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PREPARATIVE ISOELECTRIC FOCUSING

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CONTENTS

1) INTRODUCTION

Electrophoretic mobility has been used for years as a basis for separation and characterization of proteins. The resolving power of conventional electrophoresis is limited, and the characterization by mobility is not unique because of the dependence of the protein mobilities on the pH and ionic strength of the buffers.

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 molecules. IEF replaces differences in ionic mobilities with differences in isoelectric points (pI) as a separation criterion. IEF is an equilibrium method in which amphoteric compounds are segregated according to their pI's in pH gradients, formed by electrolysis of amphoteric buffer substances known as carrier ampholytes. In this system, an electrical current directed toward increasing pH sweeps a protein, which is positively charged in regions where pH < pI, toward the isoelectric zone, where it is arrested due to loss of charge. Similarly, proteins which are negatively charged in regions where pH > pI, are swept in the direction opposite to the current toward the isoelectric zone, where they come to a stop. Thus, eventually all of the amphoteric species are condensed (focused) in sharp isoelectric zones, which represent an equilibrium between electrical mass transport and back-diffusion.

The technique has now been refined to a level that permits the resolution of ampholytes whose pI's differ by as little as 0.01 pH unit or less. This resolving power cannot be achieved by conventional electrophoretic or chromatographic procedures. With these latter methods, specially adjusted conditions have to be devised for particular separations, while IEF, by virtue of being an equilibrium process, has a "built-in" resolution which allows one to

separate in the same experiment all components with measurably different pI's. IEF is particularly suitable for differentiating closely related ampholytes and provides a valuable criterion of homogeneity.

This review deals primarily with preparative procedures for IEF, in both liquid and solid media. Particular emphasis is given to continuous-flow techniques, either in gel or carrier-free systems, because they appear to be particularly valuable for enzyme purification in the gram scale, that is in a convenient level for industrial applications.

A. *Background*

As early as 1912 Ikeda and Suzuki[1], in a three-chamber apparatus, with membrane walls, were able to coarsely separate some amino acids in plant protein hydrolysates. They noticed that the amino acids tended to arrange themselves according to their pI's. Williams and Waterman[2] refined the technique by designing a multichamber apparatus in order to increase the resolving power and to reduce convective disturbances. Practical applications were limited, however, because of the variability of the field strength between the electrodes.

In 1954-56 Kolin[3-5] described the separation and concentration of proteins by electric transport in a pH gradient, stabilized by a sucrose density gradient. He used the term "isoelectric spectrum," in agreement with the use of "spectrum" to denote a distribution pattern established by a sorting process. The pH gradient was generated by placing the substance to be separated at the interface between an acid and a basic buffer, in a Tiselius-like apparatus, and allowing diffusion to proceed under an electrical field. In these "artificial" pH gradients, Kolin was able to obtain "isoelectric line spectra" of dyes, proteins, cells, microorganisms, and viruses on a time scale ranging from 40 sec up to a few minutes. These extremely rapid results, still unmatched in a field of electrophoresis, were obtained through the concomitant action of several

factors playing on the separation cell: a pH gradient, a density gradient, an electrical conductivity gradient, and a vertical temperature gradient. The main drawback was the instability of these gradients, due to rapid migration of the buffering electrolytes during electrolysis.

In the 1960's Svensson[6-8] (now called Rilbe) laid the foundations of IEF in its present form with a series of theoretical articles entitled "Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients." He introduced the law of pH monotony, and studied the protolytic properties of carrier ampholytes, their conductivity, their buffering capacity, their titration curves, and the resolving power of the technique in presence of suitable ampholytes, capable of generating stable pH gradients. These concepts were achieved in practice with the synthesis by Vesterberg[9] of carrier ampholytes with many of the properties described by Svensson. These substances, encompassing the pH region 2.5 to 11, are available from LKB Produkter AB, Bromma 1, Sweden, under the trade name Ampholine, either as a "wide pH range" (3.5 to 10) or as several narrow ranges of 0.5 to 3 pH units.

Since the general acceptance of IEF in the field of biochemistry and related areas, several international meetings have been held to sum up developments in the field: in 1972 in New York [10] and in Tübingen[11] (the latter mostly on electrophoresis, however), in 1973 in Glasgow[12], and in 1974 in Milano[13]. Several review articles have appeared by Haglung[14], Vesterberg[15,16], Catsimpoolas[17,18], Williamson[19], Wrigley[20], and Righetti and Drysdale[21]. However, since the classical review of Haglund, little attention has been paid to new developments in preparative IEF.

B. Synthesis and fractionation of ampholytes for IEF

Some of the properties required of carrier ampholytes can be summarized as follows: (*a*) good buffering capacity at the pI, (*b*) good conductivity at the pI, (*c*) low molecular weight, (*d*) good

solubility in water at the pI and hydrophylic character, (*e*) low light absorption above 260 nm, and (*f*) chemical properties different from the proteins to be separated. Vesterberg[9] ampholytes meet most of these requirements.

I feel, however, that progress in preparative isoelectric focusing, especially when using continuous-flow techniques, has been severely hampered by the high cost of Ampholine and by difficulties in recovering them after a run, particularly when using sucrose density gradient stabilized columns. This problem has spurred Vinogradov *et al.*[22] to "rediscover" the Vesterberg synthetic procedure and to obtain carrier ampholytes in the pH range 4 to 8 by coupling pentaethylene-examine (PEHA) with acrylic acid. Under the same pressure, the Vesterberg synthetic process has been discovered for a third time in my laboratory[23]. I will describe here briefly how we perform it. Suitable carrier ampholytes are obtained when appropriate amounts of acrylic acid are allowed to react with different polyethylene polyamines in water at 70°C until all the acrylic acid has been consumed. The synthesis goes via an anti-Markovnikov addition, after the mechanism of the Michael reaction, and therefore β-amino acids are obtained[23]. We use hexamethylenetetramine (HMTA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA), and PEHA; and we couple them, either singularly or in a mixture, to acrylic acid. All the reagents have to be distilled under nitrogen and reduced pressure just before use. To a 0.15 M solution of the polyamine in water, acrylic acid is added dropwise, under continuous stirring, over a period of 60 min, to a final N/COOH ratio of 2:1. After that, the flask is stoppered and transferred to a Dubnoff shaker, thermostated at 70°C, for 16 to 20 hours. The ampholytes thus obtained are diluted to 40% (w/v) and stored frozen in dark bottles. Yellow compounds formed during the synthesis can be removed by repeated charcoal treatment, as reported by Vinogradov *et al.*[22].

By several criteria[23], our product is equivalent to commercially available Ampholine. Our ampholytes can be easily synthesized,

the starting reagents are inexpensive, and large amounts can be produced in a short time. The quality of the synthetic product can be checked by inspecting focused ampholyte patterns along a gel slab, visualized by their different refractive indexes. Fig. 1 shows a picture of PEHA-ampholytes focused in a gel slab. The sharp, closely spaced peaks indicate a smooth pH gradient and good conductivity along the gel. Thus, even PEHA-ampholytes by themselves are already quite good for IEF, even though they still show three major valleys of low ampholyte concentration and even though the peaks of the most basic ampholytes become progressively spaced and shallower.

Our mixture of HMTA-, TETA-, TEPA-, and PEHA-ampholytes is useful in the "wide pH range" 3 to 9.5. However, we have described a simple method[24] to fractionate them in narrow pH ranges, encompassing one or two pH units, based on the continuous-flow isoelectric focusing principle of Fawcett[25]. Fig. 2 shows the setup for continuous-flow fractionation of carrier ampholytes in a 12 channel chamber, stabilized by a Sephadex G-100 bed. This method appears to be superior to multicompartment electrolysers, as described by Rilbe[26] and Vesterberg[9], since it avoids problems of osmotic pressure and polarization, due to membranes, and it avoids anodic oxidation of ampholytes.

There have also been other approaches to the production of ampholytes. Thus, Pogacar and Jarecki[27] have described the coupling of TEPA and PEHA to either propansulfone, vinylsulfonate, or chloromethylphosphonate. However, their "sulfonic ampholytes" do not buffer in the pH region 3.5 to 6, while they are able to cover the pH zones 2 to 3.5 and 6 to 9.5. While not useful *per se*, these compounds could be a useful addition to "carboxyl ampholytes" to extend their fractionation range down to pH 2.

Blanicky and Pihar[28] prepare ampholytes from bactopeptone, after removal of proteins by precipitation in 60% ethanol. These compounds, however, are peptides and in IEF they could give rise to some inconveniences. There have also been attempts to obtain pH

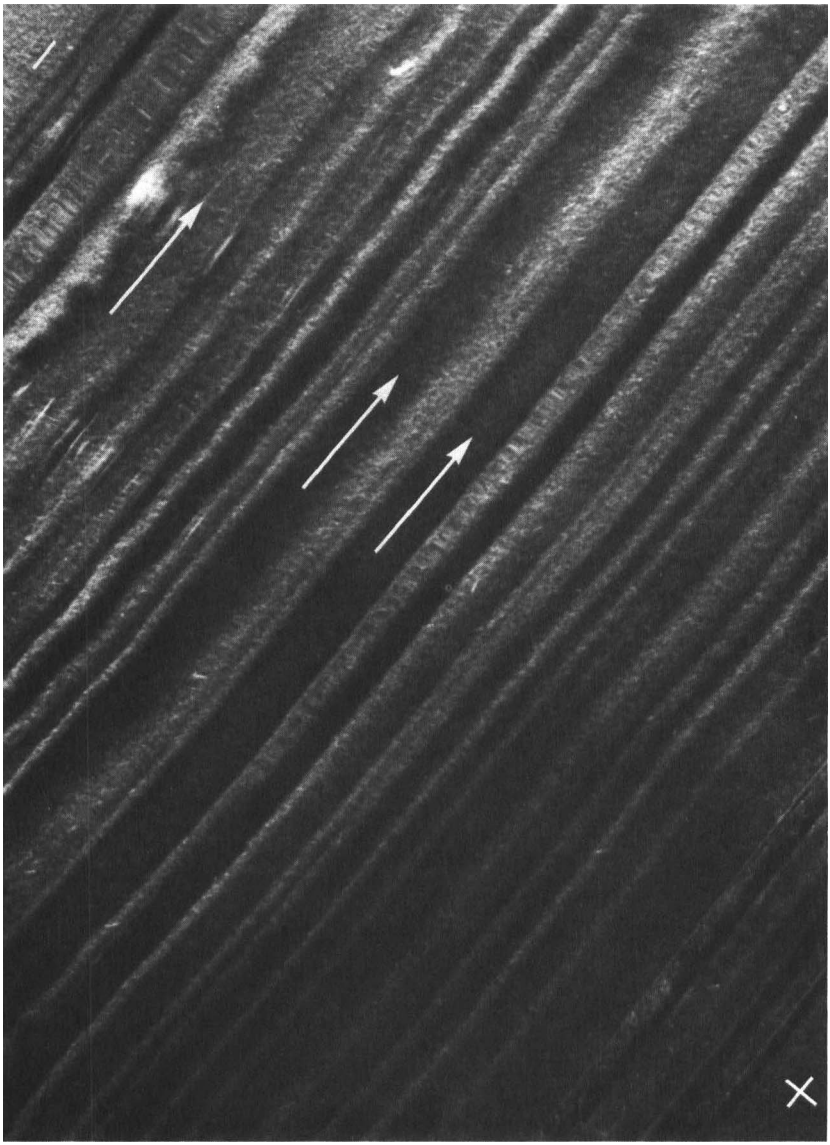

FIGURE 1

PEHA-ampholytes focused in a gel slab. At equilibrium, the gel was photographed against a black background with side illumination. The rope-like structures are clusters of focused ampholytes. This is a picture of a transparent, unstained gel and the ampholytes are detected on the basis of different refractive indexes. The anode (+) and the cathode (-) are marked. The three white arrows indicate ampholyte gaps. (By permission of Elsevier. See ref. 23.)

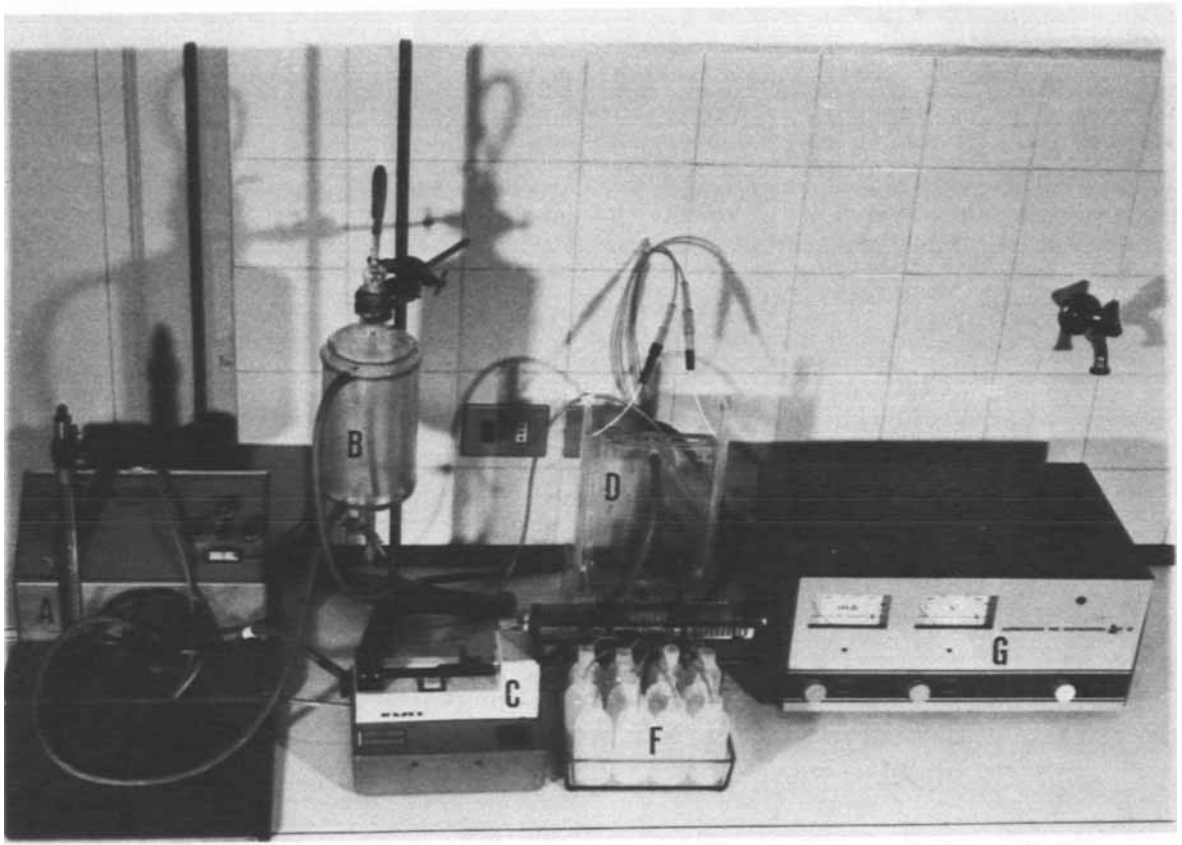

FIGURE 2. Setup for continuous-flow isoelectric focusing of ampholytes. A: thermostat. B: Mariotte flask. C: stepping motor from a Razel syringe pump, model A99. D: chamber for continuous flow isoelectric focusing. E: modules of a delta/6 pump from Watson-Marlow Ltd. F: collection bottles. G: power supply. (By permission of Elsevier. See ref. 24.)

gradients without the use of carrier ampholytes. Thus, Troitzki *et al.*[29] generate pH gradients by using common buffers in gradients of organic solvents, such as ethanol, dioxane, glycerol, or in poly-ol gradients, such as mannitol, sucrose, and sorbitol. They have shown separations of rabbit haemoglobin in a pH gradient 7 to 8.6 obtained with borate buffer in a mixed gradient of 0 to 5% glycerol and 0 to 30% sucrose.

Another interesting approach is the one by Luner and Kolin[30], who perform IEF in a "thermal pH gradient." Again, Kolin's system is characterized by quick focusing times (15 min for a complete fractionation). In this method, the pH determination amounts to a temperature measurement with a thermistor, which can yield the pH in a zone to better than 0.01 pH unit, from the known pH versus temperature curve. Broadly speaking, the approaches of Troitski *et al.* and Kolin are similar, since they are based on pK variations of buffers either in gradients of organic solvents or in temperature gradients. It is too early perhaps to assess the impact of both methods; however, Lundahl and Hjertén[31] report that they can only be of limited applicability in isoelectric focusing.

2) ANCILLARY EQUIPMENT

A. Power supplies

Since the introduction of the technique of electrophoresis, supporting media, buffer systems, electrophoretic cells, and cooling methods have all undergone extensive changes. Little attention, however, has been paid to the source of electrical potential. Among typical power sources used in electrophoresis, the only differences in output are in the range of power provided and in the type of regulation: constant voltage or constant current.

Recently, a second generation of power packs has appeared, capable of operating either in the constant voltage, or in the constant current mode, and with automatic switch-over from one mode

of operation to the other. With the advent of IEF, we have felt the need for a third generation of power supplies, operating at constant wattage. Since the migration rate of the sample and the sharpness of focused bands at their pI's is proportional to the applied voltage, it is desirable to have the applied field strength as high as possible, while maintaining the joule heating (which is proportional to the square of the applied voltage) at an acceptable level. This maximum tolerable power, defined as optimum power by Schaffer and Johnson[32], will depend on the conductivity of the system and on its efficiency to dissipate joule heating; and can be determined by inserting a thermic probe in the separation column. Once this optimum power, for a given system, has been determined, that system will run on an isotherm for the duration of the experiment.

A form of constant power can be produced by repetitively discharging the energy stored in a capacitor or in an inductor. This is the case of the Ortec model 4100 (Ortec Inc., Oak Ridge, Tenn.) and of the Bosi's pulsed power supply (patent Bosi No. 833364, Milano, Italy). However, in the case of the former Chrambach *et al.*[33] and in the case of the latter, Righetti and Righetti[34] have found that they do not really provide constant wattage when applied to IEF.

Schaffer and Johnson[32] have built a regulator which transforms an unregulated DC power supply into a constant wattage power unit. Söderholm and Lidström[35] have built a constant wattage power pack, capable of delivering a maximum of 3000 V, 300 mA, and 300 W, by applying a wattage regulator, which feeds the signal to a reversible synchronous motor (5 rpm) used as a servomotor, to a constant voltage power supply.

One system presently available is the ISCO (Lincoln, Nebraska) power supply model 492, which is capable of delivering a maximum of 1000 V, 150 mA, and 150 W (see Fig. 3). A useful feature of it is that, in addition to constant power, the instrument can also provide constant voltage with automatic current limiting and constant cur-

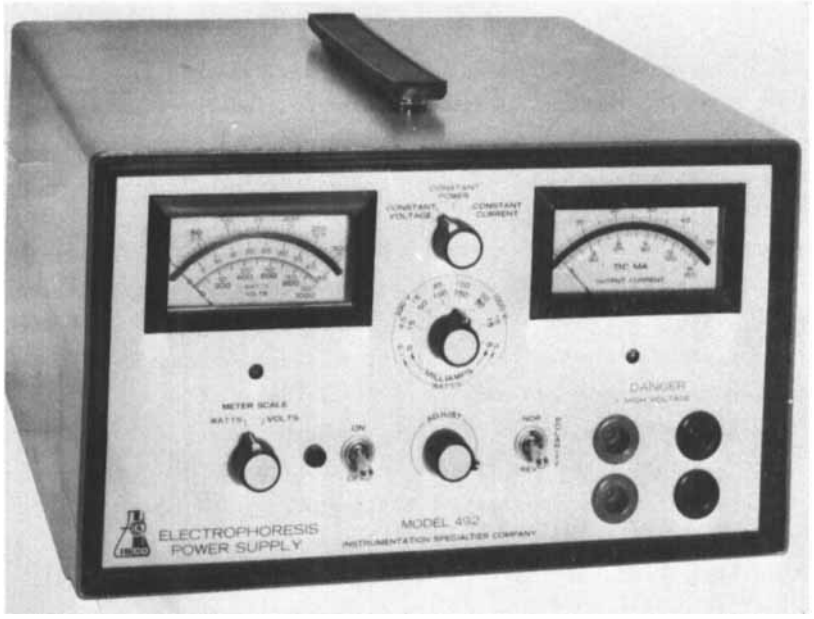

FIGURE 3

The ISCO constant wattage power supply, model 492. Specifications: 1000 V maximum voltage; 150 mA maximum amperage; and 150 W maximum wattage. The instrument can be operated either in the constant voltage, constant amperage, or constant wattage modes.

rent with automatic voltage limiting. This is particularly attractive, since this power supply can be used not only for IEF and normal electrophoresis, but also for isotachophoresis (where constant current is a must). Another constant power supply, but of the "pulsed" type, is currently available from Medical Research Apparatus (MRA, Boston, Massachusetts). It continuously monitors and regulates the average power delivered to the cell by automatic control of pulse frequency.

B. Flow cells

In preparative IEF, especially in liquid support media, combined UV and pH readings of all the collected fractions can be a lengthy and cumbersome procedure. To overcome that, Jonsson *et al.*[36] have automatized the analysis of column contents by pumping it at a constant rate through series-coupled flow cells - one for pH recording and the other for UV absorbancy determination. The advantages are obvious: direct transfer from peaks of the absorbancy curve to the pH curve gives the pI's, while integration of the UV peaks gives information on the relative amounts of the various components. This setup, however, presented some severe technical difficulties in pH determination, discussed in detail by Jonsson *et al.*[36]. More recently, Secchi[37] has described a modification of this technique, which appears to overcome many of these problems. He couples serially a combined glass electrode (Ingold type 401-M7, provided with a flow vessel and water cooling jacket) with the UV flow cell of the LKB Uvicord II. However, in this case, instead of using two separate recorders, he has built a control unit which feeds the pH signal coming from the pH meter into the same galvanometric recorder (LKB 6500) used for UV readings. Since the inner chamber of the flow pH-electrode has been reduced to 0.5 ml, and since the volume lag between pH and UV cells is only 0.35 ml, accurate and simultaneous pH and UV readings are obtained in the same recorder chart. Moreover, since the pH cell is thermostated, pH readings are performed at the same temperature applied to the focusing column. Another advantage of flow systems is that possible CO_2 absorption from the air, which might affect pI readings by lowering the pH of the solution, especially in alkaline regions, is avoided. The block diagram in Fig. 4 shows the experimental setup for the flow system described.

A similar setup has been reported also by Strongin *et al.*[38], who have built a pH flow-cell in perspex with type K-401 and type GK2320C pH electrodes from Radiometer A/S (Denmark). An interesting feature is that they have an extremely small dead volume (0.15

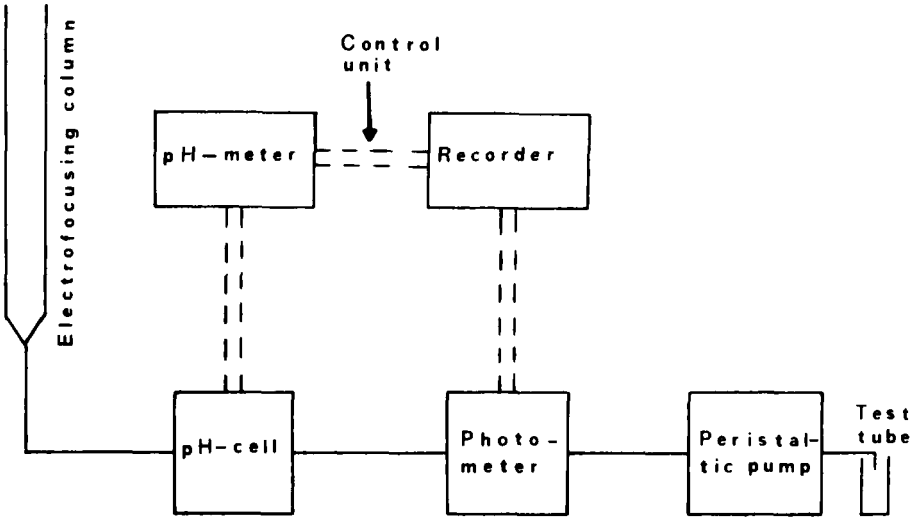

FIGURE 4

Block diagram of series-coupled UV and pH flow cells, with simultaneous reading on the same recorder. For further details, see the text and refs. 36 and 37.

ml) in their flow-cell. Drawbacks are found in the lack of temperature control for pH determinations and in the use of two separate recorders, as in the system of Jonsson *et al.*[36]. Whatever method is used, it is important to make sure that the peristaltic pump is attached after the cells in order to prevent KCl leakage from the electrode; and to keep the flow rate as slow as possible, to avoid pressure on the electrode glass membrane, which could alter the pH readings.

C. Other equipment

When performing IEF in solid support media, it might be convenient to be able to measure the pH gradient directly along the gel length. This can be accomplished using an antimony microelectrode (1 mm diameter) in conjunction with a calomel reference

electrode, as reported by Beeley *et al.*[39]. These electrodes are manufactured by Activion Glass (Kinglassie, Fife, England), according to the design of Kleinberg[40]. Measurements are made by pressing the reference electrode at any position along the gel and scanning at regular intervals with the antimony microelectrode. Readings of electromotive force are obtained on the millivolt scale and converted into units of pH by means of an appropriate calibration graph. This type of electrode might be better standardized against Ampholine solutions, which in turn are standardized against known buffers. Alternatively, on gel slabs, the pH can be measured directly on the gel surface with a flat membrane electrode, such as type LOT403-30-M8 from Ingold (Zurich, Switzerland).

It should be noted that pI values estimated by IEF are temperature dependent and usually decrease with increasing temperature[41]. The pH should, therefore, be measured at a constant, specified temperature, preferably the focusing temperature. Unfortunately, for the pI determinations of proteins reported in the literature since the advent of IEF, little temperature data are given. Even when the temperature is reported, there are still doubts as to how the experiment has been performed. In fact, some people keep the gel or the solution, for pH determinations, in ice, but they allow the electrode to be at room temperature. Others standardize the pH electrode against a buffer kept at another different temperature. I think that the best solution is to have the electrode, with saturated KCl and buffers, in the cold room, connected to the electronic control unit outside by a cable passing through the wall. In this way, since most of the time IEF is performed at 4°C in thermostated units, reliable and reproducible pH determinations will always be obtained.

Among other equipment needed, especially when working in liquid media, are fraction collectors such as the LKB Ultrorac, the ISCO model 328 Golden Retriever, or the escargot. They are compact in size and handy in the laboratory.

Gradient formers are particularly useful when working in density gradient stabilized columns. LKB has made two such mixers, one for the 110 ml (LKB 8121) and the other for the 440 ml column (LKB 8122). They are built on the basic design by Parr[42] which has been modified by Svensson and Pettersson[43]. They are made of two cylindrical containers: one with two outlets and a stirrer for the dense solution; the other with one outlet and a plunger (thickening upwards for gradients of falling densities) to hydrostatically equilibrate the light with the dense solution. Alternatively, gradients can be produced with pumps, such as the ISCO model 570 gradient former, which utilizes two syringe pumps to form linear, convex, and concave gradients in the volume range 3 to 80 ml.

Cooling baths are, of course, essential for IEF in any scale and in any support media. The heat produced by the current has to be dissipated by a liquid contact, possibly in glass apparatus or, in case plastic is used, in equipment with very thin plastic walls. Placing the electrophoretic cell in the cold room and allowing heat dissipation in air is a very poor and inefficient method, as can be seen by placing a thermic probe in the separation medium[34].

3) PREPARATIVE IEF IN LIQUID MEDIA

A. The LKB columns

The 110 ml (LKB 8101) and the 440 ml (LKB 8102) columns are perhaps the best known equipment in the field of IEF, since they are tied up with the early development of the technique (see Fig. 5). Both columns are a result of the early models of Rilbe[8]. The former allows a total protein load of a maximum of 25 mg and the latter up to four times as much. In both cases, the load should not exceed 5 mg protein/cm^2 of cross sectional area. Since experiments are usually performed in 1% Ampholine solution, 1.1 g and 4.4 g Ampholine will be needed, respectively, in each column. The column is usually stabilized by a density gradient obtained

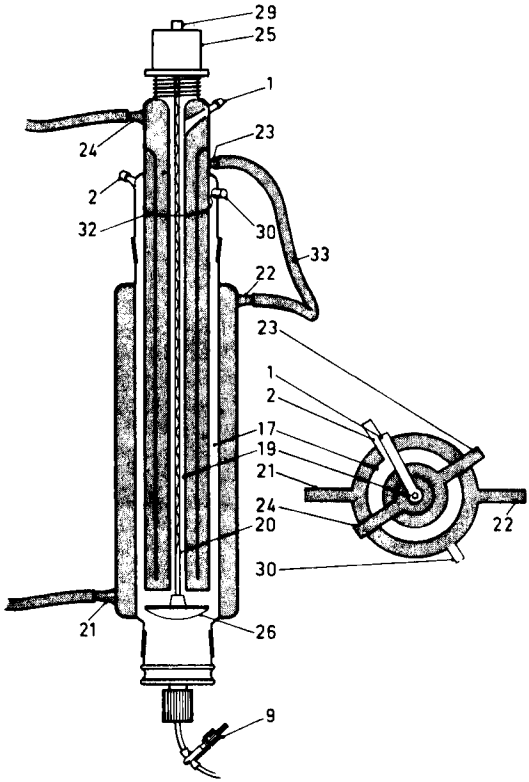

FIGURE 5

Sketch of the LKB 8101, 110 ml column. (1) gas escape for the lower electrode. (2) gradient and sample inlet. (9) elution capillary. (17) electrofocusing compartment, consisting of a hollow cylinder thermostated on both sides by cooling jackets. (19) lower electrode compartment. (20) teflon support for the lower electrode. (21) and (22) inlet and outlet, respectively, for the outer cooling jacket. (23) and (24) inlet and outlet, respectively, for the inner cooling jacket. (25) setting device for opening and closing valve (26) used to shut off the lower electrode compartment (19) from the electrofocusing chamber (17). (29) and (30) lower and upper electrode terminals, respectively, for connection with the power supply. (32) upper platinum electrode. (33) tubing connection between the two cooling jackets.

with analytical reagent grade sucrose. It is convenient to have a 50% (w/v) sucrose concentration in the dense solution, since this allows a density difference, between the light and dense solutions, of 0.2 g/cm^3, for which the characteristics of the two LKB gradient mixers have been calculated. The anodic solution is usually 1% phosphoric or sulfuric acid, while the catholyte is either 2% ethanolamine or ethylendiamine, or 8% NaOH. When possible, it is preferable to have the anode at the bottom of the column, since the electrolyte solution layered in this region must be dissolved in 60% (w/v) sucrose. If the polarity were reversed, the NaOH dissolved in the dense sucrose would ionize it, and, therefore, sucrose in this region would act as an acid[44]. The choice of the electrode position depends also on the pI of the components to be separated. It is desirable to be able to focus the sample as near to the bottom of the column as possible, to minimize diffusion effects due to long elution times in the case of components focused near the column top.

The sample can be added dissolved either in the dense or the light solutions, or in both. Alternatively, it can be introduced as a narrower zone close to the place where it is expected to focus. In this way the run will be shortened, and exposure to pH extremes avoided. The amount of salt and buffer in the sample should be low, since high salt concentration will give high current densities at the start, and generate acid at the anode and base at the cathode. A good solution is to dialyse the sample against 1% glycine or 1% Ampholine, which are known to stabilize protein solutions.

Usually 500 V is applied at the beginning of electrofocusing. The small column should not be allowed to dissipate more than 2 to 3 W at the beginning, and the large column 6 to 9 W. Since the separation column is a hollow cylinder with cooling on both surfaces, heat dissipation is very efficient. However, it is suggested that the column should not be thermostated below 2 to 4°C[14]. This is because the viscosity and conductivity of the sucrose-Ampholine mixture have a temperature coefficient which is

nearly constant down to around 1°C. Just under 1°C the temperature coefficient rises steeply. Therefore, at or below 1°C, small temperature differences between layers close to the cooling mantle and layers in the middle of the column will give rise to wavy zones, thus impairing the resolution.

Instead of sucrose, other solutes can be used, such as sorbitol or glycerol, especially when performing IEF in alkaline regions. Ethylene glycol can also be used at concentrations of 60 to 70% in the dense solution, because of its lower density. Other useful agents include mannitol and polyglycans, such as Dextran and Ficoll (Pharmacia, Uppsala, Sweden)[16].

There are some problems connected with the use of columns stabilized with vertical density gradients. One is due to diffusion during elution and remixing of the zones in the lower chamber of the apparatus, as the liquid column contained in the hollow cylinder merges in a continuous stream. Additional remixing of adjacent zones can be generated by the parabolic flow profile in the capillary tubing and in UV and pH flow cells. To minimize that, the capillary path after the column should be kept as short as possible and the volume of the flow chambers reduced to a minimum. Another source of diffusion is due to the peristalsis generated by pumps, especially when they are inserted in the capillary used to collect the eluates. To avoid that, it is suggested that the column be emptied by gravity, or that the column top be plugged (inlet No. 2 in Fig. 5) with the pump tube. As the water enters there, it displaces the column content downwards. The rate of outflow will thus be dictated by the speed of the pump.

Another great drawback is due to flocculation of less soluble proteins at their pI. The precipitates, once formed, aggregate into larger particles which begin to sediment slowly along the column. Sometimes they are redissolved as they fall into zones away from their pI, and are thus forced back. In any case, the separation is often spoiled, as the precipitate sticks to the walls and disturbes the density gradient or contaminates adjacent zones.

To avoid that, one could decrease the protein load, or increase the Ampholine percent. However, both methods are undesirable, since they lead to an economically unfavorable protein/Ampholine ratio. A better approach is to use additives[45]. It is known that urea at a concentration of 2 and up to 4 M and formamide can have solubilizing effects on proteins. For this purpose, non-ionic detergents have also been successfully used, in concentrations ranging from 0.1 to 5%, especially for membrane bound proteins. Many such detergents are now available under various trade names, e.g.: Tween 80, Emasol, Brij 39, Triton X 100 (Sigma Chemical Co., St. Louis Missouri). Serum lipoproteins have been maintained in solutions by using 33% ethylene glycol[46] or tetramethyl urea[47]. When focusing slightly soluble peptides or antibiotics, 50% dimethyl sulfoxide (DMSO) can be added to gels or liquid media[48]. Alternatively, as suggested by Rosengreen in a round table discussion at the Milano meeting[13], dimethyl formamide could be used instead, since DMSO easily undergoes redox reactions. At this same meeting, Wadström[13] has suggested the combined use of additives in the same column. For instance, a mixture of urea and Triton X 100 seems to be very useful in the analysis of protein components of the red blood cell membrane. Wadström has also stressed the importance of using highly purified, non-ionic detergents, since they often contain impurities which adversely affect the separation. In the case of Triton X 100, he suggests the scintillation grade product from Serva (Heidelberg, Germany).

As reported by Jacobs[49], additional problems may arise from the oxidation of cysteine and methionine to cysteic acid and methionine sulfoxide. This can be prevented by performing IEF in the presence of antioxidants, such as thiodiglycol or ascorbic acid. The latter seems to be more effective, and also prevents possible modifications of tyrosine and arginine residues. In the case of sulfhydryl-dependent enzymes, their activity can be preserved by working in the presence of a low concentration (about 10^{-4} M) of

thiol compounds[50]. For this purpose, 2-mercaptoethanol, 2,3-dimercaptopropanol and dithiothreitol[51] have been effectively used.

B. The ISCO columns

Instrumentation Specialty Company (ISCO) has put on the market two columns for isoelectric focusing - an analytical or small scale preparative setup (model 212), and a preparative version (model 630). The former has a bore of 1 cm and a length between electrode wells of 31 cm. Its maximum gradient volume is 23 ml and its maximum load capacity is a few mg protein. When cooled to 2°C it can be run with an electrical load of up to 15 W. The latter, built on the same principle as the model 212, can accommodate gradients up to 160 ml in volume and 32 cm in length. When cooled to 2°C it can be run at approximately 20 W. ISCO reports separation of 30 mg BSA in an 80 ml gradient (16 cm). Fig. 6 shows a complete separation setup with the model 212 column, and Fig. 7 is a drawing of the same column. An interesting feature of both systems is that the electrode chambers are built coaxially around the separation column. Two concentric membranes separate each electrode from the gradient to inhibit the introduction of electrolysis products and pH changes into the column. This also prevents net transfer of gradient components into or out of the inner column.

Another advantage is the possibility of intermittent scanning of the column. During isoelectric focusing, the current can be turned off and the entire gradient column raised past a densitometer to produce an absorbance profile at one or more wavelengths. Thus, progress on sample separation can be recorded at selected intervals and the minimum focusing time determined before cathodic drift begins[21]. In addition to that, since the sample can be inserted with a special applicator at the center of the column, at any desired time, the focused Ampholine column can be scanned without sample, to provide a baseline for Ampholine absorbance. This is achieved with an ISCO absorbance monitor mounted directly

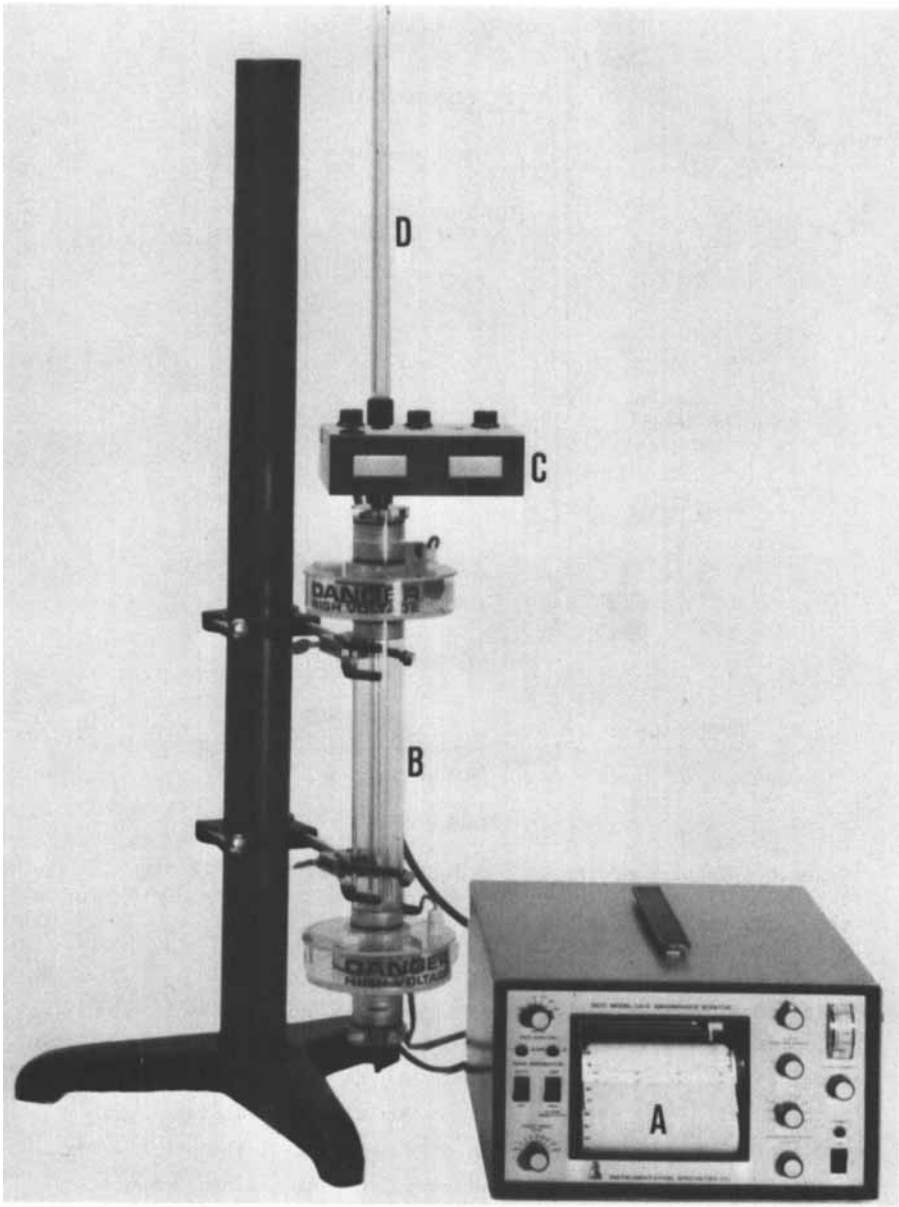

FIGURE 6

The ISCO model 212 column. A: absorbance monitor. B: isoelectric focusing column. C: UV flow cell. D: scanning reservoir tube.

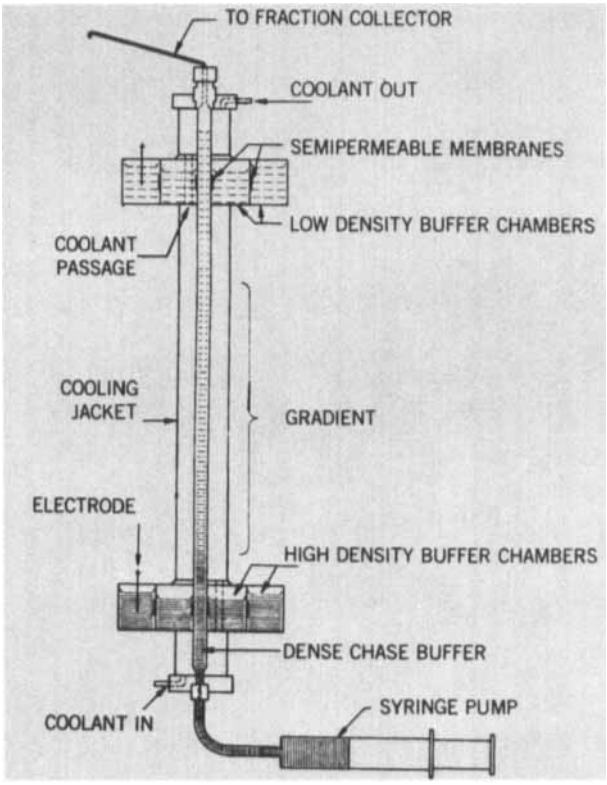

FIGURE 7

Schematic drawing of the ISCO model 212 column shown in Fig. 6. The UV flow cell and the scanning reservoir tube are not shown. This is the setup for column elution.

above the column. A flow cell and reservoir tube are attached to the column, and a dense sucrose solution is injected into the bottom of the column to raise the gradient through the flow cell and into the reservoir. The use of dual beam monitors allows scanning at one wavelength while raising the gradient, and at the other wavelength while lowering it for continued focusing. Since scanning and column unloading are performed with pulseless syringe pumps, in

an apparatus with no mixing chambers, ISCO reports sharp zone recoveries without any mixing due to convection, turbulence, or laminar flow. The principle of these columns has been described by Brakke *et al.*[52]. This type of intermittent scanning is, of course, different from the *in situ* scanning performed by Catsimpoolas[53], which allows him to obtain kinetic parameters which are the basis of a new technique called transient state isoelectric focusing[54-56].

C. The Valmet column

As early as 1969 Valmet[57] described a new apparatus, based on a new electrophoretic principle called "zone convection isoelectric focusing." The separation cell (shown in a scheme in Fig. 8 and in a built model in Fig. 9) is made of perspex with a wall thickness of 1 mm. It resembles a series of Tiselius cells with a cross section of 3 mm x 40 mm. Each "U-tube unit" is about 10 mm high. The channels are spaced 10 mm apart. The cell consists of two separate units, the trough and the lid. The trough (Fig. 8a) is a shallow rectangular box with a corrugated bottom. The lid is built with the same indentations. When the lid is lowered on top of the trough, the projections on the lid, which fit into the depressions of the trough, force the liquid into each pocket to overflow, thus closing the electric circuit on the cell. A unique feature of this apparatus is that it does not require any anticonvective medium. IEF is performed by using a mixture of Ampholine and proteins to be purified, and band stabilization is achieved by the Ludwig-Soret effect[58,59]. As a protein condenses into a pocket, it concentrates at its bottom, against the cold wall, thus generating a vertical density gradient within the channel perpendicular to the current flow. At the end of the run, as the lid is removed, the liquid flows back into each U-tube, and the trough acts at this point as a fraction collector. Moreover, in the case of colored proteins, they can be recovered from the bottom of the U-tube with the aid of a peristaltic pump, leaving in the apparatus most of the Ampholine solution, which can thus be used again for subsequent separa-

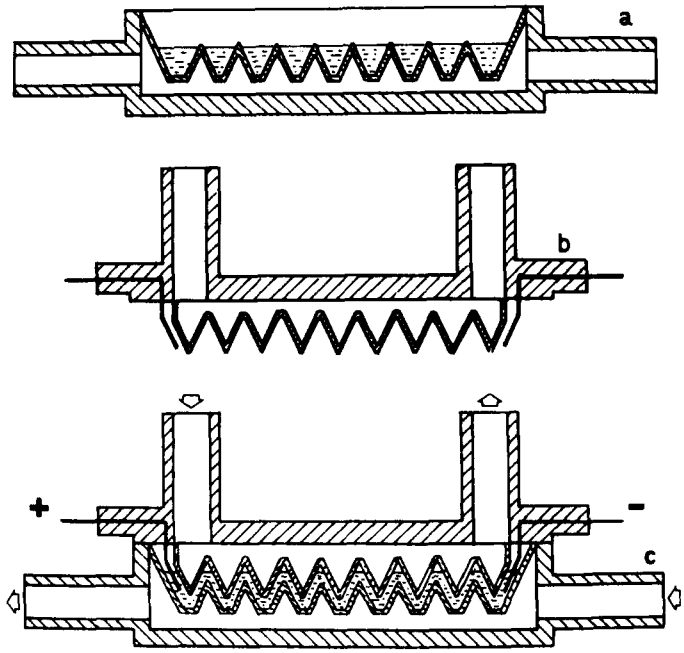

FIGURE 8

Schematic drawing of the Valmet zone convection electrofocusing apparatus. (a) the filled trough before (or after) the experiment. (b)the lid. (c) lid and trough put together, as during the experiment. (Courtesy of LKB-Produkter AB. See ref. 57.)

tions. Protein precipitation at its pI is, in the case of Valmet's trough, a great advantage, since it favors zone detection and allows sample recovery by centrifugation, as well as Ampholine recovery.

Valmet has also successfully used this apparatus for fractionation of Ampholine in narrow pH ranges. In a 30-pocket cell, he has reported separation of pH 3 to 10 Ampholine in a number of fractions each covering about 0.2 of a pH unit. The sample load in Valmet's trough is much higher than in conventional vertical density gradient apparatus. He has reported a separation of 350 mg serum

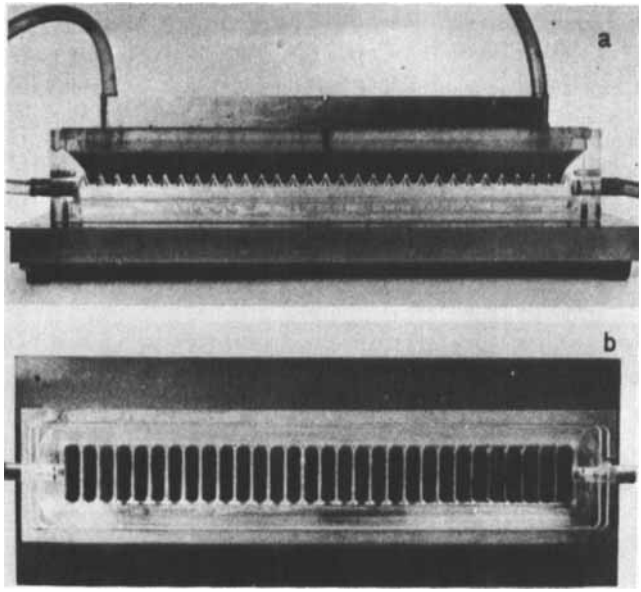

FIGURE 9

The Valmet zone convection electrofocusing apparatus. (a) front view. (b) top view of trough. (Courtesy of LKB-Produkter AB. See ref. 57.)

protein in 50 ml of 1% Ampholine. The high sample load, the economically favorable Ampholine/protein ratio, and the other unique features reported, make this apparatus particularly attractive for large-scale IEF. Unfortunately, the Valmet cell has not become commercially available, due to problems, it seems, in building a trough with an adequate number of pockets and with satisfactory cooling.

D. The Talbot trough

Luckily, Valmet's concept has not been wasted. In 1973 in Glasgow, Talbot[60] presented an improved model which embodies Valmet's idea and its advantages in a practical design. Further

exploiting Valmet's cell, this apparatus, besides eliminating difficulties associated with precipitation of focused material, provides for easy access to the pH gradient and incorporates a facility for autofractionation of the gradient at the termination of focusing. The separation trough, called MK.I, is machined into a block of perspex 1 x 2½ in wide and 24 in long, and is formed with a corrugated base and sides. It is fitted with a lid to minimize evaporation and with electrode plates at each end. The block is inserted in a supporting framework, and can be pivoted about pins and locked either in a 45° position or in a horizontal position.

For operation, 30 ml of 1% (w/v) Ampholine are poured into the trough and distributed between its 36 corrugations by tilting the whole apparatus from end to end. With the trough in the 45° position, this volume of Ampholine solution bridges the corrugations and forms a continuous electrical path along the length of the cell. 1% (v/v) phosphoric acid is used at the anode and 1% (v/v) N,N,N',N'-tetramethyl ethylene diamine at the cathode. Thus, Talbot's design has simplified the Valmet trough by eliminating the cover lid. Unfortunately, also the cooling mantle has been eliminated, and the apparatus has to be precooled in the cold room and run in the cold for a long time (72 hours or more) to reach equilibrium.

In 1974 at the Milano meeting[13], Talbot presented an improved version of his apparatus, the MK.II. This new trough, built on the same principle as the MK.I, is carved out of an aluminum block, which is then covered with a thin layer of varnish to provide electrical insulation. The block, which contains 52 pockets, is this time built with a cooling serpentine at its base. This provides very efficient cooling, since the heat is dissipated by a metal contact.

Fig. 10 shows the MK.II in operating position and Fig. 11 in autofractionation position. The milky solution contained in it clearly explains the principle of operation. Autofractionation is

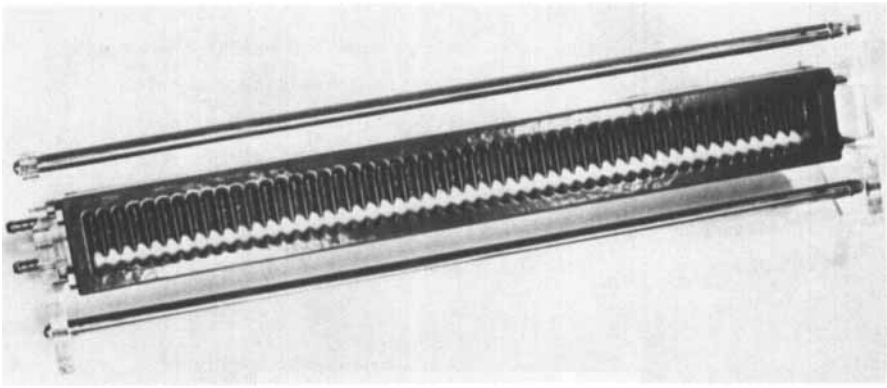

FIGURE 10

The Talbot trough (MKII) in operating position. The trough is built of aluminum and contains 52 corrugations. To the left, the inlet and outlet for cooling water are visible. During operation, the trough is tilted 45°. (Courtesy of P. Talbot (unpublished). See ref. 60.)

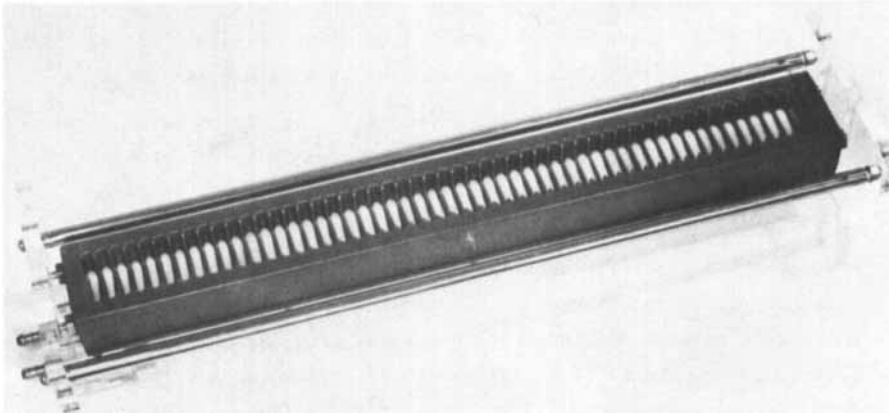

FIGURE 11

The Talbot trough (MKII) in autofractionation position. This is accomplished by returning the cell to the horizontal position. The electrodes and cover lid are not shown. (Courtesy of P. Talbot (unpublished). See ref. 60.)

accomplished simply by returning the apparatus to the horizontal position, where the Ampholine solution does not bridge the trough corrugations and the focused gradient is located as discrete fractions in the bottom of each corrugation. The pH of each fraction is measured directly in the apparatus, using a combination microelectrode. Working with viruses, Talbot and Caie[60] have reported sample recoveries of 80 to 100%. Talbot tells me that the MK.II apparatus will probably be made and sold by Shandon in the near future.

E. Rilbe's columns and multicompartment electrolysers

Rilbe's group has been working for years in the field of preparative IEF. We have already seen that both LKB columns are a result of Rilbe's concepts[8]. In the last few years this group has continued the efforts along two lines: development of two columns of the vertical type, and improvement of multicompartment electrolysers.

In 1973 in Glasgow, Rilbe[61] reported the results of preparative IEF in short density gradient columns with vertical cooling. Following Philpot's[62] early suggestion of using short and thick columns with vertical heat dissipation, Rilbe and Pettersson[61] have designed two types of columns: one with large, noble-metal electrodes serving also as heat conductors; and the other with quite small electrodes, with heat being dissipated through cellophane membranes and chilled electrolyte solutions. The former is an extremely short and thick column, the distance between electrodes being only 1.55 cm and the cross sectional area 283.5 cm^2. The electrodes are sheets of an alloy with 75% palladium and 25% silver, and they are soldered to brass plates at the bottom and the ceiling of the column. They are also used as cooling surfaces in the separation column, thus creating a vertical temperature gradient. Equilibrium is achieved in a very short time, 30 min, in a final potential gradient of 75 V/cm. Drawbacks of this type of cell are

found in the leakage of metals from the electrodes and in the necessity of charging the anode with hydrogen prior to the run.

The second type of column (shown schematically in Fig. 12), still built on the same principle of short and thick cells, has a 110 ml capacity and smaller electrodes of platinum wire. Vertical cooling is achieved through membranes and chilled, recirculating anolytes and catholytes. In this type of cell, Rilbe and Pettersson[61] have reported the separation of as much as 1.05 g of sperm whale myoglobin, focused during 3 hours in a pH 7 to 9 gradient. The main band (MbI, pI 7.6 in the ferrous form or pI 8.3 in the ferric form) contained approximately 800 mg protein, quite an appreciable amount to be carried by a density gradient. A great advantage of this column type is, therefore, its high sample load and the relatively short focusing time.

Rilbe's group has also continued along the classical line of stationary electrolysis in efforts to improve multicompartment apparatus. This field has already been reviewed by him[63] in 1948 and, more recently, in 1970[26]. In 1974 in Milano, Rilbe[64] presented the latest advances in the field. His group has worked on three types of electrolysers: a first type with open cells, internal cooling, and no stirring; a second type with closed cells, external cooling, and no stirring; and a third type with closed cells, internal cooling, and stirring. The last model embodies all the desired features of a good electrolyser. It is shown schematically in Fig. 13 (a and b). Even though this last model appears to be rather difficult to build, it has solved most of the technical difficulties connected with electrolysers, i.e., how to dissipate heat, how to cope with electro-osmosis, how to homogenize the solutions in each compartment, and how to isolate the various fractions without remixing. One of the promising applications of these types of cells is in the very large-scale fractionation of proteins. Stationary electrolysis can now be brought to completion in about 24 hours: since the volume in these 20-compartment electrolysers is usually between 500 and 1000 ml, and since rather

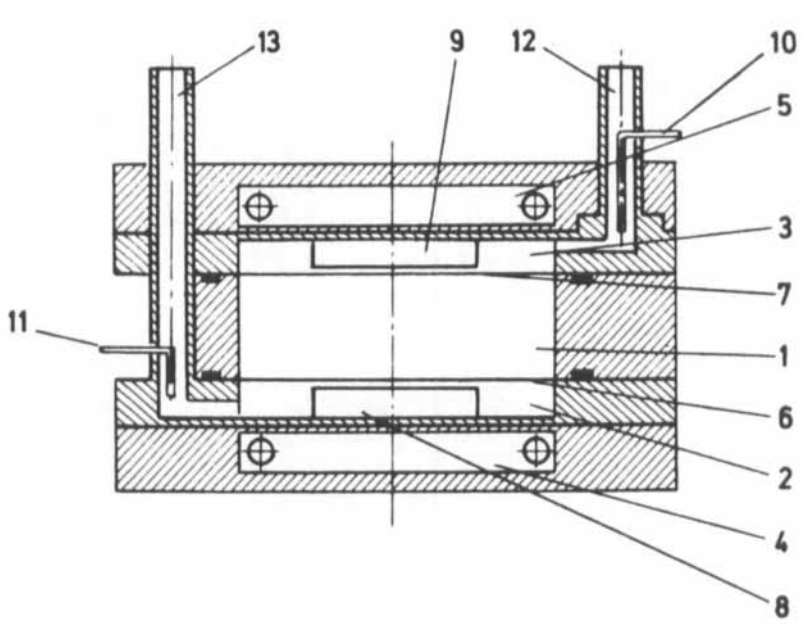

FIGURE 12

Rilbe's short column with chilled semipermeable membranes and small platinum electrodes. (1) separation column. (2) and (3) compartments for catholyte and anolyte. (4) and (5) compartments for circulating refrigeration medium. (6) and (7) cellophane membranes. (8) and (9) rotating magnets. (10) and (11) platinum electrodes. (12) and (13) plexiglass gas-escape tube. (By permission of Butterworths. See ref. 61.)

high concentrations (about 4 to 5%) can be used, the load capacity is in the order of several grams per day. These types of electrolysers are still not commercially available but it is my feeling that when they do appear on the market they will overshadow many other preparative techniques. Actually, work is still in progress in the field, and Rilbe has announced in Milano a new apparatus built on an entirely new principle for cooling and stirring.

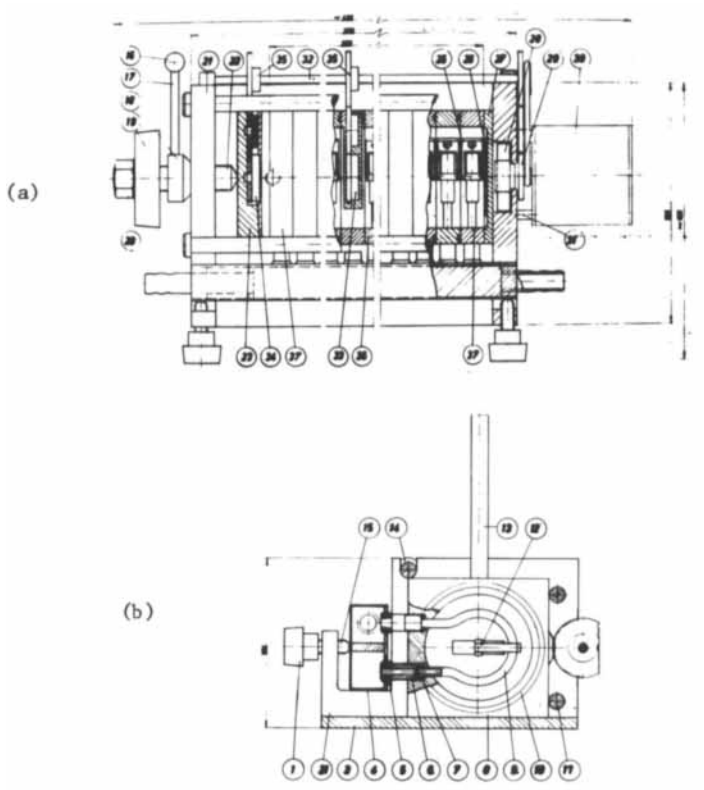

FIGURE 13

Multicompartment electrolyser with closed cells, internal cooling and stirring. (a) front view. (b) side view. (1) two knurls, which operate two screws (15) to press the cooling water distributor (4) against the perspex tubes (6) used for the cooling loops (9) of each cell. (2) and (21) end plates. (3) base plate. (5) rubber rings fitting to the perspex tubes (6). (7) O-ring-sealed holes, used to fix the cooling loops (9) of each cell. (8) one of the 20 compartments of the electrolyser. (10) O-ring connecting each cell to the adjacent one. (11) and (14) two of the three pull rods which hold together the 20 cells. (12) bearings for the stirring magnets, affixed to the cooling glass loops (9). (13) gas-escape tubes for the hydrogen and oxygen formed at the electrodes. (16) to (20) lever and locking nut system which operates the screw (22) acting upon the brass plate (23). (26) platinum net electrodes, glued to the two end walls (27) of the two end compartments (37). (28) magnet driven directly by synchronous motor (30). (32) axle used to drive the magnet (34). (33) one of the 20 internal capsulated stirring magnets. (By permission from ASP Biological and Medical Press. See ref. 64.)

F. Free-flow, high voltage IEF

The technique of continuous-flow, carrier-free IEF has been developed by Werner's group in Frankfurt. They have further developed the apparatus introduced by Barrolier *et al.*[65] and modified by Hanning[66] for free-flow electrophoresis. The cell has been described by Seiler *et al.*[67] and its first application in IEF reported by the same authors[68]. It consists of a rectangular chamber 11 cm wide, 36 cm tall, and with a buffer film thickness of 0.5 mm. A scheme of this apparatus is shown in Fig. 14. Forty-eight fractions are collected at the bottom by capillary tubings connected to a peristaltic pump. The separation chamber and the electrode cells are divided by semipermeable membranes. At the anode a solution of 5% acetic acid is used and at the cathode 1.5% ethanolamine. The electrode solutions are placed in 2-liter reservoirs and continuously pumped through the electrode compartments during IEF. The cooling system consists of a teflon coated aluminum block, cooled by circulating liquid from a thermostat at 1°C. The temperature within the separation cell is controlled by a thermistor. The sample is usually injected in the chamber by a micro pump connected with the cooling system. A typical run is performed at a field strength of 110 V/cm and at a liquid flow rate of 1 ml/min. Just *et al.*[69] have reported excellent separations of mixed red blood cell (RBC) populations and of subcellular particles using this apparatus. They were able to separate, for instance, mixtures of human, mouse, and rabbit RBC's, in pH gradients 3 to 10 and 5 to 7, at an RBC injection rate of $3x10^7$-$7x10^7$ cells/min, and with a flow-through time for RBC's of only 7 min. This separation is shown in Fig. 15. To prevent cell clumping, they used 1 mM EDTA.

Just *et al.*[69] have also reported preliminary data on the separation of the light mitochondrial fraction of rat liver into lysosomes and mitochondria. Here, to prevent aggregation, polyanionic substances (such as heparin, chondroitin sulfate, polyvinly sulfate, dextran sulfate, and polyanethol sulfonic acid) are used. This technique appears to be tremendously promising in the field

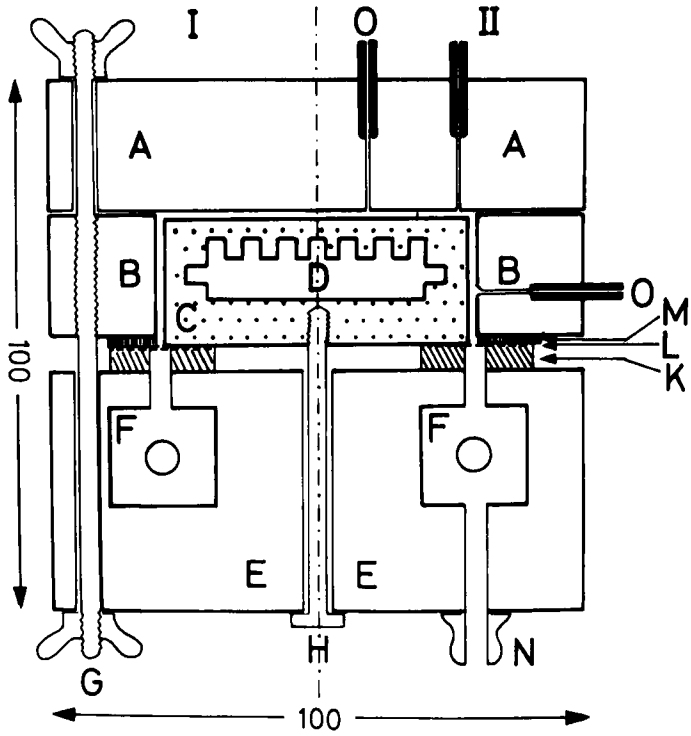

FIGURE 14

Schematic drawing of two sections of the separation chamber for free-flow isoelectric focusing. (I) vertical section 1 cm from the upper side. (II) vertical section 2 cm from the upper side. (A) front block of the separation chamber. (B) side view of the separation chamber block. (C) aluminum cooling block. (D) channel for the cooling liquid. (E) electrode block. (F) electrode chamber with electrodes. (G) holding screw. (H) adjustable screw which regulates the tightening of blocks (E) against (K). (K) silicon band. (L) electrode membranes. (M) levelling rubber band. (N) inlet for electrode buffer. (O) inlets for the buffer of the separation chamber. (By permission of Springer-Verlag. See ref. 67.)

of cell and subcellular organelle separation. The method is very mild to cells, it avoids exposures to pH extremes, it allows recovery of fully active and non-aggregated fractions, it is very

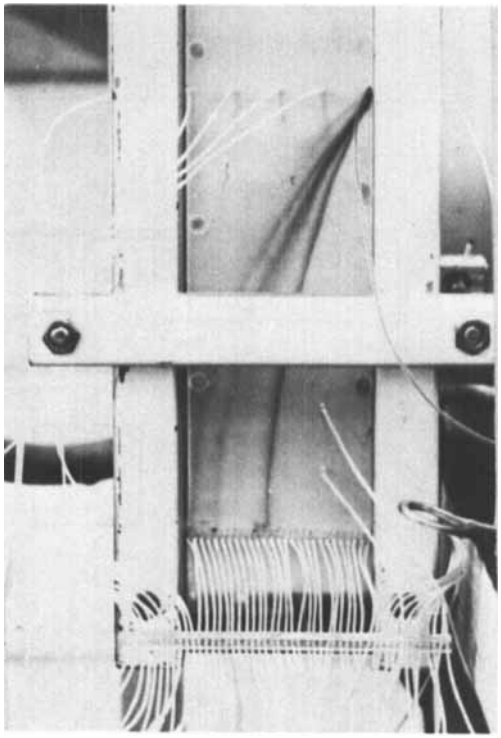

FIGURE 15

Separation of a 1:1:1 mixture of human, mouse, and rabbit RBC's by continuous-flow isoelectric focusing. 1% Ampholine, pH 5 to 7, electrical field strength 110 V/cm, total liquid flow 1 ml/min. The sample injection port can be seen on the upper right side. (By permission of ASP Biological and Medical Press. See ref. 69.)

quick (a few minutes of sample flow-through), and it has a resolving power definitely superior to conventional techniques (such as differential or density gradient centrifugation). This equipment, however, is still not available on the market.

4) PREPARATIVE IEF IN SOLID MEDIA

A. The "Righetti and Drysdale" apparatus

Because of the excellent resolution and load capacity afforded by gel media, Righetti and Drysdale[70] have scaled up their analytical apparatus for preparative purposes. The salient features of this cell include efficient cooling of cells and small electrolyte vessels. Gel tubes are held in a water-tight compartment through which coolant at 1°C is circulated. Circular platinum electrodes are positioned close to the extremities of the tubes to minimize loss of Ampholine from the gel and, thus, reduce the cathodic drift of the pH gradient. The core of this cell, which is interchangeable with the analytical one, accommodates six gels: 3 gels of 50 ml capacity (2 cm I.D.), 2 of 20 ml capacity (1.2 cm I.D.), and a control gel of 2 ml. All gels are cast in 16 cm glass or plastic tubes. The maximum sample load is approximately 200 mg protein. This apparatus, shown in Fig. 16, is commercially available from MRA, Boston, Massachusetts. A drawback of this type of cell is in the sample recovery after IEF. We have usually performed it by cutting the zone of interest, squashing the gel and eluting it serially with buffer. This leads to incomplete sample recoveries and high dilutions. To overcome that, Suzuki *et al.*[71] have described an ingenious sample retrieval after IEF. They prepare a small chamber at the bottom of each gel tube, closed at one extremity by a dialysis membrane and, slightly above it, by a short layer of polyacrylamide gel. After IEF, the sample zone is cut, the gel minced and packed into the tubes on top of the stacking gel which forms the ceiling of the chamber just described. The sample is then recovered by electrophoresis in the same apparatus used for IEF. This allows quantitative sample recovery in a highly concentrated form and Ampholine free. A similar apparatus has also been described by Chrambach *et al.*[33].

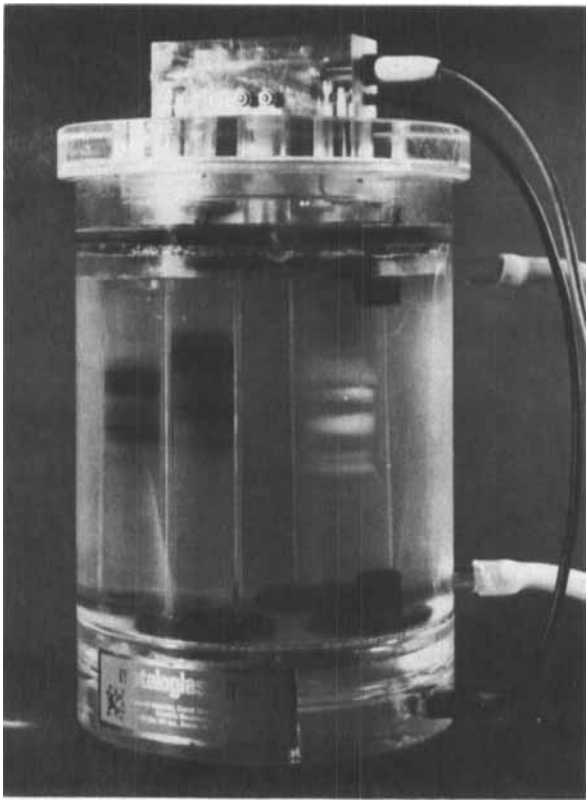

FIGURE 16

Apparatus for preparative gel electrofocusing. The apparatus contains six gels: 3 of 50 ml capacity (2 cm I.D.); 3 of 20 ml capacity (1.2 cm I.D.), and a control gel of 2 ml. (By permission of the New York Academy of Sciences. See ref. 70.)

B. Fawcett's continuous-flow apparatus

Fawcett[25] has described two types of continuous-flow apparatus. One uses a packed Sephadex bed with vertical liquid flow. The other apparatus uses a continuously flowing density gradient which is pumped horizontally through a vertical trough. Good resolution

is achieved with both, however, I will limit my description to the former, since the latter requires a rather complicated setup (pumping in of 54 gradient fractions and pumping out of the same number of fractions) and a pump with at least 108 channels. The gel stabilized layers, instead, require a rather simple experimental setup. In a very simplified version, we have fractionated synthetic carrier ampholytes in a chamber with only 12 outlets, no recirculation of anolyte and catholyte, and continuous sample input by constant hydrostatic pressure via a Mariotte flask[24]. This system can be run, practically unattended, for weeks.

Fawcett's technique is based on the continuous-flow electrophoresis method first described by Svensson and Brattsten[72] and by Grassmann[73]. The difference between the two principles is illustrated in Fig. 17. The separation chamber consists of two cooling plates 23 cm wide, 30 cm high, and 0.3 cm apart. Semipermeable membranes are fixed to the sides and separate the trough from the electrode vessels. The membranes are porous polyethylene sheeting containing polyacrylamide within the porous medium. The cell is packed with Sephadex G-100 beads (or polyacrylamide particles) supported by a filter membrane. The 54 exit tubes on the chamber floor are connected to a multichannel peristaltic pump built on the delta principle. The complete apparatus with pump units and collecting tubes is shown in Fig. 18. Continuous-flow IEF offers distinct advantages over density gradient stabilized columns. The zones are sharpened by the applied potential right up to the time of removal from the apparatus. Therefore, they are not subjected to spreading by diffusion and to remixing during elution, as encountered in density gradient columns. The method is capable of processing large quantities of material, since it can tolerate high concentrations of protein in the focused zones. Fawcett reports separations of 500 mg protein/day. Furthermore, the system can be run in a cascade form, i.e., first in a trough in a wide pH range and then, sequentially, in a trough in a narrow pH gradient. To summarize the method, I report here what Chrambach said in 1972 in

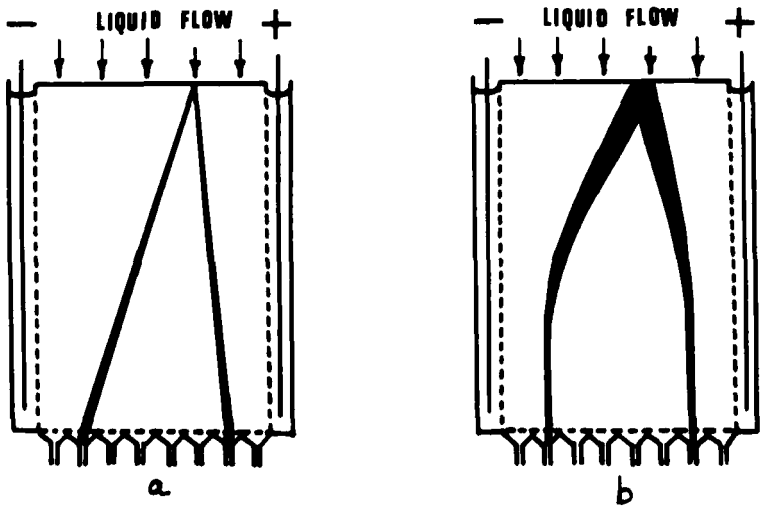

FIGURE 17

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. 25.)

New York (addressed to Fawcett)(see ref. 10, p. 126): "I think it is a fantastic advantage to be able to extract gram quantities of proteins by the continuous stacking system. This has never been done before. It would have formidable consequences for biochemists. You are certainly to be congratulated for having made this advance."

C. *The Stathakos multiphasic column*

Recently, Stathakos[74] has designed a new type of vertical column for multiphasic isoelectric focusing. I think the idea is somewhat similar to the principle of multicompartment electrolysers as described by Rilbe[64]. The column is designed on the principle of building blocks, i.e., it is composed of alternating separable gel phases and liquid interlayers extending between two electrode compartments. The column, constructed in a modular fashion, can

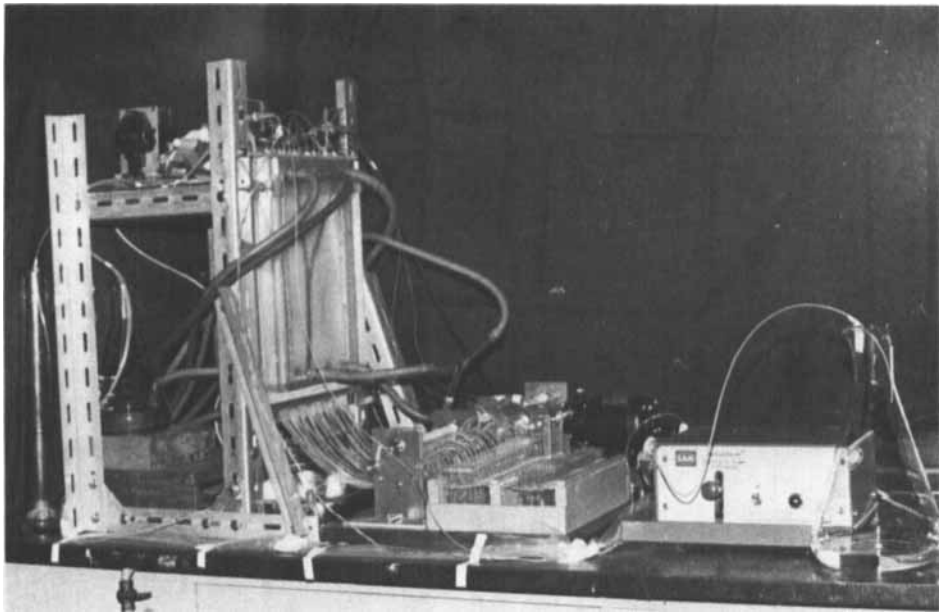

FIGURE 18

Photograph of continuous-flow isoelectric focusing in Sephadex-stabilized media. View of complete apparatus with pump units and collecting tubes. In the separation chamber two streams of haemoglobin are seen merging together toward the center of the cell. (By permission of the New York Academy of Sciences. See ref. 25.)

be shortened or lengthened according to experimental needs. The scheme of the apparatus, assembled in six units of equal size, is shown in Fig. 19. Fig. 20 is a picture of the actual setup, showing haemoglobin separation in one of the blocks. This type of column allows great experimental flexibility and offers several interesting features. From the point of view of the sample, it can be applied practically anywhere along the column: at the top, in any of the liquid interlayers (2.5 mm high) between two adjacent blocks, or in one or more of the building blocks. Through the various liquid interlayers (which form small chambers with inlet and outlet nipples, as shown in Fig. 19), the pH can be monitored at any time

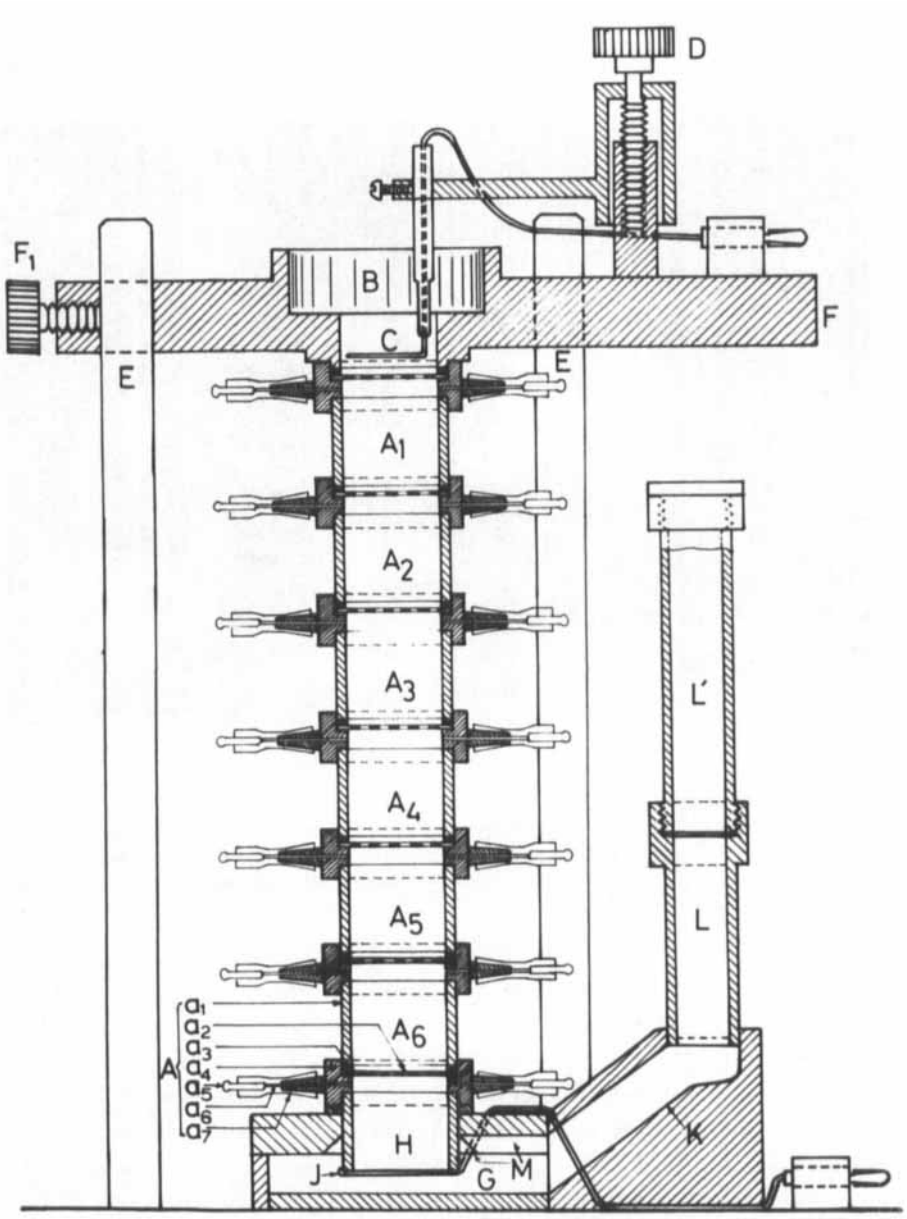

FIGURE 19 Vertical section through a six-unit column for multiphasic isoelectric focusing. A_1-A_6: separable, independent gel units. (a_1) tubular part; (a_2) supporting membrane; (a_3) silicon rubber O-ring; (a_4) structural ring; (a_5) glass plug; (a_6) tygon jacket; (a_7) outlet nipple. (B) upper electrode chamber; (C) upper electrode, movable vertically by means of screw (D); (E) metal rods; (F) upper platform; (F_1) screw for securing platform (F); (G) lower electrode chamber; (H) base cylinder; (J) lower electrode; (K) sloped connection to tube (L); (L) auxiliary counterpressure tube; (L') tube extension; (M) groove for escape of electrolysis gases. (By permission of ASP Biological and Medical Press. See ref. 74.)

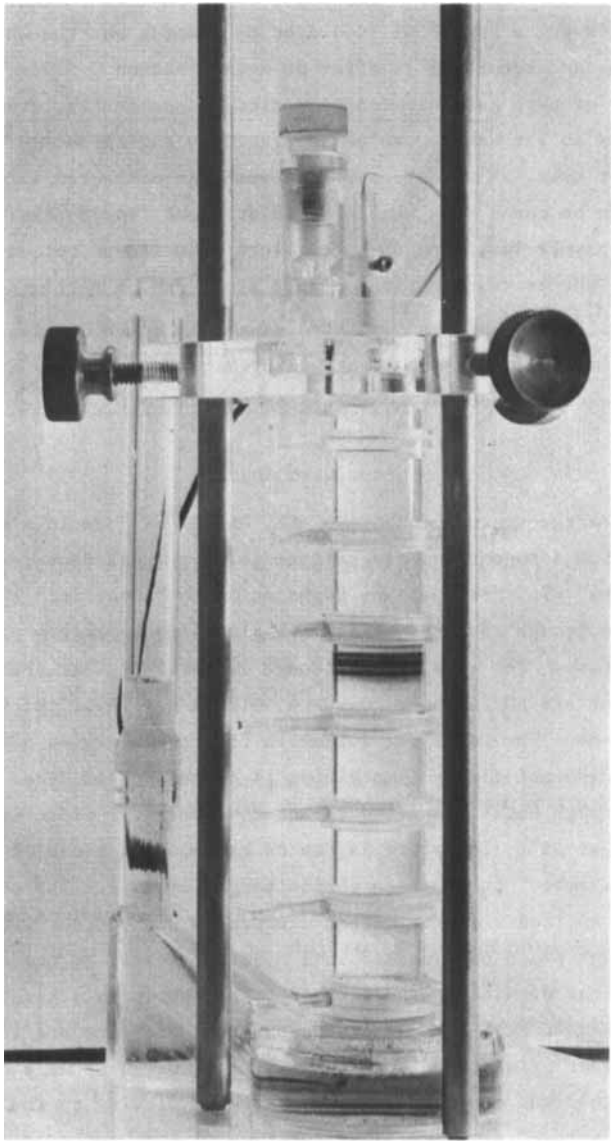

FIGURE 20

Isoelectric fractionation of partially oxidized human haemoglobin in the Stathakos column. 1 ml containing 24 mg haemoglobin was applied to the top membrane. The units contain 5% acrylamide gel. Anolyte: 0.01 M H_3PO_4 in 10% sucrose. Catholyte: 0.02 M NaOH; 1% Ampholine, pH range 3 to 10. (By permission of ASP Biological and Medical Press. See ref. 74.)

by withdrawing a few μℓ of liquid, or by pumping out the whole solution and returning it after pH determination. Since the porosity of each gel unit can be varied independently, unwanted compounds in the sample can be separated by making the gel "restrictive" for them, or vice versa. The proteins collected into a gel block can be rerun in a shallower pH gradient for further separation simply by inserting this gel block in a second column. Finally, the proteins collected in a block can be retrieved electrophoretically, and at the same time separated by Ampholine, by a method similar to the one described by Suzuki *et al.*[71]. This apparatus should soon be available on the market.

D. Radola's trough for granulated layers

For large-scale preparative IEF, Radola[75,76] employs troughs coated with a suspension of granular gels, such as Sephadex G-75 "superfine" (7.5 g/100 ml) or Sephadex G-200 "superfine" (4 g/100 ml). The trough consists of a glass plate at the bottom of a lucite frame. The area of the trough is variable, but the usual dimensions are 40x20 cm or 20x20 cm, with a gel layer thickness of up to 10 mm. The total gel volume in the trough varies from 300 to 800 ml. The gel slurry (containing 1% Ampholine) is dried in air in the trough until the gel does not move when the plate is inclined at 45°. The plate is run on the cooling block of the "Double Chamber" from Desaga (Heidelberg, Germany). The electrical field is applied via flat carbon electrodes, in contact with the gel through paper pads soaked in 1 M sulfuric acid at the anode and 2 M ethylene diamine at the cathode. This setup is schematically represented in Fig. 21. After focusing the proteins are located by the paper print technique[75], using Light Green SF as a stain. The gel gradient can be measured *in situ* by fitting to the Desaga "Double Chamber" a micro glass electrode (1.5 mm membrane diameter) and a reference electrode, both sliding on a calibrated ruler.

Radola's technique offers several advantages. IEF in granulated layers affords very high loads, ranging from 5 to 10 mg protein

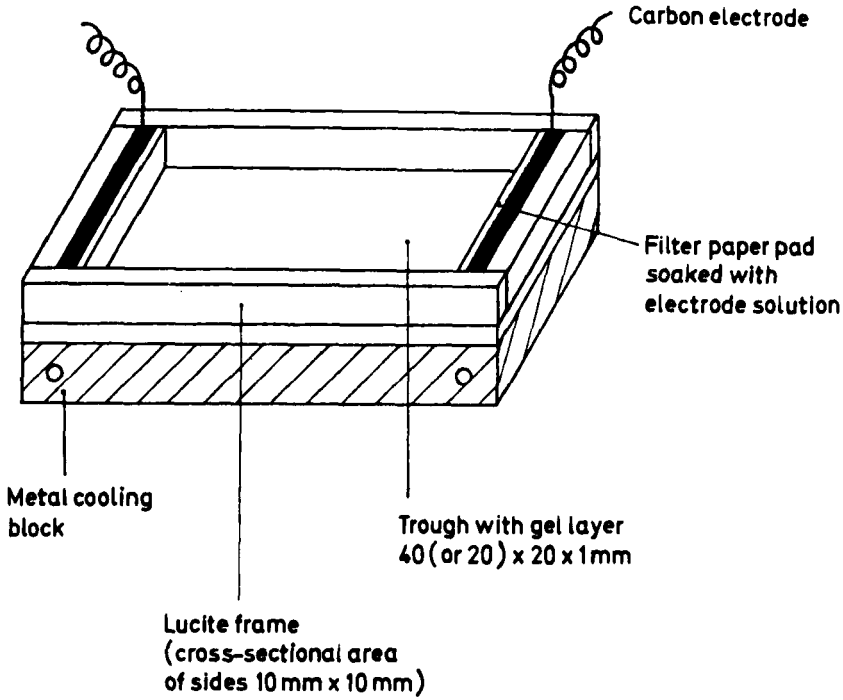

FIGURE 21

Schematic representation of preparative isoelectric focusing in a trough of granulated gels. (By permission of Butterworths. See ref. 76.)

per ml gel suspension (in the pH 3 to 10 range). By these standards, the LKB 110 ml column should be able to carry approximately 1 g protein. In a single protein zone, the load capacity is in the order of 0.3 to 1 mg/ml. Radola[76] has reported focusing of 10 g pronase E in 800 ml of gel suspension, with a load capacity of 12 mg/ml, and with an excellent band resolution. For narrow pH ranges, it seems as though even much higher loads could be applied[76]. At these high protein loads, even uncolored samples can be easily detected, since they appear in the gel as translucent zones. Recovery of proteins is high and elution is simple. The focused

proteins are usually obtained in a small volume at relatively high concentrations. The system has a high flexibility, since it allows analytical, small scale and large scale preparative runs in the same trough, simply by varying the gel thickness. For simplicity, flexibility, high resolution, and high load capacity, Radola's technique appears to be among the best presently available.

5) CONCLUDING REMARKS

From the data presented here I think we can conclude that there is no limit to human ingenuity in designing, building, or modifying apparatus to meet any need in the laboratory. Even commercially available equipment has been adapted or changed in the laboratory to meet some specific requirements. To have an idea of that, I suggest reading a recent article by Fawcett[77].

We have seen that some apparatus are able to fractionate large amounts of proteins, in the gram scale. In the field of IEF, it has generally been believed that sucrose density gradients could only carry a limited amount of protein. As a representative figure, in the 440 ml LKB column, a maximum load of only a few hundred mg protein is recommended. However, in view of some recent results and theoretical considerations by Rilbe[61], I think that this point of view should be changed. According to Rilbe, the theoretical mass content of a protein zone (m) in a linear sucrose density gradient ranging from 0 to 0.5 g/cm^3, cannot exceed the following inequality:

$$m < 0.625\ Vr^2\ g/cm^3$$

where V is the total column volume and r is the zone breadth. For a 100 cm^3 column, one has:

$$m < 0.625\ r^2\ \text{grams}$$

Thus, the load capacity rises with the square of the zone breadth. Rilbe and Pettersson[61] have experimentally verified this. In a 110

ml column, for an r value of 0.125, according to the above inequality, one should be able to load a maximum of 1074 mg protein in a single zone. In fact, working with myoglobin, these authors have loaded 1050 mg in the column, and approximately 800 mg (i.e., 76% of the applied sample and 75% of the theoretical maximum) were confined within the main peak (MbI).

The explanation here is simple. Since the load capacity depends only on the volume of the zone, broad zones will tolerate higher amounts of protein. On the other hand, broad zones (i.e., shallow pH gradients) are compatible with high resolving powers. In fact, a high resolving power requires a weak pH gradient, and a shallow pH gradient gives rise to broad protein zones capable of holding considerable amounts of proteins. This property places IEF in good contrast to all other separation techniques, in which a good yield axiomatically excludes a high purity and vice versa. On the basis of these considerations, and of the equipment presently available, I think IEF has the prerequisite to become one of the leading techniques for large-scale separations of macromolecules.

6) FUTURE TRENDS AND DEVELOPMENTS

When the new generations of preparative apparatus are built, they will have to incorporate the new developments made in the field during these years. In the case of density gradient columns, apparatus are required which can be emptied rapidly to limit zone spreading by diffusion, which incorporate UV and pH monitoring, and which allow collection of fractions with minimum disturbances of separated zones.

Rilbe's[61], Radola's[76], Fawcett's[25], and Valmet's[57] techniques should be further exploited in the field of preparative biochemistry since they all allow protein fractionations in the gram scale. In most cases, we will have to wait until the respective apparatus become commercially available; but Radola's trough is already on

the market and the technique is simple enough for anybody to play around with. The use of large amounts of carrier ampholytes should no longer be a drawback, since simple techniques have been described to synthesize[9,22,23] and fractionate[24] them in narrow pH ranges.

7) ACKNOWLEDGEMENTS

The literature survey was completed on February 15, 1975. This report is only a survey of presently available techniques and apparatus, and it is not meant to endorse any particular make or manufacturer.

I apologize to those whose work I have overlooked or been unable to include.

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