelectric points at different pH values, isoelectric focusing can be used as a separation method (Fig. 2). It also provides direct measurement of the isoionic point of a protein—in this case coinciding with the isoelectric point—which is characteristic of its chemical nature and conformation.

The main aspects involved in the study and development of the isoelectric focusing technique include formation of the pH gradient, stabilization of the focused protein zones against convection, detection of the separated components, and pH measurement.



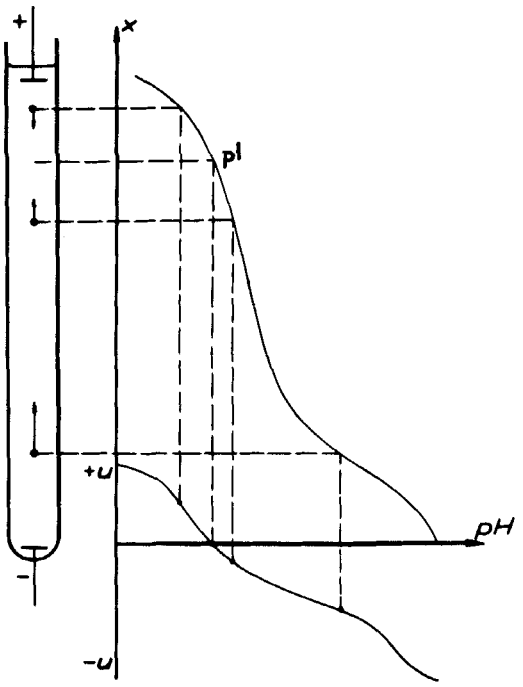



FIG. 1. The isoelectric focusing principle. Anionic electric migration is indicated by $-u$ and cationic by $+u$. pI is the isoelectric point (*138*).

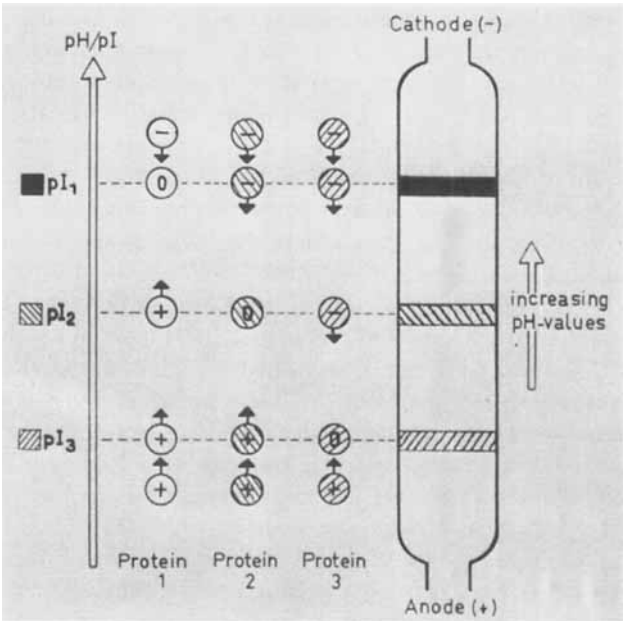



FIG. 2. Separation of proteins by isoelectric focusing. The isoelectric points of three different proteins are indicated by $pI_1$, $pI_2$ and $pI_1$ (*34*).

*History*

*Earlier Studies.* Early work on isoelectric focusing was hindered by difficulties arising from the inability to achieve a stable pH gradient during electrolysis and to prevent convection. The use of multichambered apparatuses in which adjacent compartments were separated by membranes to avoid convection was favored (*1*, *2*). Some work with these devices was performed on the separation of vitamins (*3*), peptide hormones (*4*), proteins (*5*), and amino acids (*6*, *7*). However, the separation was poor and inferior to that achieved by regular electrophoresis. Electroendosmosis and uneven field strength were part of the problem.

Kolin (*8–11*) contributed significantly to the next stage of the development of the isoelectric focusing technique. He realized that uniform field strength requires a system of electrolytes of high and uniform conductivity. He also pointed out the importance of using a pH gradient with sufficient buffering capacity. The problem of convective disturbances was overcome by the use of a density gradient system. However, the pH gradient which was formed at the interface of two buffer solutions of dif-

ferent pH values did not remain constant with time. The instability of the pH gradient in the electric field was the major technical difficulty encountered by Kolin. This was caused by the electrophoretic migration of the buffer ions. "Artificial pH gradients" made with buffers were subsequently used by other investigators (*12–15*) without any fundamental solution to the problem of the unstable pH gradient.

*Recent Achievements.* Taking into consideration the requirements for isoelectric focusing in density gradients as suggested by Kolin (*11*), Svensson in a series of theoretical studies (*16–18*) defined the conditions for the formation of stable natural pH gradients and advocated the use of carrier ampholytes of specified properties. In support of his theoretical suggestions, he used mixtures of peptides to achieve separation of proteins. However, the technique was still not practical because of disadvantages associated with the properties of the peptides. Finally, Vesterberg (*19*) was able to synthesize a mixture of aliphatic polyaminopolycarboxylic acids which met the requirements for the formation of a natural pH gradient as defined by Svensson (*16–18*). Thus the combined efforts of Kolin, Svensson, and Vesterberg resulted in the development of the isoelectric focusing technique as a practical tool for high resolution analysis and separation of amphoteric molecules.

The need for micromethods subsequently led to the development of isoelectric focusing procedures in gel media (*20*) and scanning techniques in sucrose gradients (*21*, *22*). In addition, special apparatuses were used for isoelectric focusing in free solution (*23–26*).



### *Main Applications*

Isoelectric focusing has been used for both preparative and analytical purposes. The method has mainly been applied to the separation of proteins and measurement of their isoelectric point. In certain fields, such as in clinical chemistry, comparison of isoelectrofocusing patterns of certain components is sufficient, and the isoelectric point need not be determined. In physicochemical studies the separation process is of limited interest, but the exact measurement of the isoelectric point is of paramount importance.

## The Natural pH Gradient

### *Basic Description*

Consider a stationary electrolytic system that is composed only of water ions in the presence of very low concentrations of a salt, such as

sodium sulfate. When a dc potential is applied to the system, the water ions undergo oxidation or reduction at the electrodes with consequent evolution of hydrogen at the cathode and of oxygen at the anode. Sodium hydroxide will collect at the catholyte and sulfuric acid in the anolyte. If an amphoteric compound is added to the system, it will become negatively charged at the cathode and positively charged at the anode. This causes repulsion of the ampholyte from the electrodes which migrates away from the acid and base until it becomes uncharged. The ampholyte is thus focused at some point between the two electrodes. If a mixture of ampholytes is used, these will focus at positions according to their isoelectric points. The more basic ones are concentrated closer to the base, and the more acidic ones near the acid. Thus a pH gradient is formed which is called "natural" because it is stabilized by the current itself (*16*). The nature of the pH gradient will depend on the number of ampholytes in the system, the range of their isoelectric points, their buffering capacity, and relative concentration.



*Requirements*

*Buffering Capacity.* The carrier ampholytes must have good buffering capacity at their isoelectric point. This is necessary because their presence dictates the pH gradient and this should not be changed by the presence of a focused protein or other amphoteric compound.

Svensson (*17*) expressed the buffering capacity of an ampholyte as a function of pI-$pK_+$:

$$\frac{-dQ}{d(\mathrm{pH})} = \frac{2 \log 10}{2 + 10^{(\mathrm{pI}-pK_+)}} \tag{1}$$

where $Q$ is the mean electric charge of the ampholyte, pI is the isoelectric point, and $pK_+$ is the dissociation constant of the more acidic of the two ionizable groups.

A plot of $dQ/d(\mathrm{pH})$ vs. pI-$pK_+$ (Fig. 3) indicates that the buffering capacity falls off linearly at first and then assumes an exponential decline. Thus ampholytes with good buffering capacity should exhibit pI-$pK_+$ values as small as possible. This means that they should be isoelectric between two ionizable groups with closely spaced $pK$ values since

$$\mathrm{pI} = (pK_+ + pK_-)/2 \tag{2}$$

At values of pI-$pK_+$ = 1.5 pH units, 50% of the limiting buffering capacity is still retained. Values less than 1.5 pH units are thus desirable.

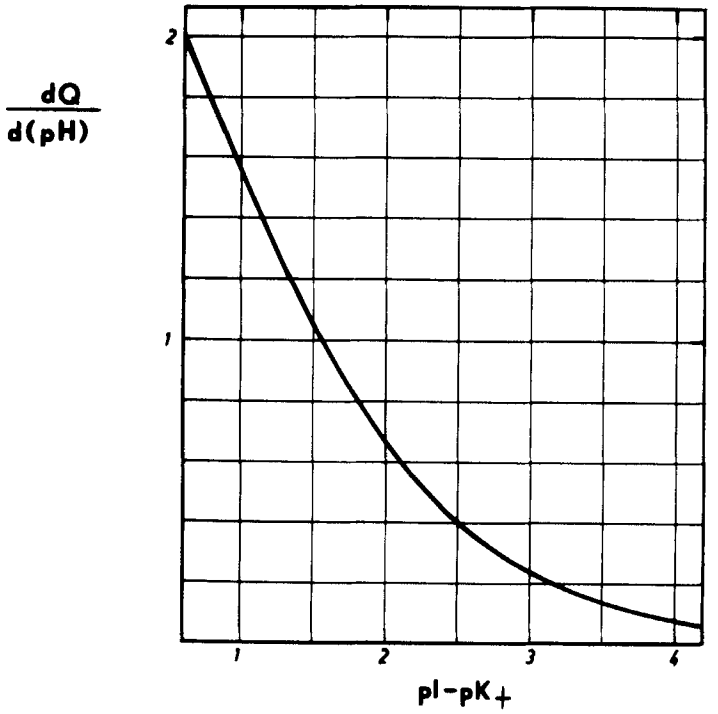



FIG. 3. Relative buffering capacity, in units of the maximum capacity of a monovalent weak protolyte of an ampholyte in the isoionic state, as a function of the p$K$ difference between the dissociation steps on either side of the isoionic point (*142*).



At pI-p$K_+$ = 3.5 pH units, only 1/10 of the buffering capacity remains and the compound can be considered a poor carrier.

*Conductance.* Conductivity is as important as buffering capacity in determining the suitability of an amphoteric compound to be used as a carrier ampholyte. This becomes obvious if we assume that a region of low conductivity is formed during electrofocusing. The Joule effect produces heat proportional to the square of the current divided by the conductivity. In regions of low conductivity, local overheating may result in thermal convection currents with consequent mixing of the pH gradient. In addition, the area of low conductivity will absorb the applied voltage, and thus field strength becomes very low in conducting zones. This may reduce the electrolytic transport and abolish focusing.

The conductivity of an ampholyte is dependent on the extent of its protolysis at the isoionic point (*17*):

$$\alpha = 2/(2 + 10^{(pI-pK+)}) \quad (3)$$

The highest value that can be obtained for $\alpha$ (which is associated with maximum conductivity) is 1/2 since pI-p$K_+$ has a lower limit of log 2. Equation (3) shows that good conductivity is associated with small values of pI-p$K_+$. This was also demonstrated to be true for good buffering capacity. The parameter pI-p$K_+$ becomes the most important factor in selecting carrier ampholytes exhibiting both good conductivity and buffering capacity (Fig. 4).

*Stability.* The contribution of good buffering capacity and conductivity to the stability of the natural pH gradient at equilibrium has already been mentioned. However, in order to achieve stable conditions, other factors have to be taken into consideration. The electrical load should not exceed certain practical limits. If the heat produced by the electrical field is not counteracted by the cooling capacity of the column, convective

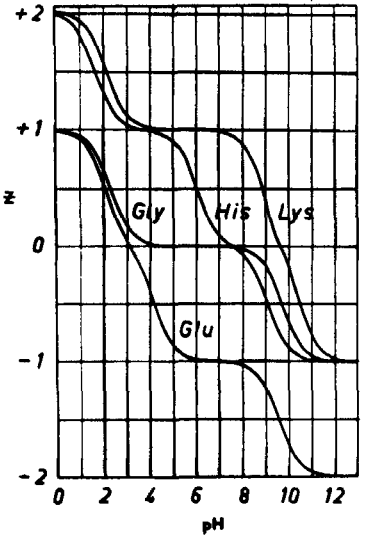



FIG. 4. Titration curves of glutamic acid, glycine, histidine, and lysine. The three amino acids with sharp isoionic points (low pI-p$K_+$ values) are useful as carrier ampholytes, while glycine, with its extended horizontal part of the curve is useless (*17*).

disturbances will take place in the gradient. Usually, gels can withstand higher field strengths than sucrose density gradient columns. Again, the more uniform the conductivity distribution of the carrier ampholytes, the less likely is the possibility of local overheating.

The presence of a high oxidation potential in the anode may lead to oxidation of susceptible compounds, thus increasing the amount of acidic components (*27*). This may cause "drifting" of the pH gradient. The use of strong inorganic acids such as sulfuric and phosphoric acid in the anode is recommended for prevention of such oxidations. Carrier ampholytes and proteins acquire a positive charge because of the low pH and are repelled from the electrode.

Basic protolytes, such as ammonium ions, will migrate to the cathode during isoelectric focusing with possible destruction or evaporation which may lead to a pH gradient drift. This can be counteracted by adding a high boiling point amine or sodium hydroxide to the cathode before starting the experiment (*27*).

*Other Requirements.* Carrier ampholytes should possess some other properties in order to be useful in protein separations. High solubility in water at their isoelectric point is necessary. Their molecular weight should be much lower than that of proteins for easy removal after fractionation. This is important in preparative work. Their UV absorbance spectrum should be such as to not interfere with the detection of proteins at 280 nm. They should not react with or denature the substance subjected to isoelectric focusing.

Since all these criteria were not met by commercially available compounds, new substances with "tailored" properties had to be synthesized.



### *Synthetic Carrier Ampholytes*

*Synthesis.* Carrier ampholytes which very closely meet the specific requirements mentioned above have been synthesized by Vesterberg (*28*). They are a mixture of a large number of aliphatic polyamino-polycarboxylic acids with different isoelectric points in the pH 3–10 range. The principle of the synthesis involves the coupling of carboxylic acid residues to polyethylene polyamines to give a series of homologues and isomers with closely spaced p$K$ and pI values. The reaction depends on the reactivity of primary and secondary amino groups to unsaturated carboxylic acids (*29*). In practice, different proportions of acrylic acid are brought to react with different polyethylene polyamines in a water solution at 70° for a few hours until all the acrylic acid has been con-

sumed. The reaction can be illustrated by

$$R_1\overset{+}{N}H_2-(CH_2)_2-\overset{+}{N}H_2-R_2 + CH_2=CH-COO^- \rightleftharpoons R_1\overset{+}{N}H_2-(CH_2)_2-\overset{+}{N}H(-CH_2-CH_2-COO^-)-R_2 \quad (4)$$

where $R_1$ and $R_2$ represent hydrogen or an aliphatic radical with additional amino groups.

The yield of ampholytes is nearly 100%. Depending on the proportions of acid to amine, one or more carboxylic groups can be introduced into the amine mixture.

This synthetic procedure has been used for the preparation of commercially available carrier ampholytes (Ampholine) by LKB-Produkter AB, Bromma, Sweden.

*Fractionation.* The ampholytes produced by the synthetic procedure described above can be fractionated into narrow pH ranges with the use of a multicompartment electrolyzer (*28*) similar to that described by Williams and Waterman (*2*). The electrolytic apparatus consists of 20 compartments divided by membranes of polyvinyl chloride paper and has 550 ml capacity. The electrolyzer has provisions for cooling water circulation and simultaneous collection of fractions from the bottom of each cell into collecting tubes by hydrostatic pressure. When electrolysis is completed (usually in 24 hr with a maximum load of 500 W), each cell contains a mixture of ampholytes covering a narrow pH range which can be as little as 0.5 pH unit.

*Chemical and Physical Properties.* Carrier ampholytes are insufficiently defined chemically because of their complexity. Some of the reactions of interest to users of the isoelectric focusing technique include complexing with metal ions, and reactions with ninhydrin, biuret, and Folin's reagent (*30*). The carrier ampholytes also form insoluble complexes with some commonly used protein stains (*20*). Reports which indicate interaction of certain proteins with carrier ampholytes have also appeared in the scientific literature (*31*, *32*).

The absorbance of the ampholytes in the UV region does not interfere significantly with the detection of proteins at 280 nm (Fig. 5). Background absorbance during isoelectric focusing is usually low in relation to the absorbance of proteins present in sufficient quantity. However, background noise becomes a problem in the detection of minor components in a mixture, especially at ampholyte concentrations above 1% and low

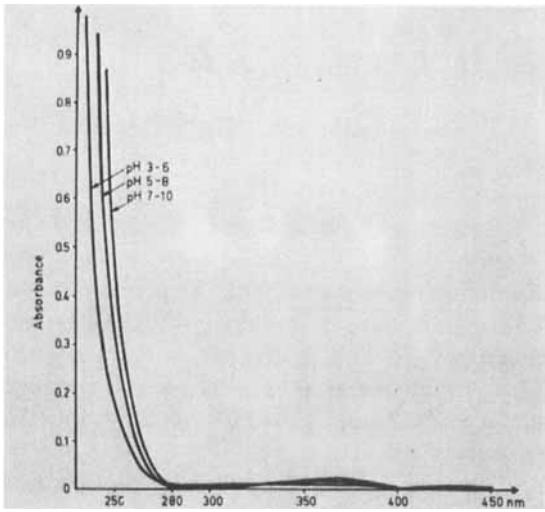



FIG. 5. UV absorbance of the Ampholine ranges pH 3–6, 5–8, and 7–10. Measured at 1% concentration, 1 cm cell (*30*).

protein content. Since the absorbance of ampholytes rises rapidly at wavelengths lower than 280 nm (*30*), detectors using interference filters of broad band width may be considerably inferior to the use of a monochromator. The ideal detector will consist of a ratio recording system using simultaneous absorption at two wavelengths, 254 and 280 nm. Elimination of the background noise can be expected by recording the absorbance ratio, $A_{280}/A_{254}$. The high UV absorbance observed at positions close to the electrode electrolytes is probably due to both the electrode reaction products and decomposition of sucrose and acid (*30*).

The conductance of carrier ampholytes has been examined at different pH ranges (*30*). In sucrose density gradients, the conductance course of the pH gradients pH 3–6 and pH 7–10 is very even.

In contrast, the pH gradients of pH 3–10 and pH 5–8 show an uneven conductance course, especially in the pH 5–8 range (Figs. 6 and 7) (*30*). Davis claims that these variations are not large enough to influence separation of proteins. However, the low conductivity exhibited around neutral pH may result in local overheating caused by a greater potential drop at this region. Variations in the conductivity course of the pH 3–10 ampholytes in polyacrylamide gels have also been noted (*33*).

As expected, the buffering capacity of the ampholytes follows their

conductance course. Thus better buffering capacity is obtained in the pH regions below pH 5 and above pH 8 (Fig. 8).

Haglund (*34*) has recommended that, when working with ampholytes far from the neutral point (e.g., pH 3–6 or pH 8–10), 10% of the total amount of ampholytes should be supplemented with ampholytes of the pH 6–8 range. This addition fills the electrolytic vacuum (low conductivity water zone) near the electrode and a reasonable field strength is obtained.

At present, carrier ampholytes covering the regions below pH 3 and above pH 10 are not available. However, it is expected that such compounds will be introduced in the near future. A method for obtaining pH

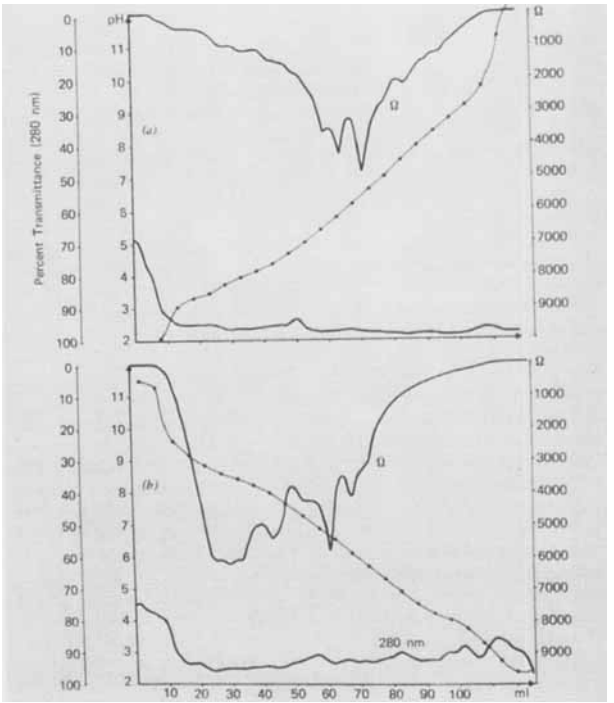



FIG. 6. The conductance course of the pH gradient 3–10 as affected by reversal of the electrode polarity. (*a*) Ampholine pH 3–10, 1%, focused at 500 V for 6 days, anode down. (*b*) Same conditions as for (*a*) but with the cathode down. The pH gradients were measured at 4°C. The resistance was measured at 35°C, cell constant 0.057 (*30*).

gradients in the region below pH 3 stable enough to permit isoelectric focusing has been described (*35*).

*Separation from Proteins.* Removal of carrier ampholytes from protein solutions is desirable in preparative procedures. This can often be accomplished by exhaustive dialysis against water or a buffer solution. Vesterberg (*36*) has recommended the use of Sephadex G-50 columns for the rapid separation of protein and carrier ampholytes. Precipitation of proteins with ammonium sulfate and repeated washings with a solution of the same degree of saturation is another method suggested for efficient removal of ampholytes from proteins that may be inactivated by dialysis or gel filtration (*37*). The use of a mixed-bed ion-exchange resin (BioRad AG 501-X8) for isolation of proteins from ampholyte mixtures has been reported to be more effective than either dialysis or gel filtration (*38*).



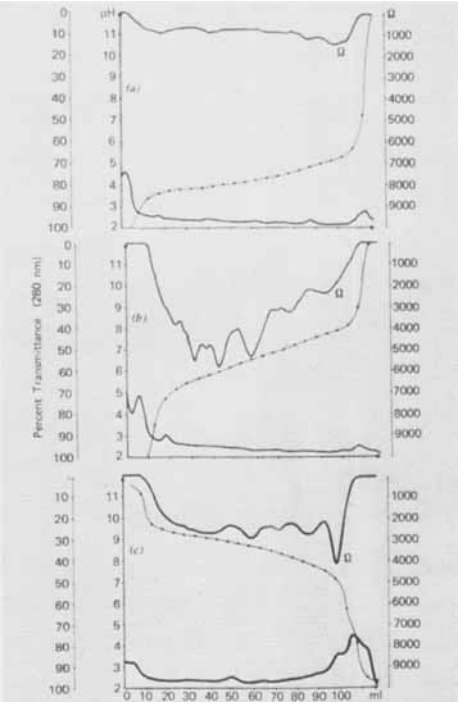



FIG. 7. The conductance course of the pH gradients pH 3–6 (*a*), 5–8 (*b*), and 7–10 (*c*). The cathode was down. The pH was measured at 4°C and the resistance at 35°C (*30*).

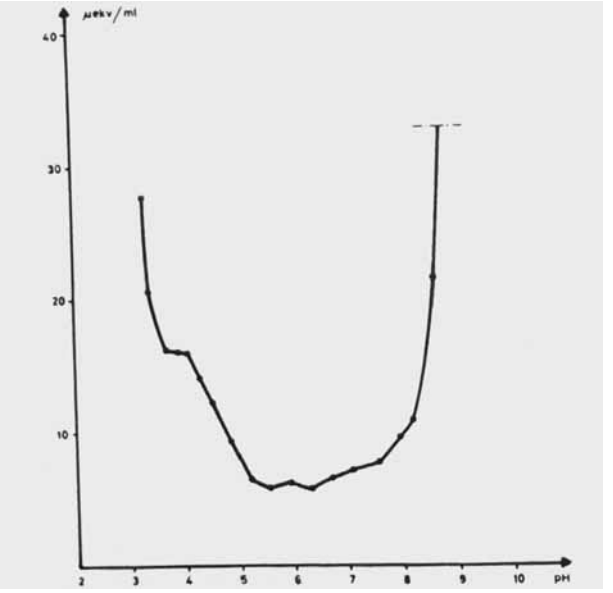



FIG. 8. Buffering capacity determined by titration in 4 ml fractions of the pH 3–10 gradient of Ampholine (with average concentration of 10 mg/ml). The anode was at the bottom of the column (*30*).



## Focusing of Proteins—Basic Concepts

### *Concentration Distribution*

Svensson (*16*) considered the concentration distribution of an electrolyte as an equilibrium between electric mass transport and diffusional mass flow. The equation describing this relationship is

$$Cui/q_K = D(dC/dx) \tag{5}$$

where:

$C$ = concentration of component and ion constituent in arbitrary mass units per arbitrary volume unit
$u$ = electric mobility in $cm^2\ V^{-1}\ sec^{-1}$ of ion constituent except $H^+$ and $OH^-$, with positive sign for cationic and negative sign for anionic migration
$i$ = electric current in A

$q$ = cross-sectional area in $cm^2$ of electrolytic medium measured perpendicularly to the direction of current
$\kappa$ = conductance of medium in $\Omega^{-1}$ $cm^{-1}$
D = diffusion coefficient in $cm^2$ $sec^{-1}$ of component corresponding to the ion constituent with mobility $u$
$x$ = coordinate along the direction of current ($x$ increases from zero at the anode toward the cathode)

Each term in Eq. (5) expresses the mass flow per second and $cm^2$, the left side being the electric, and the right side the diffusional mass flow. Assuming a constant pH gradient and a constant conductivity throughout a focused protein zone, an analytical solution describing the concentration distribution of the ampholyte can be expressed as a bell-shaped Gaussian curve with inflection points at

$$x_1 = \pm \left(\frac{q\kappa D}{pi}\right)^{1/2} \tag{6}$$

where

$$p = -\frac{du}{dx} = -\frac{du}{d(\mathrm{pH})}\frac{d(\mathrm{pH})}{dx} \tag{7}$$

and $x_1$ denotes the width of the Gaussian distribution of the focused zone measured from the top of the distribution of the focused ampholyte to the inflection point (one standard deviation). The course of the pH gradient $d(\mathrm{pH})/dx$ is determined by the nature of the carrier ampholytes. The diffusion coefficient $D$ and the mobility at the isoelectric point $du/d(\mathrm{pH})$ are characteristic properties of the protein. Successful isoelectric focusing of proteins is achieved because of their low diffusion coefficients (small values of $D$) and steep electrophoretic mobility curves and pI [large values of $-du/d(\mathrm{pH})$].

Since small values of $x_1$ indicate sharply focused bands, the degree of focusing is directly proportional to the square root of the field strength $E = i/q\kappa$. However, the field strength cannot exceed certain limits because of undesirable heating effects. The Joule heat per $cm^3$ of the medium is given by

$$W = i/q^2\kappa \tag{8}$$

Equations (6) and (8) show that the conductivity course is important in isoelectric focusing. A uniform and high conductivity course is desirable for the application of high field strength and avoidance of local overheating.

The molecular weight of the protein has negligible effect on the degree of focusing because there is a sixth-root dependence of $x_1$ on molecular size. This conclusion derives from the third-root dependence of $D$ on molecular size, and the square-root dependence of $x_1$ on $D$.

*Resolution*

From the theoretical study of Svensson (*39*) on the exact definition of minimally but definitely resolved zones, Vesterberg and Svensson (*19*) derived Eq. (9), which indicates the pH difference needed for minimal separation of two protein zones by isoelectric focusing:

$$\Delta\text{pH} = \frac{d(\text{pH})}{dx}\Delta x = \frac{d(\text{pH})}{dx} 3.07x_1 = 3.07\left(\frac{D(d(\text{pH})/dx)}{-E(du/d(\text{pH}))}\right)^{1/2} \quad (9)$$

If the pH gradient $d(\text{pH})/dx$ is known and the value of $x_1$ is obtained from the zone breadth at theoretical ordinates $e^{-1/2} = 0.61$ of the peak height, the numerical value of $\Delta$pH can be estimated. Such calculations have shown that the resolving power of isoelectric focusing can be as little as 0.01 to 0.02 pH unit (*19, 40*). Giddings and Dahlgren (*41*) have also confirmed theoretically that neighboring peaks can be resolved if their isoelectric points differ by only 0.01 pH unit.

It may be seen from Eq. (9) that the only variable experimental factors that can affect resolution are the field strength $E$ and the slope of the pH gradient $d(\text{pH})/dx$ at the area of focusing. Of course, the method of analysis of the separated components affects the resolution indirectly. *In situ* scanning methods (*21, 22, 40, 42*) are superior to other detection techniques in this respect.

*Peak Capacity*

As defined by Giddings and Dahlgren (*41*), peak capacity $n$ is the maximum number of components resolvable by a given technique under specified conditions. In isoelectric focusing (*41*) the peak capacity is given by

$$n = \left(\frac{-\mathfrak{F}E(dq/d(\text{pH}))(d(\text{pH})/dx)L^2}{16RT}\right)^{1/2} \quad (10)$$

where $q$ is the effective charge, $\mathfrak{F}$ the faraday of electricity, and $L$ the total path length. An alternative form is obtained by replacing $L(d(\text{pH})/dx)$ by $(\text{pH}_L - \text{pH}_0)$, the total pH increment. It is, of course, assumed

that a constant value of $d(\mathrm{pH})/dx$ is only an approximation. In this case we have

$$n = \left(\frac{-\mathfrak{F}E(dq/d(\mathrm{pH}))(\mathrm{pH}_L - \mathrm{pH}_0)L}{16RT}\right)^{1/2} \tag{11}$$

Thus the peak capacity is directly proportional to the square root of the electric field, path length, and total pH increment. In practice the path length can be changed by varying the length of the column, and the total pH increment can be varied by using carrier ampholytes covering different numbers of pH units.

*Isoelectric Point Determination*

The isoelectric point of a focused protein can be determined directly after the experiment by measuring the pH of the corresponding fraction. The accuracy of the measured pH will depend on the proper collection of fractions, the size of the collected fractions in relation to the range of the pH gradient, the absence of atmospheric carbon dioxide absorption by the fractions, and the model of pH meter used. Good reproducibility in isoelectric point determinations requires focusing and pH measurement at constant temperature. By definition the pH concept and the intrinsic dissociation constants of the carrier ampholytes and proteins are temperature dependent (*43*).

The isoelectric point of a protein determined by isoelectric focusing also represents its isoionic point in the absence of complex forming ions (*27*). This statement requires that the possible complexes between a protein and the ampholytes will not influence the isoelectric point. By definition the isoionic point is a measure of the intrinsic acidity of a pure protein since it is defined as that pH which does not change on addition of a small amount of pure protein (*44*). This definition is also applicable to a protein focused isoelectrically (*27*). If more of the protein is focused in the isoelectric zone, the pH remains unchanged. However, this does not apply to the carrier ampholytes because the point of maximum concentration represents their isoelectric value (*34*). The pH of the carrier ampholyte zone is dependent on the carrier ampholyte concentration.

Often there is a descrepancy in isoelectric point values of a protein obtained by isoelectric focusing and moving boundary electrophoresis. Usually higher values are obtained by isoelectric focusing in the absence of salt ions which, if present, migrate toward the electrodes (*27*). In electrophoresis, the pI value depends on the kind of buffer and ionic

strength used. Lower values may be observed because of possible complexing of proteins with anions. Thus the great advantage of isoelectric focusing involves the direct determination of isoelectric points at practically zero ionic strength. In electrophoresis, several experiments have to be performed at different pH values and ionic strength for zero extrapolation.

The determination of isoelectric points of proteins of known chemical structure in the presence of denaturants such as urea can also be valuable for conformational studies (*45*, *46*). However, since urea may cause an upward shift in the apparent p$K$ and pI values of the carrier ampholytes (*47*), correction factors have to be used for the determination of isoelectric points of unfolded proteins.

*Charge Difference Determination*

If two different molecular forms of a protein have different isoelectric points and the electrometric titration curve and molecular weight of one protein are known, then it is possible to calculate the net charge which this protein would possess at the pI of the other protein (*27*). This is important since many proteins exhibit different molecular forms. This can be caused by deamidation of susceptible groups, amino acid composition difference, presence of metals that have more than one valence (metalloproteins), subunit composition, conformational variations, mixed disulfide formation, and chemical modification during isolation or separation.



*Mass Load of a Protein Zone*

The amount of protein that a focused zone can carry depends on the construction of the specific apparatus used and the method of zone stabilization. In the density gradient technique the maximum amount of protein load depends on the capacity of the density gradient to carry a focused zone, the solubility of the protein at its isoelectric point, the height of the zone at the particular pH range of the experiment, and the cross-sectional area (*27*). The height of the zone depends on the electric field strength. The lower voltage results in broader protein zones at the expense of decreased resolution and increased time of focusing. With narrow pH range ampholytes the zones are allowed to increase their height without loss in resolution which results in higher zone capacity. Solubility of the protein at its isoelectric point can be increased by the

use of urea, nonionic detergents, disulfide cleaving reagents, and high concentration of ampholytes (above 1%).

When the zone convection apparatus is used (*23*) the mass load of the protein zone can be increased considerably because of the self-stabilizing characteristics of the system which does not require a density gradient or gel support.

## Density Gradient Isoelectric Focusing

### *Preparative Procedures*

*The Density Gradient.* Solutes suitable for preparation of density gradients must be nonionic, with good solubility in water, and must not interact with the sample. They should also produce density differences greater than 0.12 g/cm$^3$. Substances such as sucrose, glycerol, and ethylene glycol have been used in isoelectric focusing. The dense solution usually contains 50% sucrose or 60 to 70% glycols (*34*). The gradient can be prepared either manually or with gradient mixers. The manual preparation of the density gradient involves mixing of a dense and a light solution in certain proportions in a test tube so that a series of solutions of different densities are available. These are then stratified, one on top of the other, by slowly flowing them into the electrofocusing column. The density gradient is then "smoothed out" by diffusion.

The density gradient can also be prepared by using commercially available gradient mixers which save labor and reduce errors in mixing.

*Sample Application and Load.* In isoelectric focusing the technique of sample application is not as critical as in other forms of electrophoresis since a thin starting zone is not required. The sample can be either added to the entire volume of the density gradient or it can be applied as a zone at a position in the column close to the expected focusing point. In the latter case the time of focusing is shortened since the protein does not have to migrate through the entire column. However, care should be taken not to disturb the density gradient by addition of the protein sample zone. The advantage of incorporating the sample in the entire gradient volume is that dilute protein solutions can be used for focusing.

The amount of sample applied to the column depends on multiple factors. Some of these include the dimensions of the column, the range of the pH gradient, the heterogeneity of the mixture, the solubility of the proteins at their isoelectric point, and the strength of the electric field. The experimental conditions favoring applications of large amounts of

sample (1.0–0.5 g protein) are a large cross-sectional area of the column and a narrow pH gradient. The LKB 440-ml electrofocusing column has approximately the same height as the 110-ml column but has about 5 times the effective cross-sectional area. This allows for large amounts of protein to be separated.

*Carrier Ampholytes.* Ampholine carrier ampholytes are available commercially from LKB-Produkter AB, Bromma, Sweden. They are delivered in 40% (w/v) stock solution covering the pH ranges 3–10, 3–6, 3–5, 4–6, 5–7, 5–8, 6–8, 7–9, 7–10, and 8–10. The stock solutions should be stored at a low temperature (below 6°) and they should not be exposed to direct sunlight or temperatures above 20° for long periods of time (*34*).

For isoelectrofocusing the carrier ampholytes are incorporated in the solutions used to make the density gradient at a recommended concentration of 1 g of Ampholine per 100 ml total volume of the gradient. Higher concentrations may contribute to the solubility of certain proteins at their isoelectric point, but may also cause increased background noise with UV detection.

Carrier ampholytes of narrower pH range can be prepared by isoelectrofocusing and fractionation of the existing commercial products. However, closer ranges will also be available commercially in the near future as well as Ampholine covering the pH range above 10.

*Electrode Electrolytes.* Regardless of the dimensions, type, and geometry of the isoelectrofocusing column, the density gradient containing the carrier ampholytes and the sample is confined between the electrode electrolytes. The anode electrolyte (usually at the bottom of the column) consists of a strong acidic solution such as 5% phosphoric acid, and the cathode electrolyte is a strong organic base such as 5% ethanolamine.

*Isoelectric Focusing.* The pH gradient is formed by the Ampholine carrier ampholytes when a voltage (200–1200 V dc)—not exceeding 10 mA in current—is applied across the mixture. The use of a constant voltage power supply is recommended. During the experiment the current drops gradually until it reaches a minimum value. This is mainly due to the migration of the carrier ampholytes toward their isoelectric point where they exhibit considerably lower conductivity than that of their electrically charged form at other pH values.

The conductivity course along the electrofocusing column is not uniform because of variations in the conductance of the different species of carrier ampholytes at their pI. This imposes limitations on the maximum power that can be used for focusing, and also necessitates the employment of efficient cooling to avoid local overheating. The cooling tempera-

ture recommended is 14°C. Temperatures under 2°C should be avoided (*34*).

The time of focusing varies with temperature, pH range, position of protein sample, and dimensions of the column. Equilibrium is usually achieved within 24 to 72 hr.

*Collection and Detection of Separated Proteins.* After isoelectric focusing, the contents of the column are either drained or pushed through a UV-photometer which measures their absorbance at 280 nm. Fractions can be collected with a fraction collector. The pH of the fractions is determined with a pH meter, preferably at the same temperature as that prevailing during electrofocusing. A graph is then prepared showing the UV absorbance of each fraction and its pH value (Fig. 9). Alternatively, enzymatic or other biological activity of the fractions can be determined in addition to their UV absorbances (Fig. 10). Continuous pH measurements are possible but involve great experimental difficulties (*48*).

Technical suggestions for partly avoiding remixing of the protein zones during elution have been given by Haglund (*34*). Remixing due to diffusion is eliminated in the column designed by Svendsen (*49*).

*Preparative Columns.* To this date, three different models of columns have been used for preparative density gradient isoelectric focusing. The

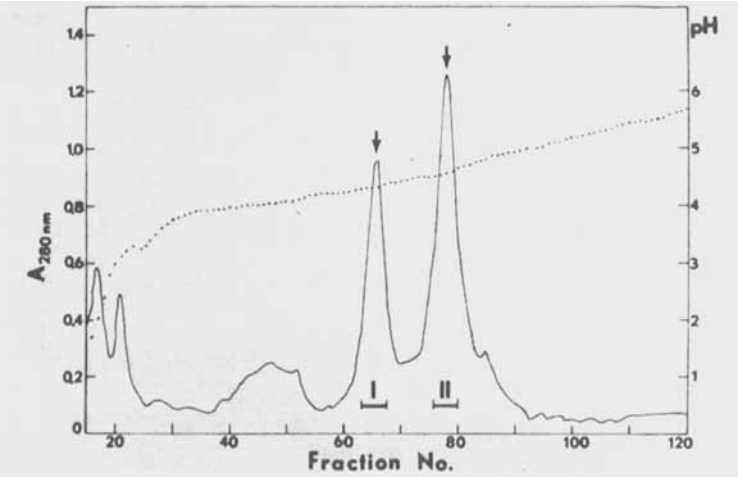



FIG. 9. Isoelectric focusing of a mixture of commercial preparations of Bowman-Birk (I) and Kunitz (II) soybean trypsin inhibitors in the pH 3–6 region: solid line, absorbance at 280 nm (1-cm cell); dotted line, pH gradient (25°); solid bars, fractions pooled to obtain pure proteins (*139*).

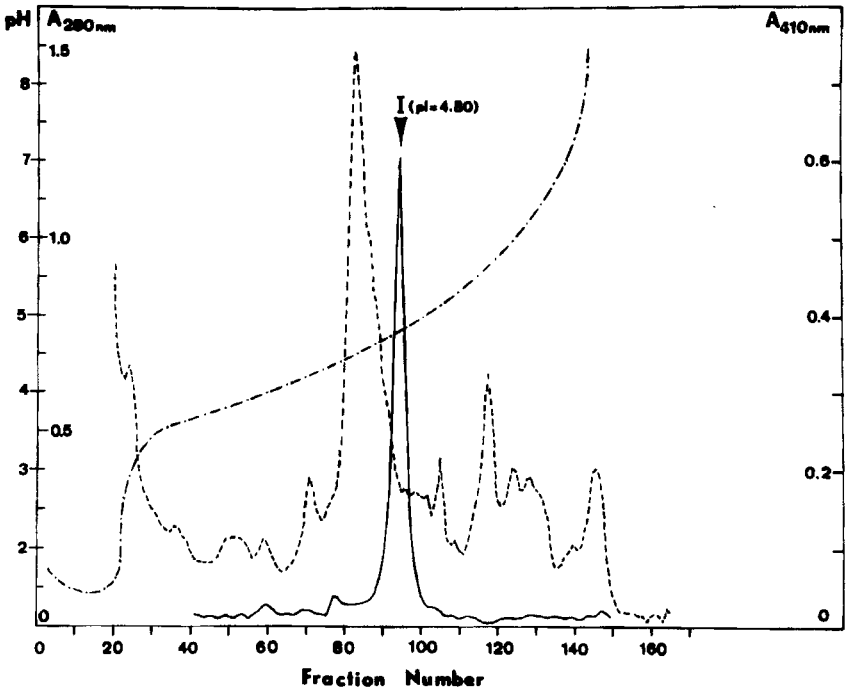



FIG. 10. Isoelectric focusing in the pH 3–6 region of the pH 4.5 soluble fraction of soybean proteins. The absorbance at 280 nm (- -) indicates the presence of total proteins whereas the absorbance at 410 nm (——) shows the enzymatic activity toward benzoyl DL-arginine *p*-nitroanilide (BAPA), a substrate of proteases. The pH gradient (- • -) was measured at 25°C (*140*).

column designed by Svensson (*18*) is manufactured by LKB Produkter AB, Bromma, Sweden, in two sizes of 110 and 440 ml capacity. The compartment where the electrofocusing takes place has an annular cross section and is thermostated from both sides by cooling water compartments. The platinum electrodes are located in such a position that no gas bubbles disturb the electrofocusing process. A valve is used to shut the central tube (where the lower electrode is located) from the electrofocusing compartment during draining of the column (Fig. 11).

In the column designed by Svendsen (*49*), elution is performed without interruption of the current. This is desirable in order to avoid zone spreading by diffusion (Fig. 12). However, by using this column, determination of isoelectric points is not possible because an acidic or basic

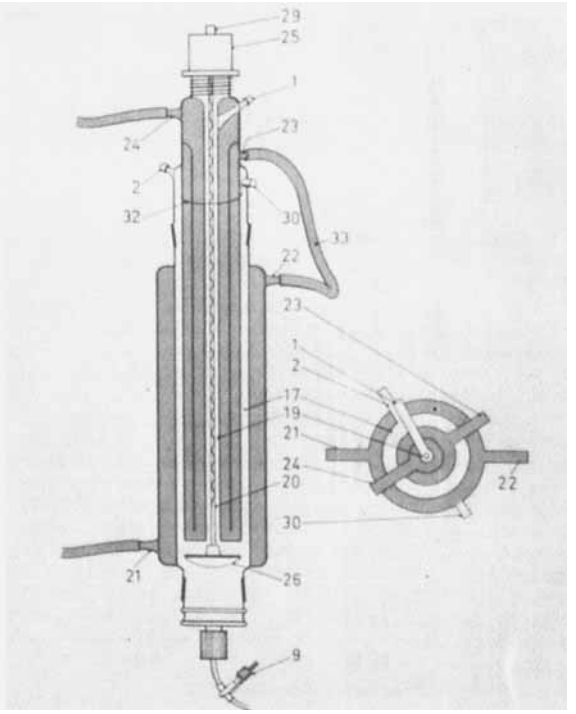



FIG. 11. The LKB isoelectric focusing column for density gradient experiments. Compartment (17), where the electrofocusing takes place, has an annular cross section and is thermostated from both sides by cooling water compartments interconnected with tubing (33). The electrodes, made of platinum, are so located that no gas bubbles disturb the electrofocusing process. The upper electrode (32) consists of a loop located at the top of the focusing compartment (17) and connects to a plug (30). The central electrode is wound on a Teflon rod (20) in central tube (19) and connects to plug (29). This rod serves both as carrier for the electrode and as a lever for valve (26) which makes it possible to shut off central tube (19) from compartment (17) by means of a setting device (25) at top of the column when emptying. An electrofocusing experiment is performed as follows: With valve (26) in the open position, cooling water is turned on. The electrode solution of the central electrode is then filled into central tube via nipple (1), by means of a pump or through a funnel (gravity feed). The sample, Ampholine carrier ampholytes and sucrose, are mixed together (by means of gradient mixer or manually) so that the solution gives a density gradient when filling compartment (17) via nipple (2) with the aid of a funnel or a pump. When this electrofocusing solution has filled compartment (17), the remaining space at the upper electrode (32) is filled with the other electrode solution.

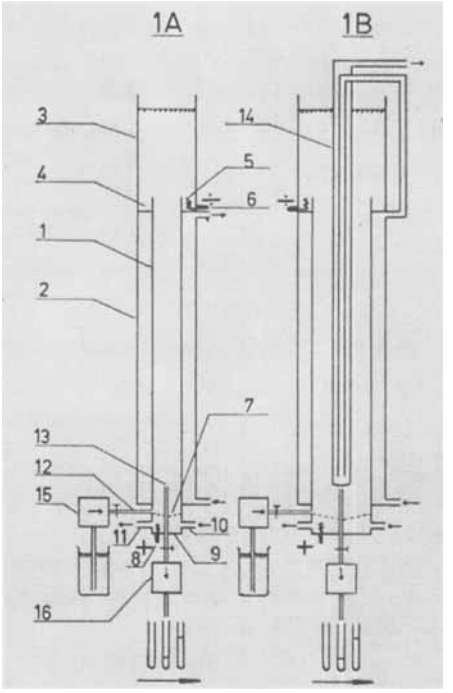



FIG. 12. Schematic diagram of the apparatus designed by Svendsen. (A): (1) Separation tube, (2) cooling jacket, (3) funnel, (4) ditch, (5) platinum electrode, (6) connection bushing, (7) semipermeable membrane, (8) electrode and connecting bushing, (9) electrode housing, (10) inlet for circulating buffer, (11) outlet for circulating buffer, (12) inlet for filling and elution, (13) outlet for elution, (15) peirstaltic pump for elution inlet, (16) peristaltic pump for elution outlet. (B): (14) Cold finger (*49*).

The voltage is now applied to the column via plugs (29) and (30), and the electrofocusing procedure is started. When the electrofocusing procedure has finished (16 to 72 hr), the voltage is turned off and valve (26) is then closed to prevent the electrode solution from mixing with the effluent. Clamp (9) is now opened and the column is emptied by means of a peristaltic pump or gravity feed. The effluent can be analyzed with a continuous flow absorption meter, the fractions are collected in test tubes, and the pH of fractions is measured at the same temperature as used during electrofocusing (*34*).

sucrose solution is continuously mixed with the eluted material by counterflow.

An apparatus (ISCO, Lincoln, Nebraska) originally designed for density gradient electrophoresis (*50*) has been used recently for isoelectric focusing experiments (*51*, *52*) (Fig. 13). The feature of this apparatus is

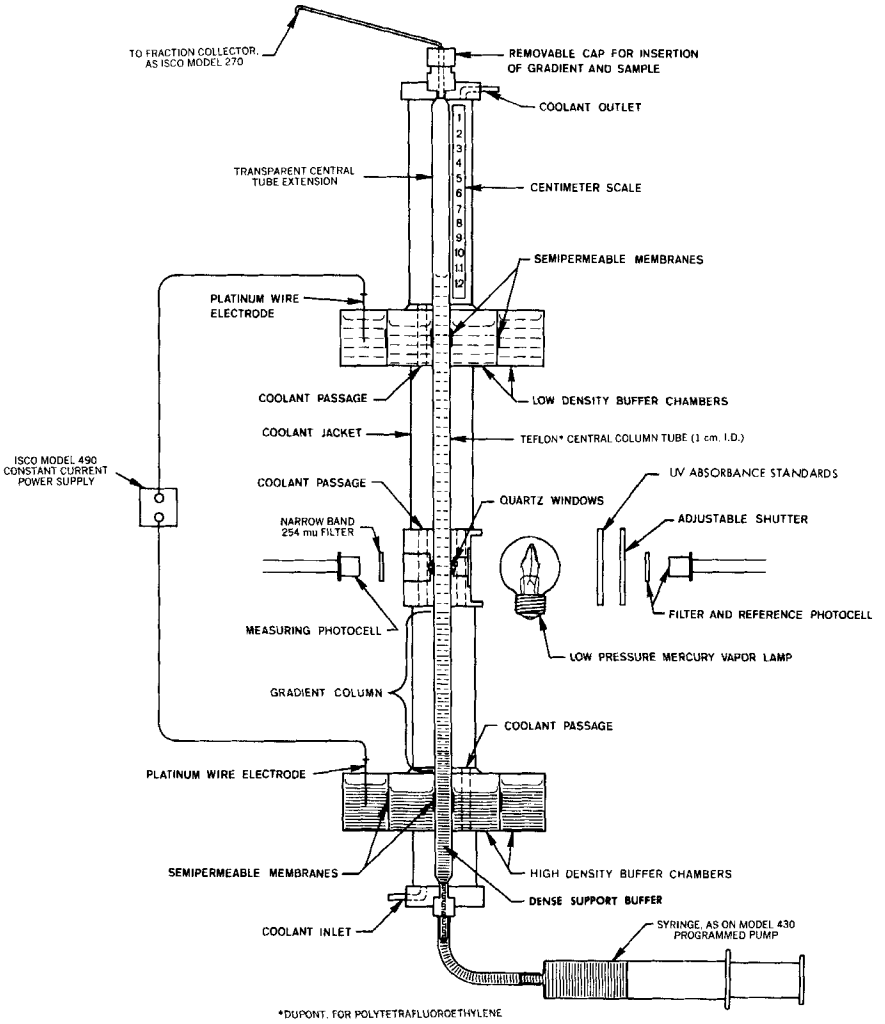



FIG. 13. Schematic diagram of ISCO Model 210 Density Gradient Electrophoresis Column used for isoelectric focusing (*50*, *51*).

that the contents of the column are raised and lowered through a UV detector by using dense chase solutions. The method is not truly scanning but rather a type of elution procedure since the separated components are forced to move inside the column mechanically and the current is interrupted during this process. However, it offers certain advantages over both Svensson's (*18*) and Svendsen's (*49*) columns in that remixing is diminished, the isoelectric point can be determined, higher resolution can be obtained, and the progress of focusing can be evaluated intermittently.

*Analytical Procedures*

*Small Columns.* In attempts to scale down the density gradient isoelectric focusing technique to the analytical level, several types of small columns have been designed (*53–56*). These are usually U- or J-shaped tubes of small capacity (7 to 14 ml). A dummy arm is used to achieve hydrostatic equilibrium of the contents of the miniature column. A polyacrylamide gel plug (*57*) and a semipermeable membrane (*21*) have also been used to support the density gradient in small columns. Detection of the separated components is performed either by forcing the density gradient upward and recording the UV absorbance, or by visual inspection using colored proteins. Spectrophotometric scanning methods (*21, 57*) have also been employed.

In Situ *Scanning Method.* An *in situ* scanning apparatus (Fig. 14) for analytical isoelectric focusing experiments has been designed and constructed (*22*). The apparatus is capable of direct optical scanning of protein zones separated in a quartz microcolumn. Sequential scans can be obtained in the presence of electrical current until equilibrium conditions are achieved. Temperature control has been provided for effective cooling. Flow of reagents and sample through the column facilitates the filling procedure which is performed with a specially designed apparatus (*58*) (Fig. 15). The scanning method offers excellent resolution of components (Fig. 16) but lacks the capability, at present, of direct recording of the pH gradient which is obtained with a "calibration curve" using marker proteins and peptides of known pI. The method appears to be promising for clinical and other routine applications since it has the capability for completely automatic operation in future models.

*General Remarks*

The main disadvantage of the density gradient isoelectric focusing technique is that the density gradient often fails to support proteins

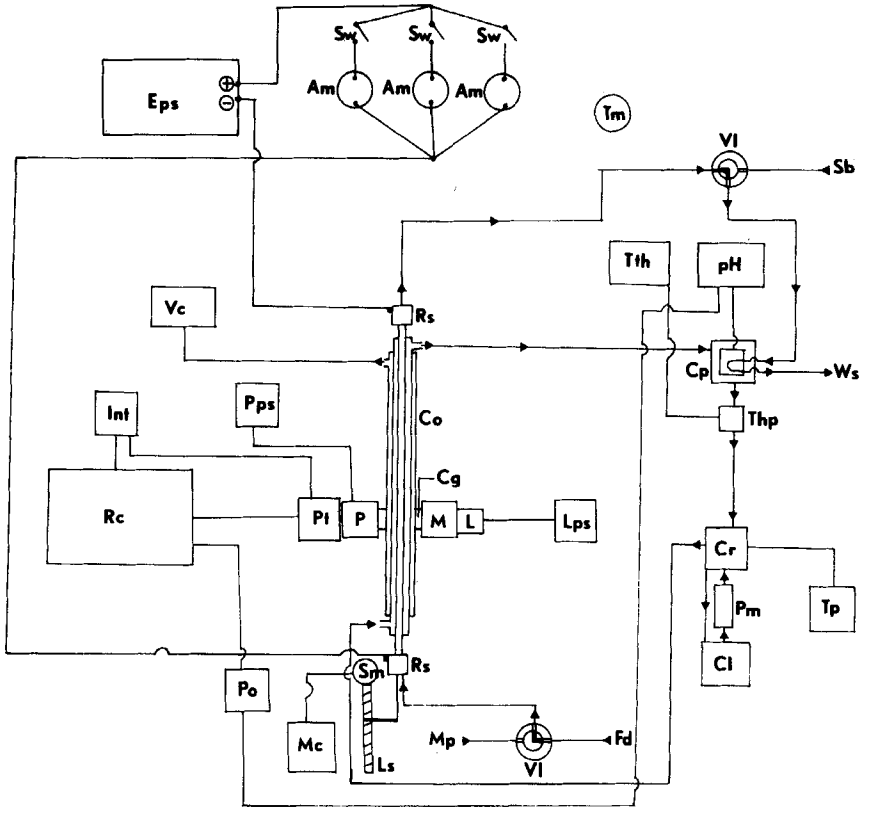



FIG. 14. Schematic diagram of the analytical isoelectrofocusing scanning apparatus (AISA): Am = ammeter, Cg = column guide, Cl = cooler, Co = column, Cp = capillary pH electrode assembly, Cr = circulator (thermostated), Eps = electrophoresis power supply, Fd = filling device, Int = integrator, L = UV lamp, Lps = lamp power supply, Ls = lead-screw, M = monochromator, Mc = motor control box, Mp = metering pump (for autoburet), P = photomultiplier, pH = pH meter, Pm = pump, Po = potentiometer, Pps = photomultiplier power supply, Pt = photometer, Rc = recorder, Rs = reservoir, Sb = standardization buffer, Sm = synchronous motor, Sw = switch, Thp = thermistor probe chamber, Tm = elapsed time indicator, Tp = temperature programmer, Tth = telethermometer, Vc = vacuum pump, Vl = valve, Ws = waste (*22*).

precipitated at their isoelectric points. Elimination of precipitation may be achieved by incorporation in the gradient of such compounds as nonionic detergents (Triton X-100, Brij 35, Emasol, Tween 80), ethylene glycol (*59*), and urea in the absence or presence of disulfide cleaving re-

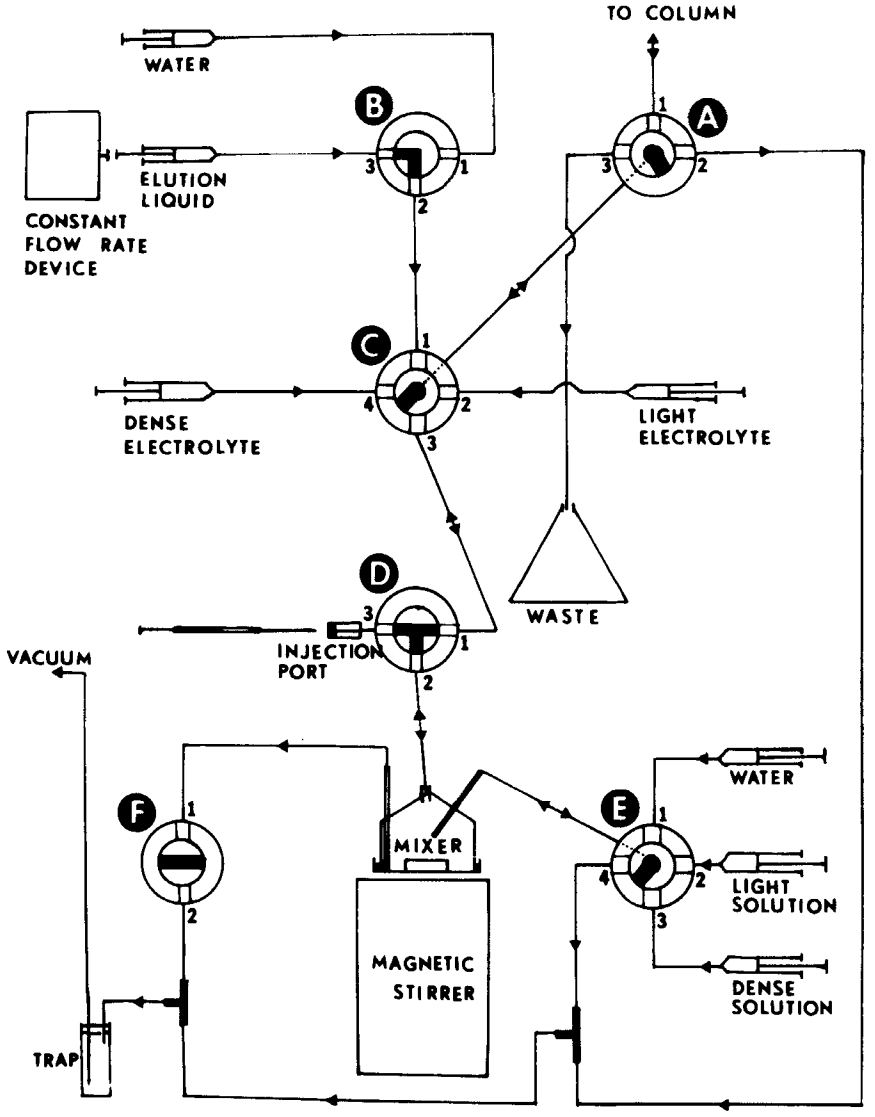



FIG. 15. Schematic diagram of the column-filling apparatus (CFA). Valves are represented by letters A through F. Terminals of each valve are numbered (*58*).

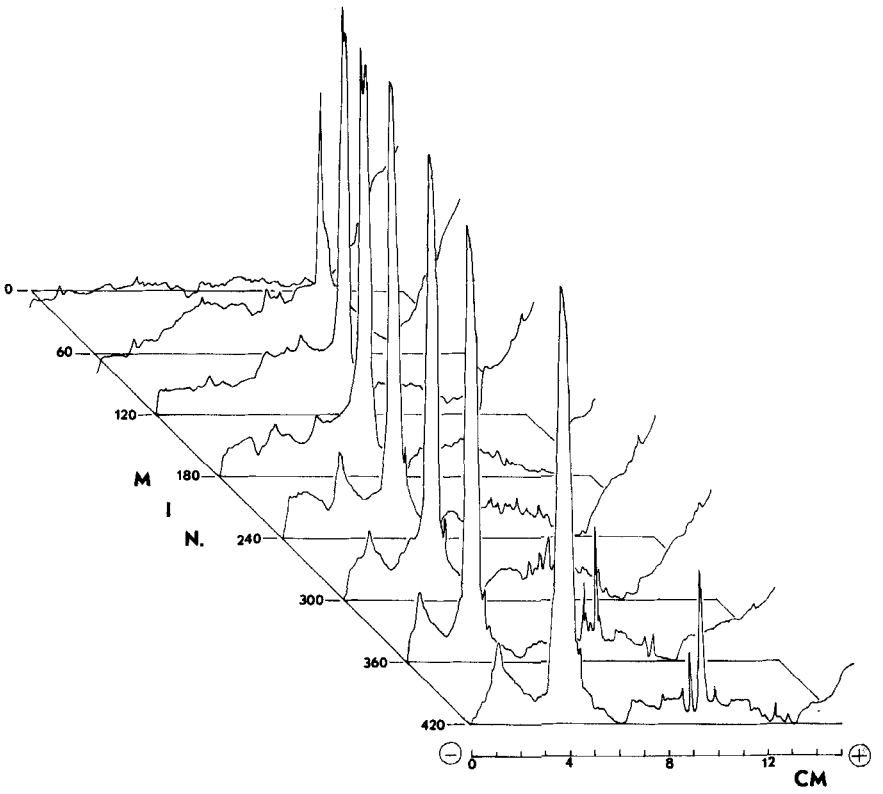



FIG. 16. Kinetic scanning isoelectrofocusing pattern of horse heart myoglobin (200 $\mu$g) in pH 3–10 ampholyte range at 300 V (16°) (*22*).



agents (*60, 61*). Brij 35 has been shown to prevent precipitation without binding to the protein (*62*). High concentration of carrier ampholytes (above 1%) and a decreased protein load also helps in stabilization of protein zones at their pI. The use of urea cannot be recommended in all cases since it may denature the protein. However, this denaturation is sometimes desirable, for instance in the study of dissociated polypeptide chains.

When carrier ampholytes of a narrow pH range remote from pH 7 are used for focusing, it is recommended that 10% of the total amount is supplemented with pH 3–10 Ampholine (*34*). This avoids the risk of a zone with low conductivity around pH 7. Artificial pH gradients covering the pH range below pH 3 and above pH 10 with the use of acids and

amines have been reported (*63*, *64*). These are not as stable as the natural pH gradient, but do serve a purpose.

In respect to the polarity of the applied voltage, it is advisable to place the cathode at the top of the column for the pH 3–10 range and for pH ranges under 6 (*34*). The anode should be at the top of the column for pH ranges over 6. The high concentration of sucrose at the bottom of the column lowers the conductivity and exerts a balancing effect (*30*). This is because the conductivity of Ampholine has a minimum in the pH 5–7 range.

Desirable developments of the density gradient technique would be the shortening of focusing time and automated operation in analytical work, and simultaneous avoidance of zone remixing and diffusion spreading in preparative procedures. A continuous flow apparatus will also be useful for large-scale purification.



## Gel Isoelectrofocusing

### *Introduction*

The isoelectric focusing technique in gel media (*20*, *65*) was originally developed because of the need for a micromethod for analytical separation of proteins. However, it was soon realized that the method had other advantages besides conserving sample and carrier ampholytes. Some of these desirable features include: (a) resistance to convective mixing, (b) dramatic shortening of the focusing time, (c) employment of a simple apparatus, (d) simultaneous separation of several mixtures, (e) employment of specific stains, (f) combination with electrophoresis in two-dimensional separations, and (g) visualization of separated proteins by use of immunodiffusion techniques. Another major advantage of the gel isoelectrofocusing technique is that the gel matrix is able to support protein precipitates which may be formed at the isoelectric point. However, most gel isoelectrofocusing methods have the inherent disadvantage of restricting the migration of large molecules because of sieving effects. An additional drawback of the utilization of gels as supporting medium is that only discrete sample analysis is possible, and this imposes limitations in possible automated operations.

### *Isoelectric Focusing in Polyacrylamide Gel Columns*

A number of techniques (*66–73*) have been developed recently for performing isoelectric focusing in small columns (Fig. 17) similar to those

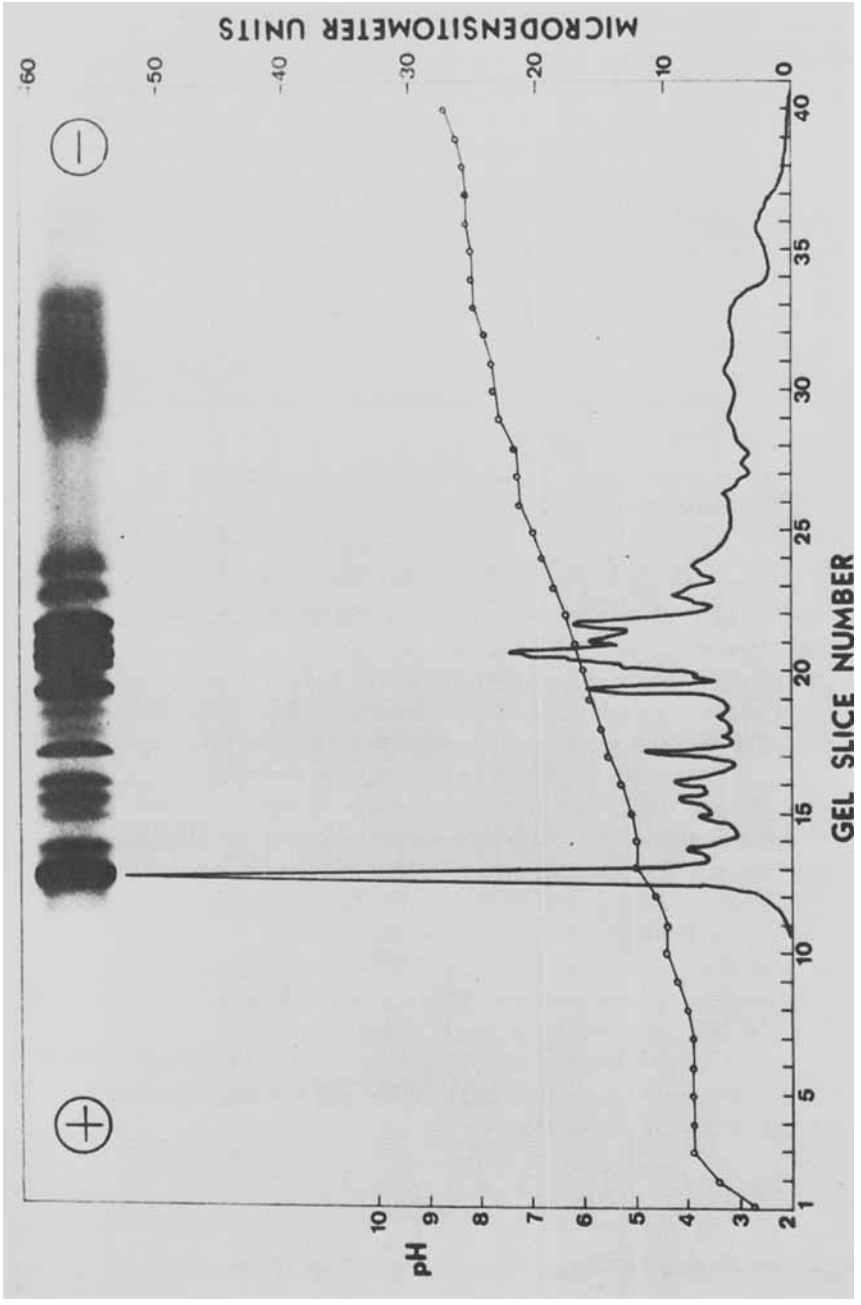



FIG. 17. Gel electrofocusing of soybean whey proteins in the pH range between 3 and 10. The densitometer tracing of the stained bands was obtained with a Canalco model F microdensitometer. Open circles represent the pH gradient along the polyacrylamide gel column after electrofocusing (*66*).

used for electrophoresis (*74*). The choice of apparatus, gel formulations, dimension of columns, electrode solutions, current applied and time of run, mode of sample application, acrylamide concentration, staining procedures, and other experimental details varied among the reported methods.

Both commercial and specially designed apparatuses (*67*, *68*, *72*, *73*) have been used. Cooling of the gels and small-volume electrolyte reservoirs are desirable features for optimum resolution (Fig. 18). Formulations for both photopolymerization and chemical polymerization of the gels are available (*66–73*). Chemical polymerization should be avoided if sensitivity of the proteins to ammonium persulfate catalyst (*75*) is suspected. However, in the presence of certain carrier ampholytes (e.g., pH 7–10) photopolymerization with riboflavin as catalyst can be inhibited, therefore requiring the use of chemical polymerization. In this case, loading of the sample on top of the gel and underneath a protective



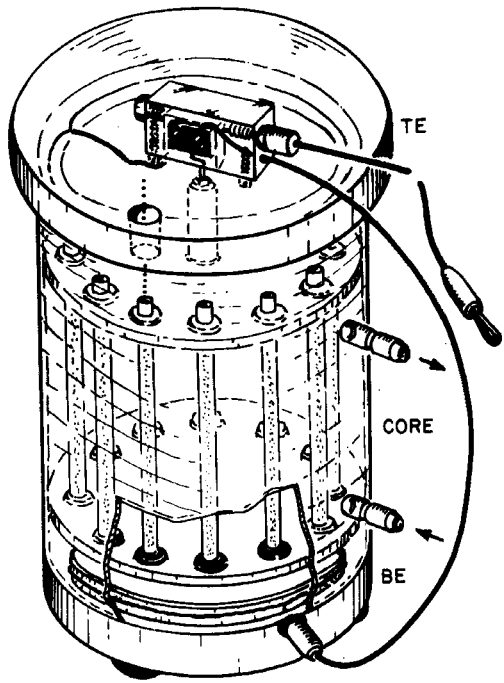
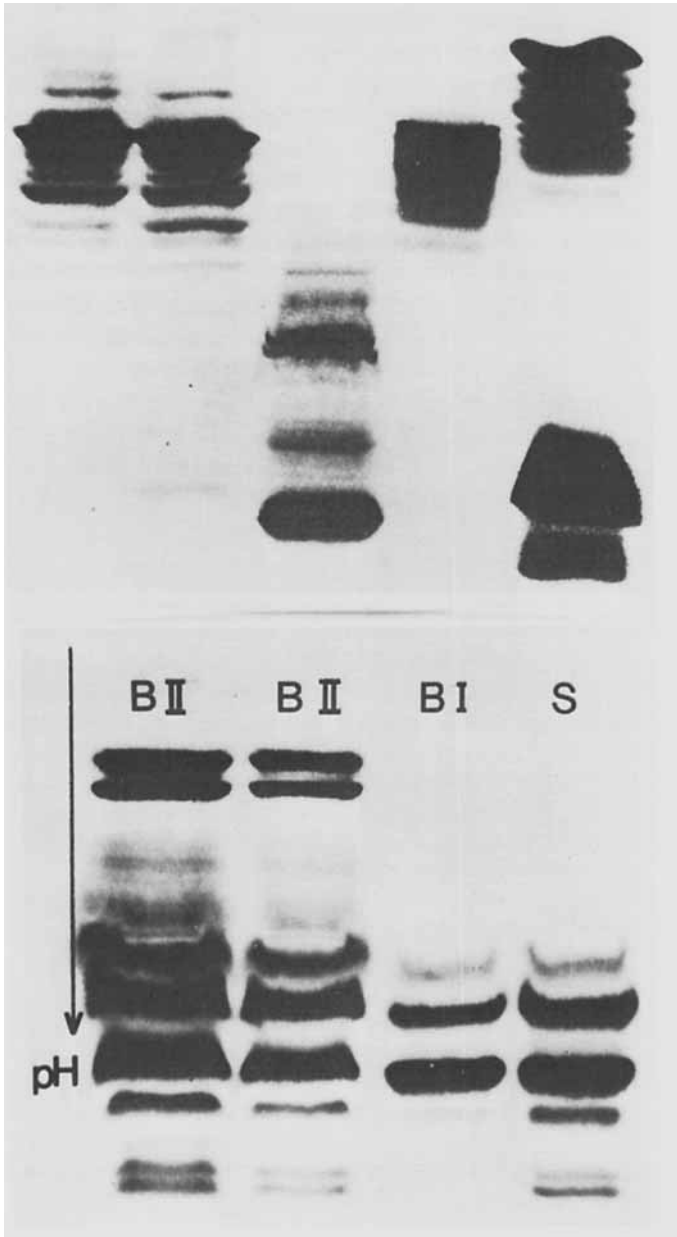



FIG. 18. Gel isoelectrofocusing apparatus made by Metaloglass, Inc. (*72*).



layer of carrier ampholyte is recommended. If photopolymerization is used, the sample can be incorporated directly in the gel formulation.

A 3.7% concentration of acrylamide has been recommended (*73*) in order to minimize sieving effects. Voltage values of 200 V/6 cm for 8 hr at 0–5°C are representative of typical runs (*73*). However, higher voltages and shorter duration of runs have been used successfully with a number of proteins (*20*). It should be kept in mind that there is a direct relationship between voltage, gel instability, and the appearance of a "plateau phenomenon," i.e., the progressive flattening from the gel center of the pH against distance curve with time (*73*). This results in a greater concentration separation of protein bands with pI's near the pH of the plateau and a compression of the distal bands. At constant time, the "plateau phenomenon" occurs in direct proportion to the potential applied.

Direct staining of the separated protein bands (*76, 77*) is now possible. A number of stains that required prior removal of ampholytes from the gels have been described (*20*). When specific antibodies are available, proteins can be detected by immunological precipitation (*78–80*). Enzymes can likewise be located by a suitable histochemical stain. The pH gradient can be measured by slicing of the gel and elution of each slice in water or with surface microelectrodes.

A new procedure for scanning *in situ* protein zones subjected to polyacrylamide gel isoelectrofocusing with a recording spectrophotometer has been developed (*40*). The method offers excellent zone resolution and



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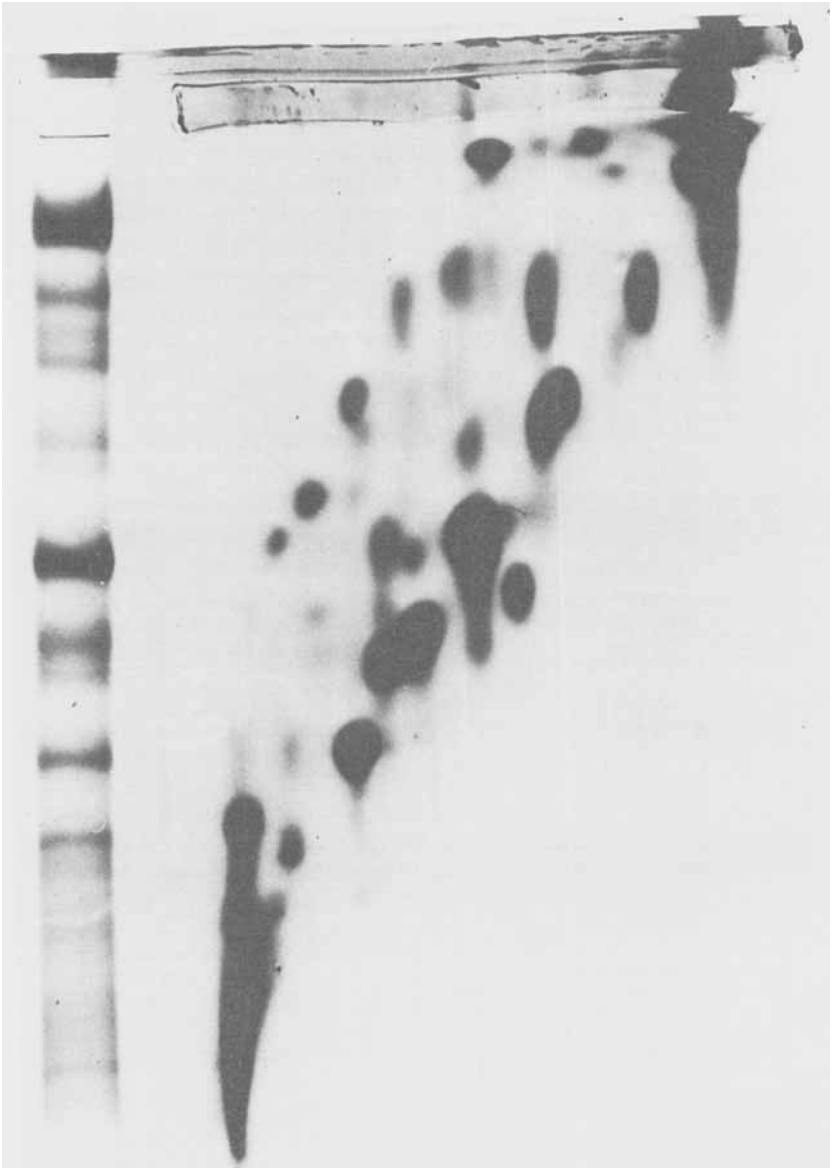
FIG. 19. (Upper): Thin-layer isoelectric focusing in a pH 3–10 ampholyte system using Sephadex gel. From left to right: horse myoglobin (Koch and Light); horse myoglobin –Calbiochem); ovalbumin 1X crystalized (Serva); beef hemoglobin (Serva); and a mixture of (from top to bottom) sperm whale myoglobin (Koch and Light), bovine serum albumin (Bechringwerke), and horse spleen ferritin (Koch and Light). All proteins were used as 5% solutions, in mixture the concentration of each was 5%; 30 μl of the protein solutions were applied with a cover slip at the middle of the plate. Plate: 40 × 20 cm, 0.75 mm layer. Focusing at 5–6 V/cm for 18 hr at 4–6°. Staining with Coomassie Blue. (Lower): Thin-layer isoelectric focusing of commercial horseradish peroxidase in a pH 3–10 ampholyte system. From left to right: B II (applied in two different amounts) and B I, peroxidase (Bochringer) with absorbance ratios of 0.6 and 2.8, respectively; S, peroxidase (Serva) absorbance ratio 2.6. About 100–200 μg of the preparations were applied. Plate: 20 × 20 cm, 0.75 mm layer. Focusing at 10 V/cm for 7 hr at 4–6°. Enzyme activity was detected by urea peroxide and *o*-toluidine (*83*).



the advantage of scanning proteins at different wavelengths. The separation and focusing processes can be observed as the proteins progress until optimum resolution is obtained.

*Thin-Layer Isoelectrofocusing*

The use of thin-layer plates of polyacrylamide or Sephadex gel as the stabilization matrix for isoelectrofocusing (*81–83*) was described at the same time that gel column techniques became available. One of the advantages offered by the thin-layer method is that several samples can be compared simultaneously on the same plate under identical conditions. Many similarities exist between thin-layer polyacrylamide gel and disc isoelectrofocusing in respect to the mode of polymerization of the gel and the procedures for staining and pH measurement. However, the apparatus used is different (*20, 81–83*). Sephadex G-75 gels have also been used successfully for isoelectrofocusing (Fig. 19). Details of the method can be found in the original paper by Radola (*83*).

*Isoelectrofocusing–Electrophoresis*

Two-dimensional separations by a combination of gel isoelectrofocusing and electrophoresis are possible as has been successfully demonstrated recently (*84–89*). A large number of components can be separated and "maps" can be compared (Fig. 20). The technique will find very useful applications in the typing of genetically polymorphic material and in clinical biochemistry. This method also offers exceptional versatility since separations in the second dimension (electrophoresis) can be performed by a variety of available electrophoretic methods and supporting gel media. An additional advantage is that the carrier ampholytes are removed from the "map" by the electrophoresis step and thus they do not interfere with common protein stains.

FIG. 20. Electrofocusing-electrophoresis pattern of proteins from potato tuber, variety Voran. The main part of the picture shows the "map" of protein spots obtained after polyacrylamide electrophoresis (pH 8.9, buffer 15.125 g Tris and 1.15 g boric acid per liter) of the electrofocused proteins (ampholine pH 5–7). The empty gel (barely visible on the picture) which has served as the starting line for the electrophoresis, is shown below the strip; it demonstrates that nearly all of the proteins entered the slab. On the left: the electrophoretic pattern run simultaneously (*84*).

*Immunoisoelectrofocusing*

Immunochemical techniques which involve either immunodiffusion or immunoelectrophoresis after gel isoelectrofocusing (Fig. 21) have been described (*20, 69, 78–80, 90–96*). When isoelectrofocusing is performed in agarose gel, certain precautions (*97*) should be taken to minimize electroosmotic flow effects. These involve the use of anion-exchange resin purified agarose, high concentration of carrier ampholytes, avoidance of a strong acid at the anodic electrolyte, and incorporation of inert substances, e.g., sucrose in the gel.

## Free Solution Isoelectrofocusing

Several methods have been described for performing isoelectrofocusing without support. "Zone convection isoelectrofocusing" (Fig. 22) is performed in the apparatus described by Valmet (*23*). The apparatus consists of two separate parts, the trough and the lid. The trough is a shallow rectangular box with a corrugated bottom. The lid is also corrugated. When the lid is placed over the box, a series of "U-tube units" are formed, as the projections on the lid fit into the depressions in the trough. The current forms a natural density gradient in each U-tube due to thermal diffusion. During focusing, the proteins are collected at the bottom of each trough because of their higher density. Protein precipitates do not disturb the separation, and zone remixing is eliminated because communication between the different compartments is cut off as soon as the experiment is finished. The pH of the fractions can be directly measured in the apparatus. Apparently the apparatus can handle large amounts of protein and the prospects for continuous flow operation seem to be good.

Carrier-free isoelectrofocusing in a coil of polyethylene tubing has been described (*26*). Some successful experiments also have been performed with a "rotating tube" free-zone electrophoresis apparatus (*98*). Continuous separation in a carrier-free stream has been reported with a special apparatus (*99*). The use of multimembrane apparatus with 20 closed compartments has been discussed by Rilbe (*100*). Another apparatus utilizing a sea-serpent shaped tube has also been applied to the separation of proteins (*24*).

There is no doubt that other types of apparatuses will be designed in the near future for the free solution isoelectrofocusing technique. The results that have been obtained by this method to this date do not seem to

be superior to density gradient or gel isoelectrofocusing methods. Rilbe (*100*) has expressed a similar opinion.

### Thermal Isoelectrofocusing

A very promising and interesting method of isoelectrofocusing was reported recently by Luner and Kolin (*101*). The pH gradient is generated by utilizing the temperature dependence of pH without the use of

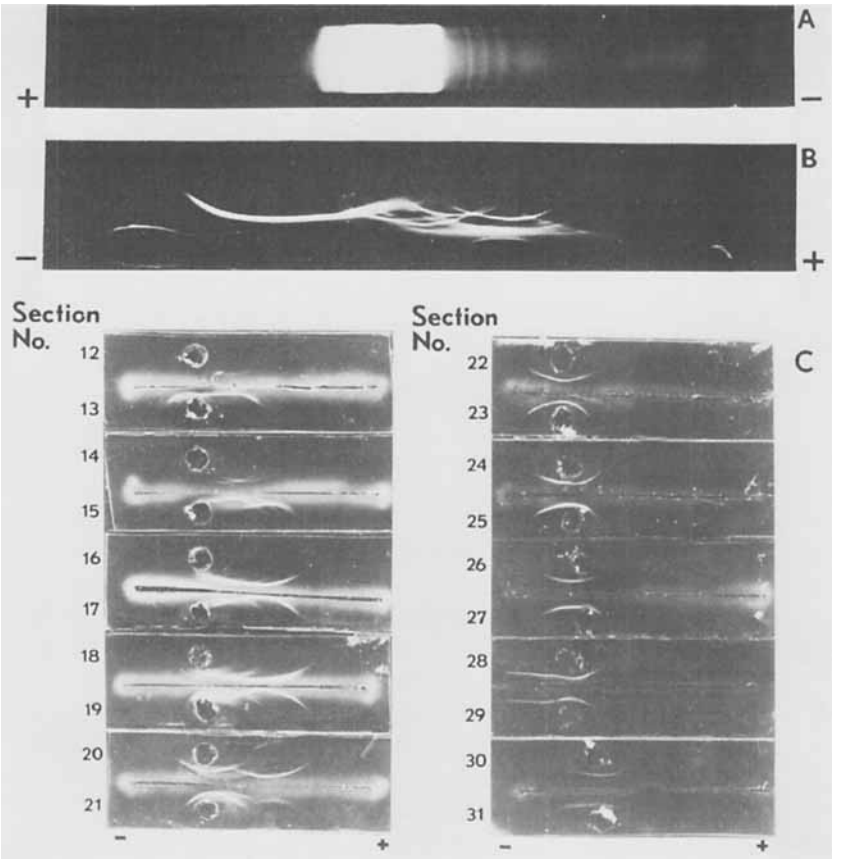



FIG. 21. Immunoelectrofocusing-electrophoresis patterns developed with goat antiserum to rabbit serum (C). The polyacrylamide gel column (A) shows the disc electrofocusing pattern (pH 3–10) of rabbit serum proteins. The immunoelectrophoresis pattern (B) was obtained from total rabbit serum proteins developed with goat antiserum to rabbit serum (*93*).

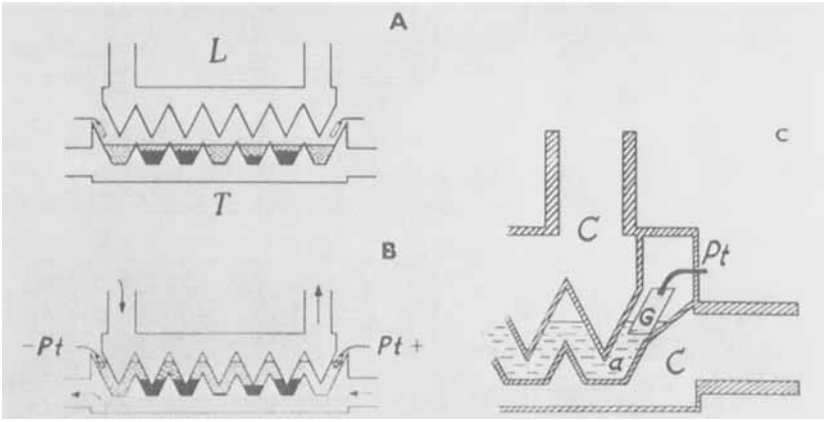



FIG. 22. Schematic drawing of the zone convection electrofocusing apparatus. (A) The filled "trough" (*T*) before or after the experiment. The separated protein zones are illustrated by the dark areas. The "lid" (*L*), lifted up as before or after the run. (B) Both parts put together, as during the experiment. Notice the different positions of the ampholyte solution level. Both parts are water-cooled. The arrows show the circulation of cooling water. *Pt*, the electrode. (C) The electrode. *Pt*, platinum wire; *G*, sintered glass filter; *a*, ampholyte solution; *C*, cooling water (*141*).



carrier ampholytes (Fig. 23). By establishing a temperature gradient between 0 and 50°C in an electrophoretic column, a stable pH gradient, in both the presence and absence of current, can be maintained extending over 1 pH unit. The pI of the focused zones can be determined indirectly by measuring the temperature of the zone with a thermistor. Isoelectrofocusing is achieved very rapidly and with very good resolution. The two main drawbacks of this technique appear to be the limited pH range of the experiment and the possible adverse effects of heat on proteins. However, compounds that are not susceptible to heat denaturation could be analyzed rapidly if their pI values are within a 1-pH unit range.

## Conclusions

The high resolution of the isoelectrofocusing technique offers a definite advantage in separation and characterization of proteins and other amphoteric substances. The method in its different variations has found many applications in the fields of biochemistry, genetics, immunology, and clinical analysis. In addition to its separation capabilities, it provides information on the isoelectric point of a compound which is not readily obtainable by other methods. Determination of the pI is in turn useful in

evaluating other properties such as conformational changes and chemical modifications.

New developments in respect to apparatus construction, nature of the pH gradient, stabilization media, combination with other techniques

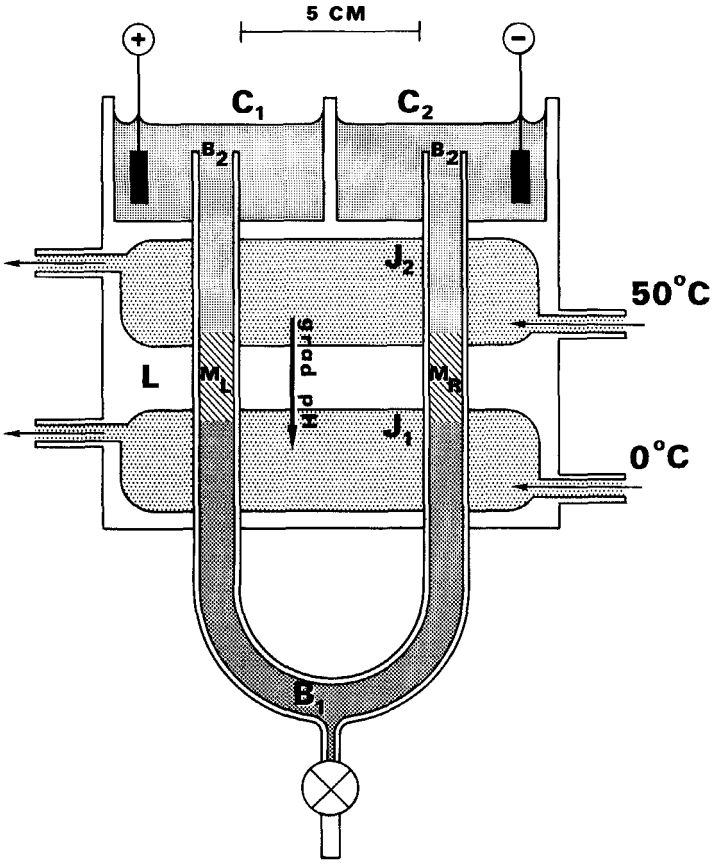



FIG. 23. Apparatus for isoelectric focusing and fractionation in a thermal pH gradient. The clear sections represent a framework milled from a 3-in. Lucite block. $J_1$, $J_2$: Water jackets through which water of 0 and 50°C, respectively, is circulated. The glass U-tube passes through the water jackets and the channels drilled for it through the solid Lucite partition $L$. $C_1$, $C_2$: Electrode compartments containing the electrode + and −. $B_1$: Sucrose-buffer solution of maximum density; $B_2$: sucrose-free buffer. $M_L$, $M_R$: Solutions of intermediate density containing the ampholyte mixture. The arrow indicates the direction of the thermally generated pH gradient (*101*).

such as fluorescence and laser Raman spectroscopy, and complete automation of certain procedures can be expected.

## ISOTACHOPHORESIS WITH SPACER IONS

### Introduction

*Principle*

Isotachophoresis is an electrophoretic method in which all ion species of the same sign having a common counterion move with the same speed at equilibrium. They are separated into consecutive zones arranged in the order of their constituent mobilities (i.e., ionic mobility × fraction ionized). The zones are in immediate contact with each other and exhibit different pH and temperature in a constant current electrical field (dc potential). The concentrations of the zones are adjusted to the concentration of the first ion zone, called the leading electrolyte, which must contain the leading ion having the same sign as the sample ions to be separated and a mobility higher than the other ions. The last ion zone consists of the terminating electrolyte having a terminating ion with mobility lower than any other ion in the system.

Theoretical treatments of steady-state-stacking (SSS) have been developed by Kohlrausch (*102*), Ornstein (*134*), Jovin (*144*), and Everaerts and Routs (*132*). Isotachophoresis can be described as SSS in the presence of spacer ions.

*Studies in Other Areas*

Kendall and co-workers applied the SSS method to the separation of isotopes (*103–106*). Further developments in terms of theory, instrumentation, and applications of the technique in areas other than protein chemistry were achieved in several laboratories (*107–132*). Haglund (*133*) has presented a comprehensive review of these studies and he has also supplied me with a complimentary reference list which has been used in this work.

*Separation of Proteins*

The present review is concerned with a particular type of isotachophoretic separation of proteins where carrier ampholytes are used as "spacer ions" of intermediate mobility. This technique was first reported

by Svendsen and Rose (*136*) as a preparative method and it was further explored by Griffith and Catsimpoolas (*77*), Arlinger and Routs (*137*), Catsimpoolas and Kenney (*143*), and Griffith et al. (*145*) as an analytical tool.

## Basic Theory and Consequences

### *The Regulating Function*

Consider two negative ions $A^-$ and $B^-$, and the common positive ion $R^+$. The equation expressing the conditions obtained at the boundary between the two salt solutions $A^-R^+$ and $B^-R^+$ migrating in an electrical field is given by

$$\frac{(B)}{(A)}\frac{x_A c_B}{x_B c_A} = \frac{u_B z_A (u_A - u_R)}{u_A z_B (u_B - u_R)} \tag{12}$$

where:

(A) = total concentration of substance containing ion $A^-$
(B) = total concentration of substance containing ion $B^-$
$x$ = fraction of dissociation
$c$ = concentration, mole liter$^{-1}$
$u$ = mobility, cm$^2$ V$^{-1}$ sec$^{-1}$ ($u_A > u_B$)
$z$ = charge

If the two solutions $A^-R^+$ and $B^-R^+$ are layered one on top of the other so that $A^-R^+$ (containing the ion with the higher mobility) is below $B^-R^+$, the boundary between the two anions will be sharply maintained during migration in an electrical field because the two species (on either side of the boundary) will be arranged to move at the same speed at equilibrium (*102*, *134*). In the case of a density stabilized leading ion migrating ahead of a trailing ion, the migration velocity of the leading ion regulates that of the trailing ion; i.e., the ion with the lower constituent mobility is accelerated to the constituent mobility of the faster ion by a change in voltage gradient. Sharp boundaries are formed between ionic species because the concentration of the ions on each side of the moving boundary are fixed (*133*).

### *The Use of "Spacer Ions"*

If one or more ions of intermediate mobility are present in the system, these will also reach equilibrium and form sharp boundaries with the ad-

jacent ions. Two narrow zones that are in immediate contact with each other may be difficult to recover separately. They can be forced apart, however, by inclusion in the sample of an ion with intermediate mobility acting as a "spacer." Ampholine carrier ampholytes can be used as "spacer ions" for the separation of proteins because of the large number of components present in the mixture with different p$K$ values and mobilities. However, a number of carrier ampholyte components may have mobilities similar to the proteins in the sample so that spacing is not always achieved.

*Conditions at Equilibrium*

Since different phases separated by moving boundaries in isotachophoresis exhibit sharp changes in temperature, thermocouples can be used to detect their position. Temperature changes occur because the field strength varies from one phase to the other (in constant current conditions) inversely proportional to their mobilities.

Jovin (*144*) and Everaerts and Routs (*132*) have derived a set of equations to describe isotachophoretic electrolyte systems in the steady state. They concluded that the pH chosen for the leading electrolyte is important for the separation of the sample ions. The original pH in the terminating electrolyte is not decisive for the pH created in the isotachophoretically moving terminator zone. Mobilities and p$K$ values are the most important parameters for the separation of ions. When working in a pH range near the p$K$ values ($\pm 1$ pH unit) of the intermediate ions, small differences in p$K$ values will influence the isotachophoretic separation more than small differences in mobilities. It also should be mentioned that the order of the net mobilities of two ions can be changed by varying the pH of the leading electrolyte.

A particularly disturbing effect is that although one normally expects that the pH in an anionic system always rises from one zone to the next, it is possible for the pH in two succeeding zones not to rise. Indeed, a pH drop can be expected in a system containing weak and strong electrolytes with comparable mobilities. In the case that strongly acidic spacers are used for the separation of compounds with high p$K$ values (e.g., proteins), the mixture can migrate in one zone without separation.

Griffith and Catsimpoolas (*77*) and Catsimpoolas and Kenney (*143*) have shown that some of the features affecting gel isotachophoresis of proteins using carrier ampholytes as "spacers" include: (a) the pH range of carrier ampholytes, (b) the amount of carrier ampholytes pres-

ent in the sample, (c) the pH and concentration of the leading electrolyte (gel buffer), (d) the mobility of the leading ion in the pH of the leading electrolyte, (e) the current intensity and time of run, (f) the gel porosity, and to a minor extent the ratio of protein to carrier ampholytes in the sample.

The multiplicity of factors that affect isotachophoretic separations offer a challenge for intelligent planning of experiments to fit a particular separation problem. There is no doubt that isotachophoretic conditions

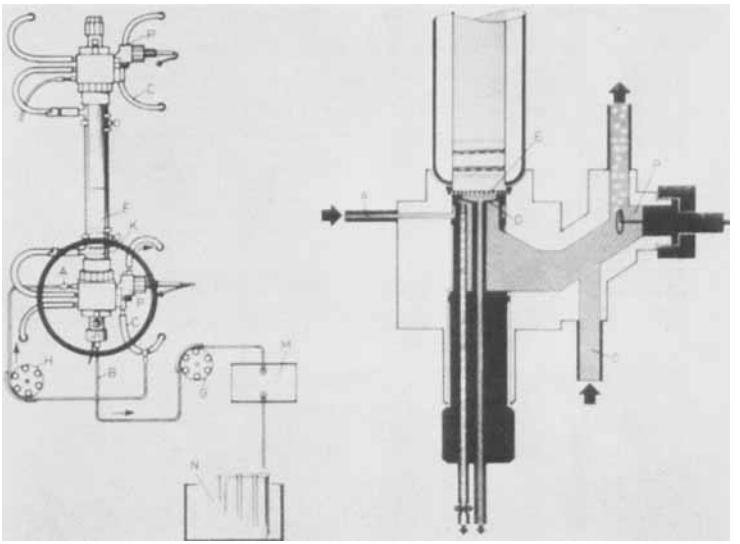


FIG. 24. The LKB Uniphor Column Electrophoresis Equipment adapted for preparative isotachophoresis according to Svendsen and Rose (*136*). Components of the sample migrate downwards in the polyacrylamide column, *F*. Separated zones, *K*, spaced with Ampholine carrier ampholytes, leave the column through the membrane, *E*, that supports the gel column, and enter the washing chamber formed by the membranes *D* and *E*. A peristaltic pump, *H*, forces electrode buffer at a rate of 5 ml/hr through the tube *A* into the washing chamber. A second peristaltic pump, *G*, sucks electrode buffer at a rate of 15 ml/hr away from the washing chamber through the tube *B*. A net flow of 10 ml/hr will thus pass from below through membrane *D* and carry away the zones as they enter through membrane *E* and take them through tube *B* to a UV detector, *M*, and eventually to a fraction collector, *N*. All the time buffer is circulated through the electrode chambers, *P*, via tubes *C*, from buffer reservoirs (not shown) (*133*).

derived with the use of computer programs will greatly facilitate separations of simple mixtures, and they may aid in the optimizations of experimental parameters for more complex ones.

## Experimental Approaches

### *Preparative*

Preparative isotachophoresis of proteins with carrier ampholytes as "spacers" has been carried out in a vertical-column electrophoresis apparatus with polyacrylamide gel as supporting medium (*136*). The leading electrolyte is incorporated into the gel matrix. The protein sample is mixed with the carrier ampholytes and is layered on top of the gel followed by the terminating electrolyte. Haglund (*133*) has shown that the same type of fractionation can be performed in the LKB 7900 Uniphor column electrophoresis system equipped with an end piece specially designed for this purpose (Fig. 24).



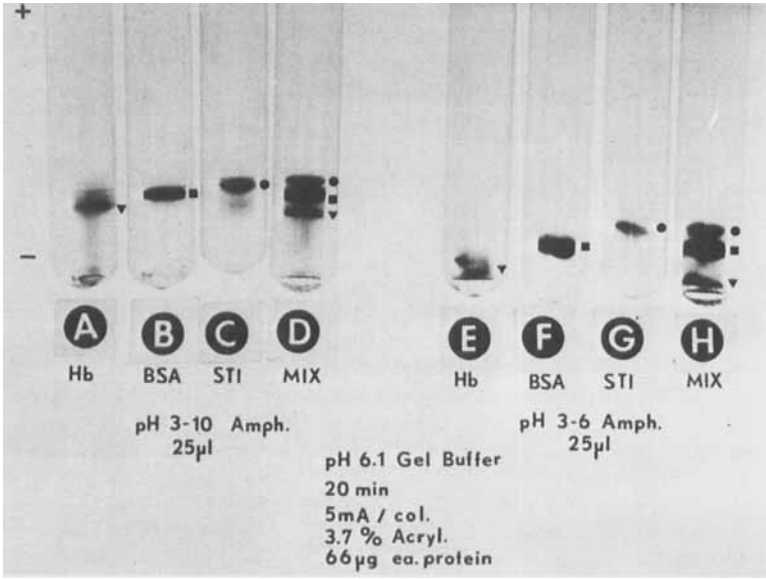



FIG. 25. Analytical gel isotachophoresis in polyacrylamide gels of human hemoglobin (Hb), bovine serum albumin (BSA), soybean trypsin inhibitor, Kunitz (STI), and mixture of the three proteins (MIX) in two different pH ranges of carrier ampholyte (*77*).

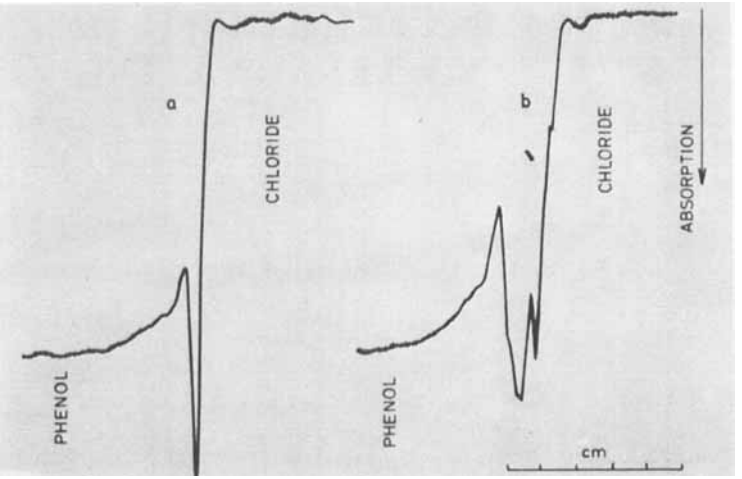



FIG. 26. Separation of ceruloplasmin in a capillary isotachophoresis apparatus. (*a*) No Ampholine added in sample. (*b*) Ampholine pH 4–6 was added to the sample. Leading electrolyte was 0.01 *M* ammediol, 0.007 *M* hydrochloric acid, and 0.002 *M* barium chloride, pH 8.4. The terminating electrolyte was 0.01 *M* phenol brought to pH 9 by the addition of ammediol. Current 60 μA, counterflow 60 μl/hr. Total analysis time was 2 hr (*13*).

Isotachophoresis can use any preparative polyacrylamide gel electrophoresis apparatus (*135*) if and only if it lends itself to the mechanical and/or hydrostatic support of very labile gels. The Buchler Polyprep and the Canalco Prep Disc apparatus do as well in this regard as the Uniphor, and all of them probably do considerably worse than their homemade prototype (*146*).

Suggestions for minor modifications of the LKB isoelectric focusing column for density gradient isotachophoresis experiments have been made by Everaerts and Routs (*132*). Apparatuses designed for other types of electrophoresis could also be modified for use in isotachophoretic experiments.

*Analytical*

Separation of proteins on a microscale in polyacrylamide gels using spacer ampholytes has been described (Fig. 25) (*77*). Equipment designed for disc electrophoresis (*74*, *134*) was used in these experiments although it has certain drawbacks.

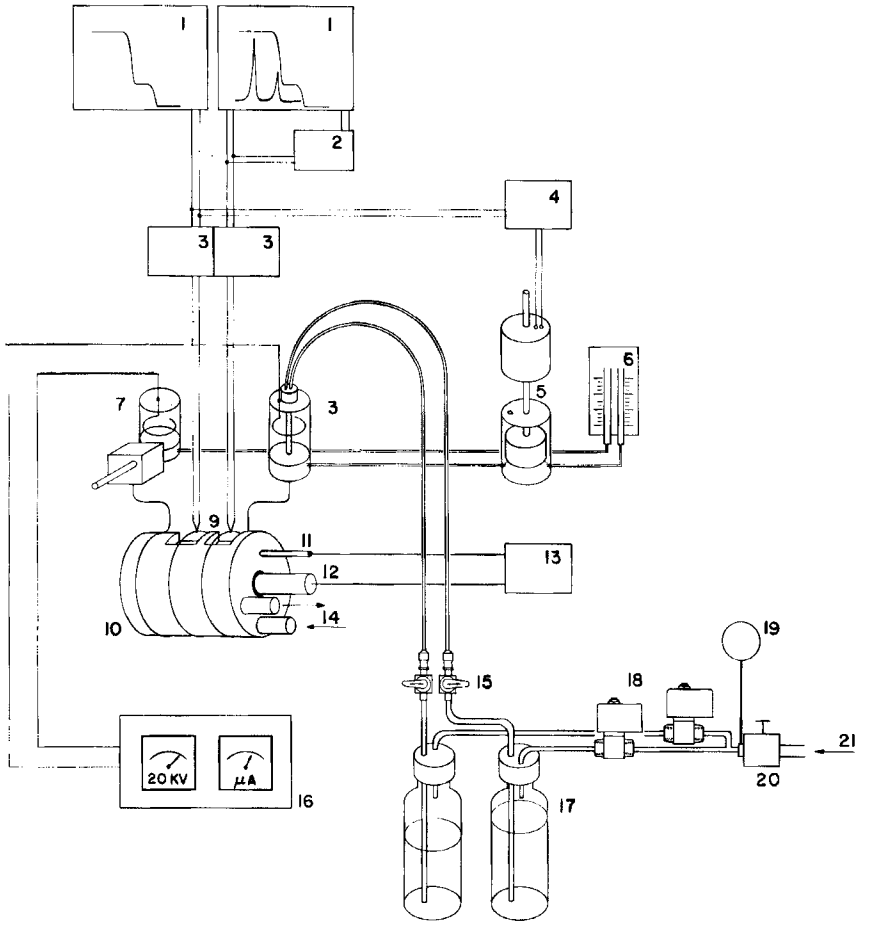



FIG. 27. Block diagram of the isotachophoresis equipment. 1 = recorders; 2 = differentiator; 3 = Knick amplifiers, type A; 4 = regulator for the counterflow; 5 = equipment for the counterflow; 6 = level control; 7 = injection bloc; 8 = counter electrode; 9 = thermocouples; 10 = Al bloc with capillary tube; 11 = Pt sensor; 12 = load; 13 = regulator for thermostating; 14 = thermostated water; 15 = Teflon-lined valves; 16 = current stabilized power supply; 17 = reservoirs; 18 = magnetic valves; 19 = manometer; 20 = pressure regulator; 21 = air (2 atm) (*127*).

A different experimental approach was taken by Arlinger and Routs (*137*). Separation of proteins with carrier ampholytes (Fig. 26) as spacers was carried out in the capillary apparatus of Everaerts and Verheggen (*127*) equipped with a UV detector (Fig. 27).

The gel isotachophoresis technique has the advantage that many samples can be analyzed simultaneously with higher sensitivity of detection. However, the capillary technique involves fewer manipulations and may become automated.

## Conclusions

The high separation capabilities of the isotachophoresis technique with spacer ions have already been established. Definition of isotachophoretic conditions for specific protein separations will be a fruitful area of work in the near future. Experiments with model or marker proteins of known isoelectric point may be helpful in establishing general guidelines for separation of other proteins. Elucidation of the "spacer" behavior of carrier ampholytes in isotachophoretic systems in mixture with proteins appears to be highly desirable.



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*Received by editor July 5, 1972*