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PREPARATIVE PROTEIN PURIFICATION IN A MULTI-COMPARTMENT ELECTROLYSER WITH IMMOBILINE MEMBRANES

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SUMMARY

Preparative electrophoresis in gel phases, including isoelectric focusing, is characterized by low load limits (only a few milligrams of protein per millilitre of matrix), low recoveries (rarely exceeding 70%) and heavy contamination from neurotoxic gel material (unreacted monomers and ungrafted oligomers). A multi-compartment electrolyser is described based on the principle of highly buffering Immobiline membranes of well defined isoelectric points (p*I*). Each chamber is delimited by isoelectric membranes having p*I*s encompassing the p*I* value of the components of interest in the mixture to be separated. Such components are transported electrophoretically from chamber to chamber until they collect in the chamber defining their p*I* values. As the sample feed is kept in a number of reservoirs equal to the number of chambers in the electrolysers, at the end of the purification process each reservoir will contain, under ideal conditions, a homogeneous protein fraction. Examples of the purification of r-DNA Eglin C and of monoclonal antibodies are given.

INTRODUCTION

Isoelectric focusing (IEF) in immobilized pH gradients (IPG) was introduced as a method with an extremely high resolving power for protein characterization and analysis[1]. In fact, with a resolving power (Δp*I*, expressed as the p*I* difference between two just resolved proteins) of 0.001 pH unit (compared with $\Delta pI = 0.02$ in conventional IEF)[2], it was shown to separate easily even neutral mutants bearing no charge difference at the substitution site[3]. Initially, the technique was used only for analysis in narrow pH ranges, but subsequently recipes for wide pH ranges (up to 7 pH units)

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were made available through a computer program developed for this purpose[4]. It was later realized that IPGs coupled a high load ability with their high resolving power[5–7].

However, most preparative techniques in gel phases are bound to fail, as it has been demonstrated that the load ability (in terms of milligrams of protein per millilitre of gel phase) is strongly dependent on the strength of the supporting matrix: only dilute gelatins afford moderately high protein loads. As the %T (grams of total monomers/ml) is increased above 3%, the load ability rapidly decreases as the gel matrix and the protein zone contained therein have to compete for water of hydration[7]. An additional, severe problem is that, when the supporting phase is a polyacrylamide gel, the protein will be eluted heavily contaminated with unreacted monomers and ungrafted oligomers, to such an extent that, at low sample loads, the amount of material recovered is represented mostly by gel contaminants rather than by the applied macromolecule[8]. An exception to this is IEF in granulated gel layers, in which the supporting Sephadex phase can be extensively washed prior to use, and the protein recovered contaminated solely by the soluble amphoteric buffers[9].

Thus, in principle, only free liquid systems could support high protein loads and yield a protein free from gel impurities. In addition to the early, vertical density columns for preparative IEF[10], two systems have been extensively described over the years: (a) multi-compartment electrolysers, as developed by Rilbe's group[11–14], and (b) continuous flow, recycling IEF chambers, as proposed by Bier's group[15–17]. As we are mainly discussing focusing systems, we shall not deal in detail with other preparative systems, such as the continuous flow apparatus of the Hannig type[18] or the Bio stream[19], which work mainly within the framework of zone electrophoretic separations.

Jonsson and Rilbe[14] described a large electrolyser containing 46 separation compartments, having a total volume of 7.6 l and a length of 1 m. The compartments are closed, and internal cooling and stirring are effected by slow rotation of the whole apparatus in a tank filled with cold water. The apparatus is run with an electric load of up to 5 kV, and isoelectric focusing takes 2–3 days. Owing to its very large size and the high cost of the chemicals needed to operate it, this instrument has never become popular. At the opposite extreme, Egen *et al.*[17] developed a small-scale apparatus, available commercially under the trade-name Rotofor (Bio-Rad Labs.), which is a compact, 20-compartment electrolyser, cooled by a central cold finger and also rotating on its axis for zone stabilization (this principle of stabilization of zones by rotating the chamber on its axis was a remarkable achievement of Hjertèn well before the Space Shuttle became available for elimination of gravitational effects in free zone electrophoresis)[20].

Ideally, such multi-compartment electrolysers should contain membranes that are chemically and mechanically resistant, flow-tight, free of electroosmosis and thin. Flow-tight membranes are desirable because (a) they prevent convective remixing of the contents of adjoining compartments during fractionation due to differences in density, (b) they prevent liquid flow forwards and backwards through the electrolyser caused by uneven pressure (*e.g.*, as generated by electrode gases) and (c) they facilitate loading and emptying of the electrolyser. In practice, owing to inherent difficulties with the membrane technology available at the time, neither Rilbe's nor Bier's electrolysers contained flow-tight membranes. In the former instance, a porous poly-

(vinyl chloride) paper membrane was adopted, and in the Rotofor the membrane was simply a nylon net.

In 1987, in a series of papers[21–24], we reported a new technique, called "segmented immobilized pH gradients", with which we opened a "window" in an IPG gel, designed to keep isoelectric in a liquid stream a major component of a mixture under purification. Only the impurities were focused in the lateral gel phases delimiting the "window", whereas the component of interest was kept isoelectric at all times during the focusing process in the initial sample feed, which was continuously recycled orthogonally to the electric field. As originally described, the sample flow cell was flanked by two segments of an immobilized pH gradient; subsequently, these two IPG segments were reduced to membranes of well defined p*I* values, able to buffer and titrate a single protein to its p*I*, while allowing all other impurities to migrate through and simply be lost in the electrodic compartments[25].

We have now realized that such a mono-compartment electrolyser is not ideally suited in practice, as researchers often have a need to isolate and characterize more than just a single fraction in their preparations. We therefore describe here a multi-compartment electrolyser based on the principle of Immobiline membranes. As the unique feature of such a system is the Immobiline membrane technology, in that such membranes possess a buffering power, an extremely well defined p*I* value and are able to quench electroosmosis[26], we shall give here guidelines on the successful preparation and characterization of such membranes.

EXPERIMENTAL

Materials

Repel- and Bind-Silane, Gel Bond PAG, the Multiphor 2 chamber, Multitemp thermostat and Macrodrive power supply were from LKB (Bromma, Sweden), Pharmalyte and Ampholyte buffers and the protein p*I* marker kit were purchased from Pharmacia (Uppsala, Sweden), the multi-channel peristaltic pump was from Ismatec (Zurich, Switzerland), the glass microfibre filters (GF/D) were purchased from Whatman (Clifton, NJ, U.S.A.) and light paraffin oil (Art. 7160) from Merck (Darmstadt, F.R.G.). Acrylamide, N,N′-methylenebisacrylamide (Bis), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulphate and Coomassie Brilliant Blue R-250 were from Bio-Rad Labs. (Richmond, CA, U.S.A.), dithiothreitol (DTT) and urea from Merck and r-DNA N-acetyl Eglin C (*ca.* 90% pure) from Ciba Geigy (Basle, Switzerland). Monoclonal antibodies against the gp41 from AIDS virus were prepared and purified by Jungbauer *et al.*[27].

Analytical IPGs

To follow the progress of the purification of monoclonal antibodies, analytical IPG gels in the pH range 8.5–10 were made according to Gelfi *et al.*[28]. The samples were loaded in pockets precast at the anodic side in a pH 8.5 plateau gel segment (2 cm long). About 40 μl, with up to 50 μg of protein, were loaded and focusing was continued for 25 000 V h as described by Sinha and Righetti[29]. The gels were stained with Coomassie Brilliant Blue R-250 in Cu^{2+}.

Analytical IEF

For monitoring the purification of Eglin C, Ampholine PAG plates in the pH range 3.5–9.5 (4%T, 3%C, 2.2% Ampholine concentration) were used. A total of 200 μg of protein was applied (in volumes up to 20 μl, at the anodic side) and then focusing was performed at 10 W limiting, 10 mA and 1000 V at equilibrium (at 10°C). The analytical runs were usually terminated within 2 h and then the gels were stained with Coomassie Brilliant Blue R-250 in Cu^{2+}.

Description of the apparatus

Basically, the multi-compartment electrolyser consists of a stack of chambers sandwiched between an anodic and a cathodic reservoir. Fig. 1 shows one of these chambers, provided with two inlets and outlets for sample recycling, an O-ring for ensuring flow-tight connections and four holes for threading four long metal rods which can be tightened by hand-driven butterfly nuts for assembling the apparatus. We have built several versions of these cells, capable of housing Immobiline mem-

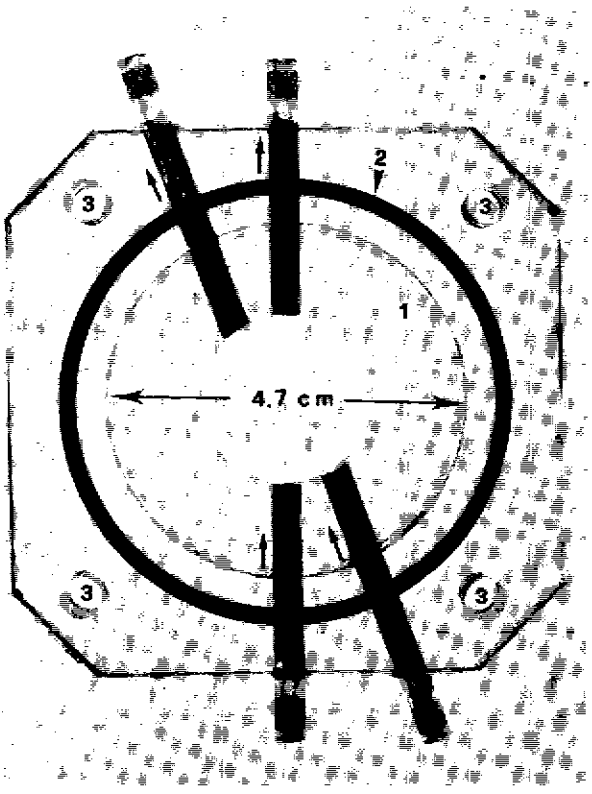

Fig. 1. A chamber of the multi-compartment electrolyser. A Perspex square of dimensions 7.5 cm with a central depression of 4.7 cm is used for housing the Immobiline membrane sandwiched between two flat caoutchouc rings of 1.2 cm width. 1 = Membrane housing; 2 = O-ring for a flow-tight connection when assembling the entire apparatus; 3 = four holes for threading four metal rods used to hold together the electrolyser. The arrows indicate inlets and outlets for sample flow. All chambers, including the electrodic reservoirs, have identical cross-section and hold equal volumes (5 ml).

branes from 4.7 cm (the present apparatus) up to 9 cm in diameter. The pH-controlling membranes are housed in the central depression between two 1-cm-wide rings of caoutchouc. After assembling and tightening the apparatus, each compartment is flow-tight, so that no net liquid bulk flow ensues (except, when applicable, as generated by electroosmosis).

Fig. 2 shows one of the terminal parts of the electrolyser, housing the platinum wire bathed by the electrolyte solution. This rests on a rectangular Perspex mounting, which will act as one of the legs of the electrolyser, once the entire stack of cells is assembled (see Fig. 3). The distance between adjacent cells is only 10 mm, so that each chamber holds *ca.* 5 ml of liquid. Fig. 3 shows the entire assembled apparatus; in this particular experiment, four fractions were collected, so that the electrolyser is built with a stack of six cells, the two extreme ones acting as anodic and cathodic chambers. All solutions, including anolyte and catholyte, are continuously recycled from larger reservoirs. Two additional chambers, not needed in this experiment, are shown assembled outside the electric field, to the left of the apparatus. Two of the four metal

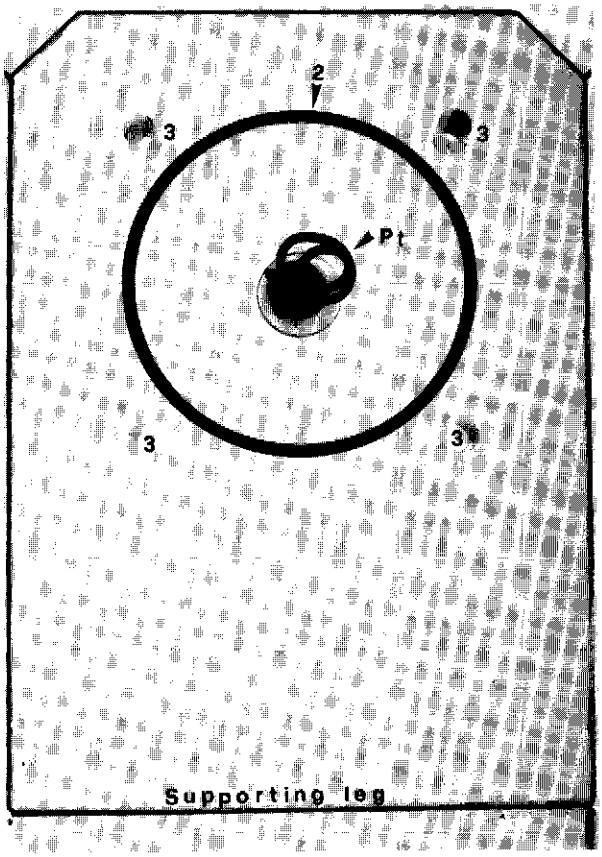

Fig. 2. Terminal part of the electrolyser and electrode support. The platinum wire is mounted on a rectangular (10 × 14 cm) Perspex slab which also acts as one of the two legs supporting the apparatus (see Fig. 3). When piling the different chambers on it (see Fig. 3), the first one will become the electrode reservoir. Numbers as in Fig. 1.

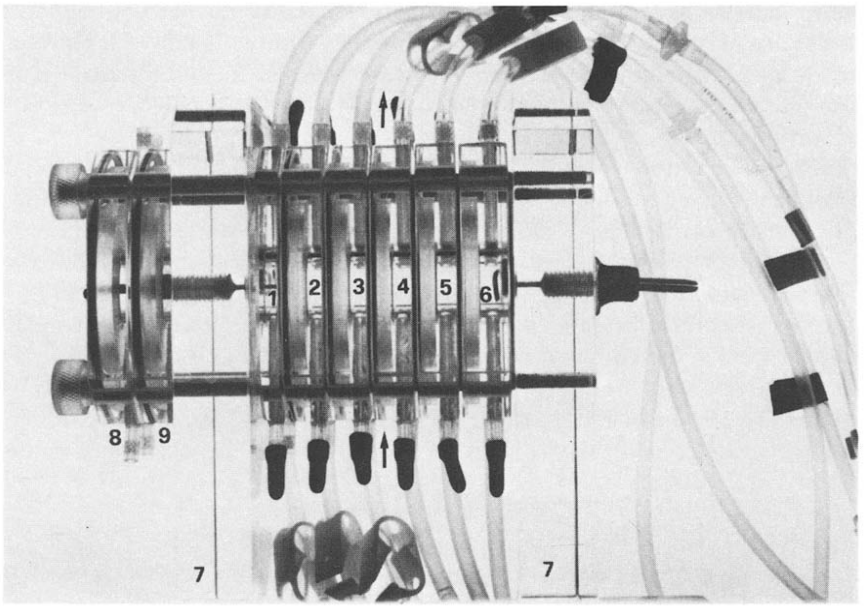

Fig. 3. View of the mounted multi-chamber electrolyser. 1, 6 = Electrode compartments; 2, 5, 8, 9 = sample flow chambers (two, not in use, are mounted outside); 7 = electrode mounting units and supporting legs. The arrows indicate sample and electrolyte flow.

rods (with two nuts), needed for assembling and tightening the electrolyser, are visible in front of the apparatus. Inlets and outlets not in use are stoppered by black caps.

Fig. 4 shows one of the sample reservoirs that we have built. They consist of three-necked flasks, bearing holes in the lateral caps for insertion of sample recycling tubing. The one shown in Fig. 4 holds a total of 25 ml, but vessels of any size can be built according to experimental needs. A magnetic bar is placed at the bottom of the vessel for keeping the solutions under gentle, continuous stirring to avoid electrode-cantation. An important feature of these sample reservoirs is the small outlet at the bottom of the vessel. This is kept plugged during the run, but it is opened to collect the sample at the end of the run, so as to avoid collection of lighter solutions floating on the liquid surface during the run. In fact, for fractionation in alkaline pH ranges, the sample liquid surface is flooded with a thin film of light paraffin oil, which will prevent adsorption of carbon dioxide (which will ruin the separation by introducing a new "Immobiline", carbonate, with pK 10.5 and 6.3)[30]. Thus, when harvesting the sample from the bottom outlet, the paraffin oil is left behind.

Fig. 5 shows the operational assembly (not including the power supply): to the left is the multi-compartment electrolyser, in the centre a multi-channel peristaltic pump and to the right the sample and electrolyte reservoirs resting on a large-surface magnetic stirrer. As very little joule heating is generated during the run in an IPG gradient we find it adequate, for proper temperature control, to run the entire assembly in a cold room. At a standard temperature of 4–5°C in a cold room, the solutions during the run will be prevalently at 8–9°C. Once assembled, the apparatus can be run for long periods without any damage to the membranes or any leakage. We have performed runs continuously for up to 10 days.

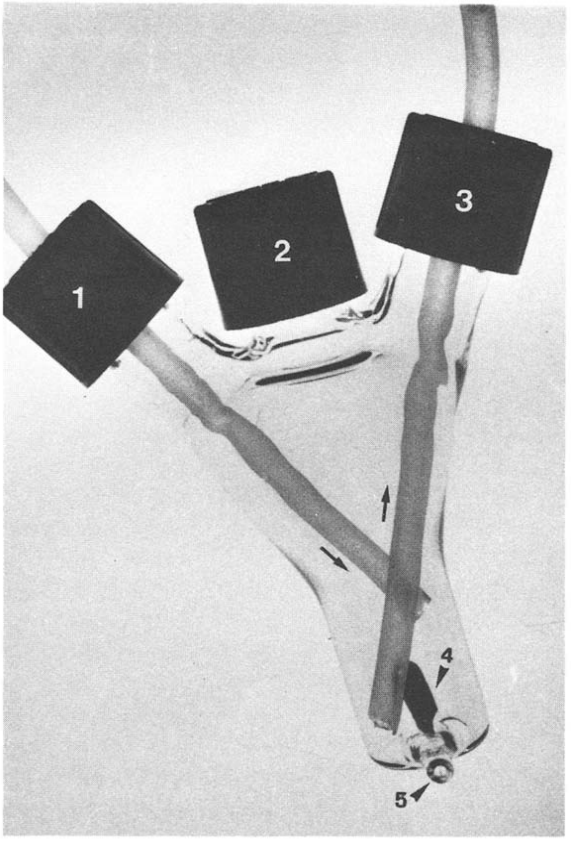

Fig. 4. Sample flow chamber. 1, 3 = Inlet and outlet for sample flow; 2 = opening for sampling; 4 = magnetic bar; 5 = bottom outlet for collecting paraffin-oil-protected liquid. The tubes are secured in place via a hole in the stoppers.

Membrane preparation

This is perhaps the most delicate part of the entire apparatus. The real novelty of this instrument is the concept of isoelectric, highly buffering Immobiline membranes. Hence the separation will only be as good as the membranes that have been prepared and will act by buffering and keeping isoelectric any desired protein within each chamber of the electrolyser. The membranes *per se* are made with the Immobiline technology, *i.e.*, by mixing neutral monomers (acrylamide and Bis) and charged species (the weak acids and bases constituting the Immobiline family; we have recently described the properties of nine such chemicals, plus two strong titrants)[31,32], in such ratios as to define unequivocally a given pH value in the entire membrane. This pH value will be the isoelectric point of the membrane, which, by virtue of its considerable buffering power, will be able to titrate macroions in contact with it. There is only one safe way to define the desired p*I* of a set of membranes: it consists in running an analytical IPG gel containing exactly the amount and types of Immobiline chemicals which will later be utilized in the preparative run. If any additives are needed to keep the protein in solution (*e.g.*, glycerol, neutral or zwitterionic detergents, urea),

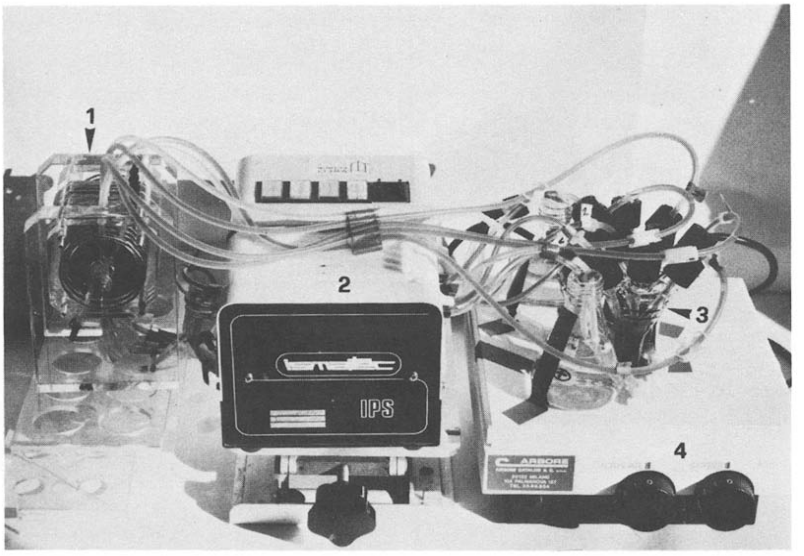

Fig. 5. View of the experimental assembly. 1 = Multi-chamber electrolyser; 2 = multi-channel peristaltic pump; 3 = block of sample flow chambers; 4 = magnetic stirrer (power supply not shown).

they should also be present in the analytical IPG run. After focusing and on staining the gel, the positions of the bands of interest will be noted and their p*I* values measured accurately by linear interpolation of the pH value along the gel matrix. pH points will then be selected just above and below the p*I* of the protein of interest, in such a way as to exclude the p*I* values of the nearest contaminants. These pH points (or values) will then be used to calculate the composition of the anodic and cathodic membranes (in terms of molarity of buffering and titrant Immobilines) needed to keep isoelectric that particular protein band. This calculation will be repeated for any other band of interest whose purification is sought (as an example of such an interpolation, see Fig. 11 in ref. 33).

Such polyacrylamide membranes are not self-supporting, and would tear apart during the run. We have investigated a number of fabrics for buttressing the buffering, isoelectric gel stratum (see also Wenger *et al.*[26]). We have found the best candidate to be glass microfibre filters from Whatman (see also Discussion). A housing is machined in a Perspex block, just 1 mm deep and having the same diameter as that of the membrane (see Fig. 6A). This housing is filled with the gelling solution having a given p*I* value and then the glass filter is gently lowered on to it, so that it will absorb the liquid from its lower face while expelling any trapped air from its upper surface. After placing the filter in position, the chamber is closed with a 3-mm thick glass and the assembled polymerization cell (see Fig. 6B) is placed in a forced-ventilation oven for a 1-h polymerization cycle at 50°C. After gelling, the chamber is gently pried open, any excess gel trimmed from the filter perimeter with a surgical blade and the membrane subjected to several washing cycles in distilled water until all unpolymerized material and catalysts have been removed. As the total membrane thickness (gel phase and glass filter) is barely 1 mm, ten washing cycles (each 1 h long) are

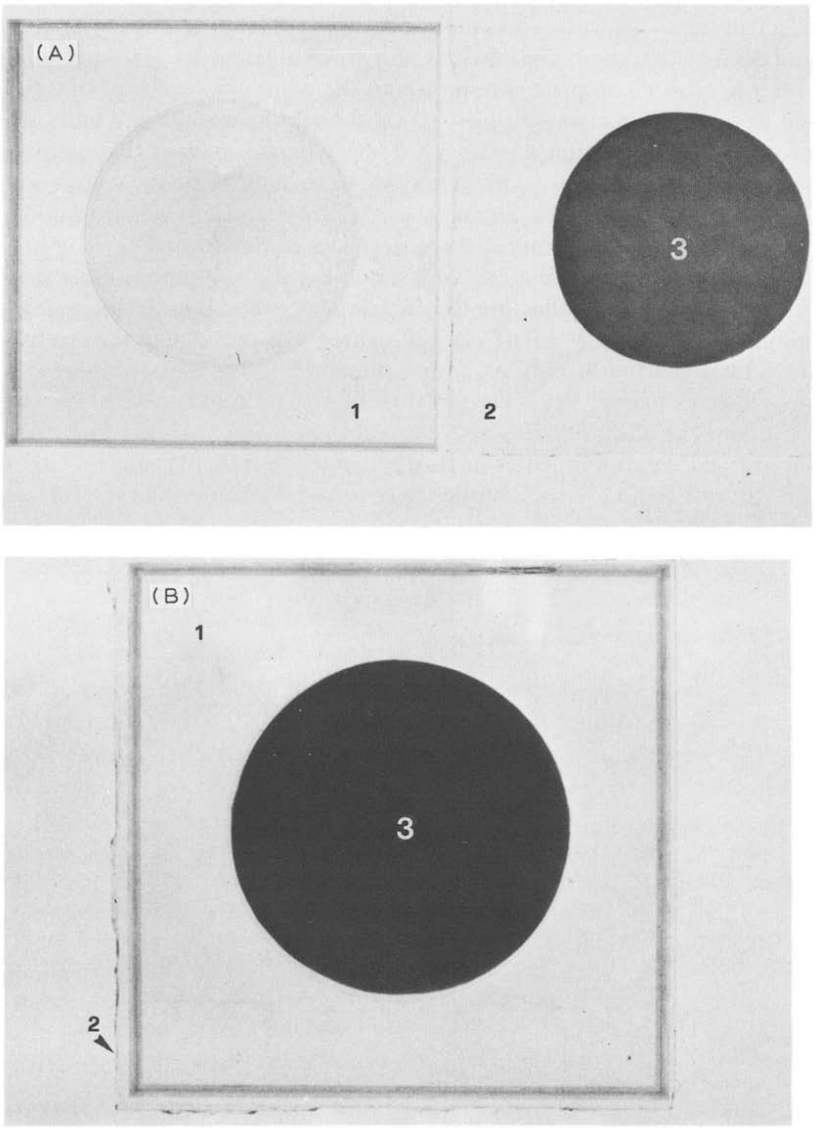

Fig. 6. Membrane polymerization cassette. (A) A Perspex block (7.5 × 7.5 cm) is machined to accommodate a central, circular housing (1 mm deep) of 4.7 cm diameter (1); 2 = cover-glass; 3 = Whatman GF/D glass microfibre filter. (B) Assembled cassette during Immobiline membrane polymerization.

sufficient to ensure virtually complete elimination of unpolymerized material (no free acrylamide could be detected in the washings down to the femtomole level).

RESULTS

Purification of N-acetyl Eglin C

This protein was produced in *E. coli* by recombinant DNA (r-DNA) techniques at Ciba Geigy[36] and was *ca.* 90% pure when given to us to be treated in the multi-

compartment electrolyser. This was a special purification problem because, by analytical IEF, we had seen that some contaminants had ΔpI values of *ca.* 0.01 pH unit. Therefore, in order to ensure complete elimination of these impurities, the p*I* of Eglin C was measured in several runs ($pI = 5.5 \pm 0.01$) and both the anodic and cathodic membranes were made so as to define a pH = 5.5. Thus, whereas in most IPG runs we work under the condition $pI_{am} < pI_p < pI_{cm}$, here we have defined the most stringent restraint, $pI_{am} = pI_p = pI_{cm}$, where the subscripts am, p and cm denote anodic membrane, protein and cathodic membrane, respectively. In this instance, we used a large apparatus with a membrane diameter of 9 cm and with only one sample flow chamber, as we had no interest in collecting the anodic and cathodic impurities. A 5-g amount of Eglin C, dissolved in 250 ml of "Baker" water, was recycled in the electrolyser at a constant 12 W in a cold room. At hourly intervals, 100 μl were sampled and kept at 4°C for subsequent analysis. The experiment was terminated with the last sampling after 5 h and the aliquots were analysed in a pH 3.5–9.5 Ampholine PAG plate. As shown in Fig. 7A, all impurities having p*I* values $\geqslant$ 0.01 pH unit on either side of the p*I* 5.5 value of Eglin C were completely removed. Considering that, during

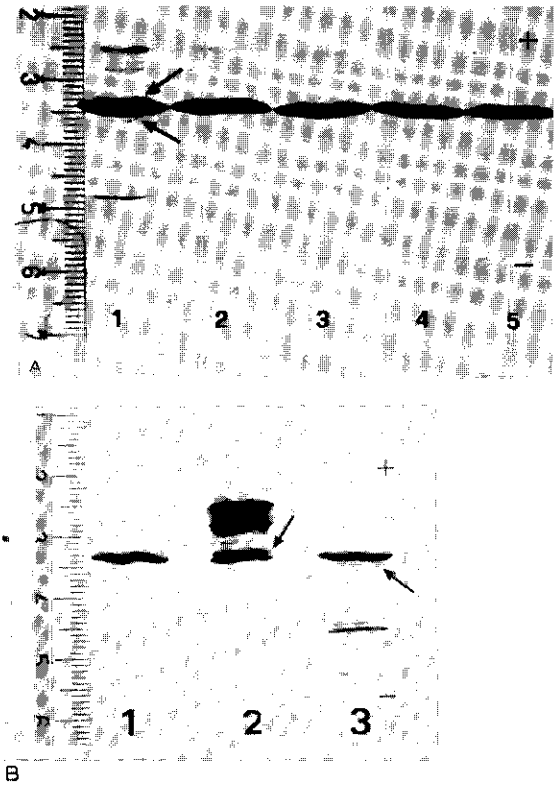

Fig. 7. Purification of N-acetyl Eglin C. A 5-g amount of Eglin C (in 250 ml of distilled water) was recycled in a single-chamber apparatus (9 cm diameter) at 12 W, 10°C for 5 h. (A) Analytical CA-IEF gel (pH 3.5–9.5) of 200 μg of protein at (1) time zero (control); (2) 1 h; (3) 2 h; (4) 4 h; (5) 5 h of recycling. (B) Analytical CA-IEF gel of (1) 5-h aliquot, (2) anodic chamber content and (3) cathodic chamber content (the last two samples representing 500-μl aliquots dialysed and lyophilized). The arrows indicate one anodic and one cathodic contaminant with a ΔpI of only 0.01 pH unit removed from Eglin C.

this time period, a total of 5 g of Eglin C were treated, this amounts to a processing rate of 1 g/h of protein, a load ability rarely reported, if at all, in any electrokinetic process ever described.

It might be asked what the mass recovery of Eglin C is, considering that the two boundaries had the same p*I* as the protein under purification. By protein and biological assay, it was found that the amount recovered in the sample flow chamber was 4.750 g, *i.e.*, 95% of the total initial mass input. Aliquots of the anodic and cathodic electrolytes were also collected, dialysed, liophylized and then analysed by analytical IEF. As shown in Fig. 7B, some Eglin C was lost in the two electrolyte reservoirs; most important, it can be shown (see the two arrows) that the two anodic and cathodic impurities (having a ΔpI of *ca.* 0.01) originally present in the Eglin C preparation have now been completely removed and have leached out in the electrolyte compartments.

Purification of human monoclonal antibody isoproteins

Human monoclonal antibodies, made in a hybridoma cell line, against the transmembrane protein gp41 from the immunodeficiency virus (HIV-1) were prepared by Jungbauer *et al.*[27]. By analytical IPGs, they were found to consist of a family of at least six isoproteins, three major and three minor (see Fig. 8A, last two tracks to the left), with p*I*s of 8.93, 9.04 and 9.18 (the three major) and 8.84, 9.40 and 9.60 (the three minor). This was a challenging separation, as it is known that focusing in alkaline pH ranges, both in CA-IEF and IPGs, is besieged with problems (carbon dioxide absorption, electroosmosis, potential hydrolysis of both the matrix and the Immobiline chemicals). We assembled the apparatus in Fig. 3 with six chambers, the two extreme ones for electrolyte solutions and four sample chambers, delimited by five membranes having isoelectric points of 8.87, 8.98, 9.11, 9.29 and 9.50. A 200-mg amount of protein dissolved in 80 ml of 25% glycerol and 0.5% Tween 20 was recycled for a total of 75 000 V h. The content of each chamber was then analysed by analytical IPGs in the pH range 8.5–10. As seen in Fig. 8A, there is clearly an enrichment of the various isoforms in the different chambers, but not a single homogeneous fraction. The content of chamber 4 was then used in a "cascade" fashion and re-run in the multi-chamber apparatus. As shown in Fig. 8B, fraction 3 now contains an almost pure component. Work on the purification to homogeneity of these monoclonal antibodies will be reported elsewhere.

DISCUSSION

Membrane preparation

The most delicate aspect of multi-compartment electrolysers utilizing isoelectric Immobiline membranes is the production and quality control of such membranes. We shall therefore describe in details the manipulations involved. An extensive study was first made of the properties of different support materials. When first described, Wenger *et al.*[26] had already tried a number of porous sheets, including polyethylene, polypropylene, nylon nets and cotton fabrics. We finally adopted GF/D glass microfibre filters from Whatman, as they appear to be the best supports for isoelectric Immobiline gels in preparative protein purification. First, they are highly resistant to chemical attack (except by hydrofluoric acid and strong alkalis). Resistance to all

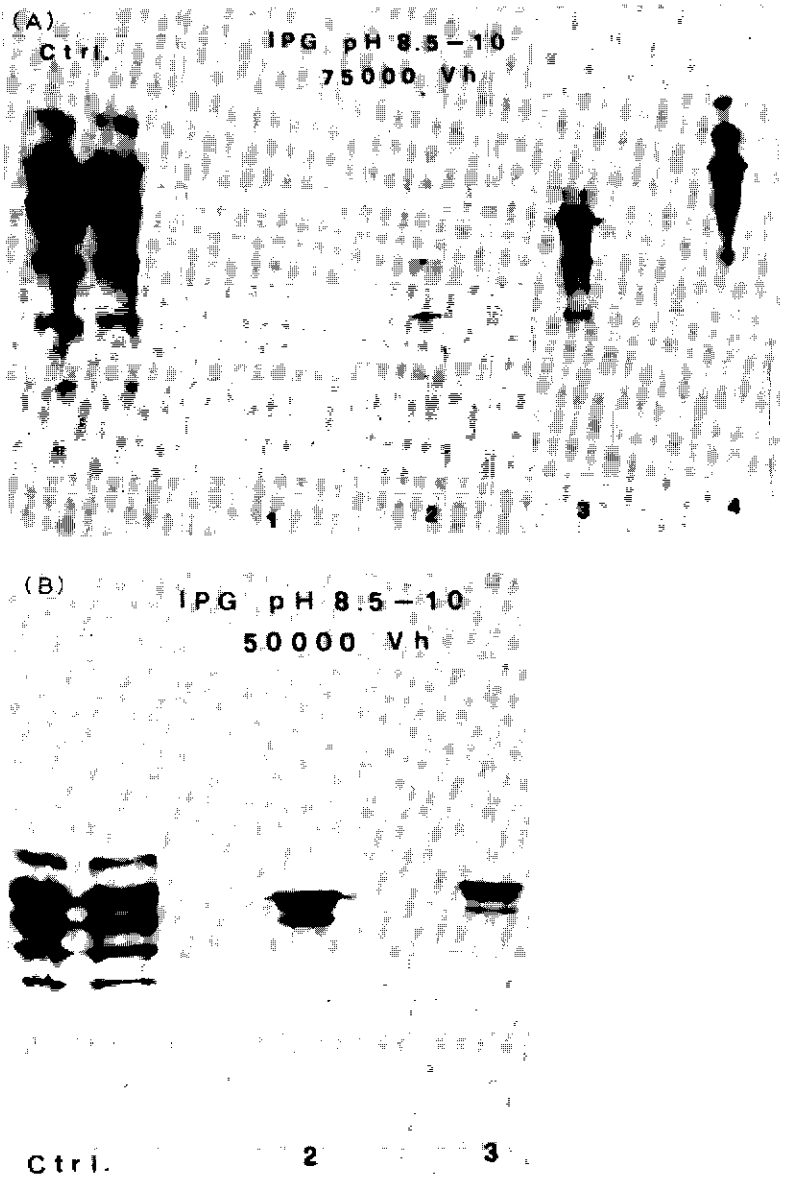

Fig. 8. Purification of human monoclonal antibodies. A 200-mg amount of total antibodies (in a total volume of 80 ml) was purified in an electrolyser with four sample chambers mounted. (A) Ctrl. = starting material; 1–4 = 30-μl aliquots from chambers 1–4, respectively. Sampling taken after 75 000 V h and analysed in an IPG pH 8.5–10 gel. Anodic sample application; Coomassie Brilliant Blue R-250 staining. The cathode is uppermost. (B) The content of chamber 4 in A (50 mg total protein) was distributed over four chambers and re-run for 50 000 V h. Ctrl. = unfractionated, starting material; 2, 3 = aliquots from chambers 2 and 3 of the electrolyser. Even though the fractions are still not homogeneous, note the extensive purification achieved.

other acids is excellent up to 5 *M* concentration. In addition, glass microfibres do not swell in organic solvents or water and do not leach out impurities, as they do not contain any binders. Because of its fine capillary structure, a glass microfibre filter will absorb larger volumes of liquids than cellulose papers of equivalent weight. This is important for the production of Immobiline membranes, as it ensures that more gel volume will be held within the glass fibres, thus allowing a higher buffering capacity of the membrane per unit weight. In addition, Immobiline gels have a very strong adhesivity to glass surfaces, this guaranteeing that no detachment between the gel phase and the glass support will occur. In fact, even on prolonged use (more than 10 days at pH > 9) we have never experienced problems of membrane destruction or even perforation. The glass microfibre filters have remarkable tear strength and burst strength, which probably result from the very large number of weak bonds between the hydrated silica surface of adjacent fibres. Being composed of glass, these filters are virtually non-ageing material and are not subject to weakening and embrittlement even for extreme periods of storage. The composition of borosilicate glass fibres, as given by the manufacturer, is SiO_2 57.9%, B_2O_3 10.7%, Al_2O_3 5.9%, Na_2O 10.1%, K_2O 2.9%, CaO 2.6%, MgO 0.4%, BaO 5.0%, ZnO 3.9% and F 0.6%. The content of leachable material is negligible except for sodium (1710 ppm extracted at 27°C for 24 h).

Membrane composition and storage

For most applications, a 5%T matrix seems to be adequate; it is porous enough to allow transmembrane migration of most proteins (at least up to 200 kDa), yet robust enough to prevent undesirable swelling of the gel due to the content of charged species. In terms of Immobiline content, we prefer to use *ca.* 20 m*M* buffering ion (plus the amount of titrant needed to define the desired p*I* value); under these conditions, and according to the relative p*I* − pK value, the membranes will have a β power ranging from 6 to 10 mequiv. l^{-1} pH^{-1}, which is more than adequate to buffer any amount of protein tangent to the membrane or crossing it. In particular instances up to 50 m*M* buffering ion can be used, but it should be remembered that at high Immobiline molarities there will be two major problems: (a) the gel matrix could swell to the point of bursting and (b) the resulting p*I* value could be erratic because, at high concentrations, the p*K* values in the Immobiline membrane could change.

In the separation of immunoglobulins, we have adopted additional strategies: the membrane was made to contain 5%T, but the amount of cross-linker was increased to 8%C (from a standard value in all gels of 4%C); the higher %C renders the membrane more porous, thus facilitating the transmigration of large proteins, and also tends to reduce swelling of the gel phase, which is more pronounced in alkaline pH ranges. In addition, the two lateral membranes in contact with the electrode solution were made even more robust (10%T), again to prevent swelling due to different osmotic pressures generated by pH and conductivity steps at the separation chamber/electrolyte boundaries. When making alkaline membranes, there could be the problem of how long to store them; if not utilized immediately, the membranes can be stored for at least 3 weeks in 20 m*M* acetate buffer (pH 5). It is known that mildly acidic pH values ensure maximum stability of all Immobiline chemicals[34]. Then, just prior to use, they are extensively washed in distilled water (or in any additive needed for the separation).

When casting the membranes, the following precautions should be taken: (a) coat the cover-glass extensively with Repel-silane so that, on opening the cassette, the Immobiline membrane will not adhere to it, with the risk of tearing the gel phase; (b) avoid trapping air bubbles; even when minute, air bubbles embedded in the filter can swell during the run and bore holes in the membrane; (c) degas the gelling solution (conventional IPG gels are not degassed, but here it is essential) and prepare enough of it so that it slightly overflows when poured into the polymerization chamber (see Fig. 6). An excess of gelling solution will reduce the risk of trapping air bubbles when lowering the glass cover into the chamber to seal it against atmospheric oxygen.

Running the electrolyser

Some precautions should be taken here also. In alkaline pH ranges, avoidance of carbon dioxide absorption is essential. If not properly shielded, the apparatus will act as a carbon dioxide trap, with a continuous background of HCO_3^- ions migrating from the cathode through the intermediate sample chambers to the anode, where they would be discharged back in the atmosphere at pH < 6. To prevent this phenomenon, we float light paraffin oil (density = 0.88) on the surface of the liquid pumped in each chamber (including catholyte and, when necessary, also anolyte). For this reason, we have made special sample reservoirs containing an outlet at the bottom (see Fig. 4), so that at the end of the experiment the sample can be collected from this outlet while leaving the paraffin oil behind. In alkaline pH ranges, we have found it to be important, for correct maintenance of pH in each chamber, to have electrode solutions with pH values approaching as close as possible the pH range in which fractionation takes place. For this reason, we have used as the anolyte a 1 m*M* solution of HEPES + pH 8.5–10 carrier ampholytes to pH 6.8 and as the catholyte a 0.1 m*M* solution of sodium hydroxide (pH 10). Alternatively, isoelectric Lys (pH 9.8) could be used. In general, dilute acids and bases should be used in the electrode compartments, *e.g.*, in the Eglin C run, 1 m*M* acetic acid and 1 m*M* sodium hydroxide solution were used as anolyte and catholyte, respectively.

Sample pretreatment

For optimum performance, the way in which the sample is treated before the IPG run is important. We had already demonstrated[23] that, in IPG runs, any amount of salt present in the sample is deleterious to the fractionation process. In particular, salts made from strong acids and bases should be avoided (*e.g.*, sodium chloride, but also a phosphate buffer) because, on passage of the current, the anions and cations present therein produce strongly acidic and basic boundaries which can rapidly denature proteins by a pH shock[35]. Thus, ideally, an already desalted sample zone should be preferentially used as the initial feed. If that is not possible, the sample should be equilibrated in buffers formed from weak acids and bases (*e.g.*, Tris–acetate will be acceptable, but Tris–HCl will not), or if strong species are present, additional weak counter ions should be added (typically carrier ampholytes, able to buffer in the transient state any strong anion or cation migrating out of the sample zone). In any event, the amount of salts present in the initial sample feed should be kept to a minimum, as the macroions will not be able to migrate until all the small ions have vacated the sample zone[23,35].

From this point of view, it is of interest to note the effect of carrier ampholytes

(CA) on the progress of separation. At least in alkaline pII ranges, and with immunoglobulins, we have noted that, rather than helping the separation, CA chemicals are in fact detrimental. They seem to induce a strong electroosmotic flow and in addition they could bind to the macroions, thus altering their p*I* values. In our early experiments, when the immunoglobulins were fractionated in the presence of small amounts of CAs, we had to recalculate the p*I* values of the different membranes, as the proteins had altered their apparent p*I* values and were focusing in the wrong chambers. There is definitely an effect of CAs on the p*I* values of alkaline proteins, which usually results in a lowering of the apparent p*I* of the protein, as measured in an IPG gel in the absence of CAs. The Δp*I* in presence or absence of CAs is, in our case, as much as 0.2 pH unit, but it could be even higher. Given the small differences in isoelectric points in the various immunoglobulin fractions, such p*I* alterations are deleterious to the fractionation process. Thus, we have recently come to the conclusions that, whenever possible, addition of CA buffers should be avoided altogether. If the protein input is high enough (concentrations of the order of 10 mg ml^{-1}), the macroions will act as buffering species, so that no extra buffers need to be added to the liquid phase for pH control; the isoelectric protein will take care of this. For example, we had calculated that, at a concentration of 10 mg ml^{-1}, and at pH = p*I*, human haemoglobin as a β power = 1 μequiv. l^{-1} pH^{-1}, which, albeit low, is adequate to ensure pH control in the absence of small ions moving through the system[6].

It is in general believed that an isoionic protein would have a very low solubility. However, in most instances, this solubility is high enough to avoid protein precipitation. So far we have not experienced any isoelectric protein precipitation in our membrane apparatus. Often, slightly changing the composition of the liquid phase is sufficient to keep the protein in solution. For example, in the case of immunoglobulin fractionation, the proteins are fully soluble, even at high concentrations, in the presence of 25% glycerol and small amounts of neutral detergent (0.5% Tween 20). In some instances, though, addition of urea might be needed. It is true that, in conventional IEF, severe protein precipitation has often been observed (for a review, see Righetti[2]). However, on close scrutiny, we can observe that in those instances the initial sample input consisted of a highly unpurified protein mixture. In any focusing system, in the absence of buffer or salt ions, acidic and basic proteins present initially will tend to form aggregates which will neutralize each other and induce massive precipitation. In our system (and we believe as a general rule in electrokinetic separations), the input protein should already be at an advanced stage of purification (it should be at least 50% pure, preferably 70–80%). Under these conditions, risks of precipitation with unlike proteins will be minimized.

Electrical conditions

The speed of the separation process will depend on, among other factors, the voltage applied and the lateral cross-sectional area of the membrane (see the mass transport equation in ref. 25). Because, for any multi-compartment electrolyser built, the geometry is fixed, given a constant membrane surface area, the other way to speed up the purification process will be to increase the voltage gradient over the separation chamber. At the beginning, in the "segmented IPG" technology utilizing pH gradients on both sides of the flow chamber, we were limited, by the joule heat that developed and by the need to perform the run in the presence of CAs, to barely 50 V

cm^{-1}. In the present membrane apparatus, and in the absence of foreign ions except the protein macroions, considerably higher voltage gradients can be applied. For example, in the purification of monoclonal antibodies, when the sample feed was devoid of salts and carrier ampholytes were not added, once residual traces of ions had been removed in an initial low-voltage run (100–150 V cm^{-1}), much higher voltages could be applied (up to 800 V/cm). Even at such high voltages, the total wattage did not exceed 5 W, and the temperature rise in the liquid compartment, simply by air cooling in a cold room, was barely 4–5°C above the standard cold-room temperature of 4–5°C. However, salt-containing samples should be run initially at low voltage gradients until the ion boundaries have been eliminated, otherwise two undesirable phenomena will occur: (a) thermal sample denaturation due to joule heat and (b) pH denaturation due to the formation of strongly acidic and basic ion boundaries[35].

CONCLUSIONS

We have accumulated 2 years of experience first with the "segmented IPG" principle and now with multi-compartment electrolysers exploiting isoelectric Immobiline membranes. We are confident that this could become one of the leading techniques in the near future for purifying r-DNA proteins to homogeneity and we hope that these experimental observations will serve as guidelines for scientists eager to exploit this novel technique. We emphasize that, so far, IPGs appear to be the only technique coupling an extremely high load capacity with an extremely high resolving power, which is not lost in the scaling-up process.

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REFERENCES

1 B. Bjellqvist, K. Ek, P. G. Righetti, E. Gianazza, A. Görg, R. Westermeier and W. Postel, *J. Biochem. Biophys. Methods*, 6 (1982) 317–339.
2 P. G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983.
3 G. Cossu and P. G. Righetti, *J. Chromatogr.*, 398 (1987) 211–216.
4 P. G. Righetti and E. Gianazza, *Methods Biochem. Anal.*, 32 (187) 215–278.
5 K. Ek, B. Bjellqvist and P.G. Righetti, *J. Biochem. Biophys. Methods*, 8 (1983) 134–155.
6 C. Gelfi and P.G. Righetti, *J. Biochem. Biophys. Methods*, 8 (1983) 157–172.
7 P. G. Righetti and C. Gelfi, *J. Biochem. Biophys. Methods*, 9 (1984) 103–119.
8 P. G. Righetti, K. Ek and B. Bjellqvist, *J. Chromatogr.*, 291 (1984) 31–42.
9 B. J. Radola, *Ann. N.Y. Acad. Sci.*, 209 (1973) 127–143.
10 O. Vesterberg, T. Wadström, K. Vesterberg, H. Svensson and B. Malmgren, *Biochim. Biophys. Acta*, 133 (1967) 435–445.
11 H. Rilbe, *Protides Biol. Fluids*, Proc. Colloq., 17 (1970) 369–372.
12 H. Rilbe, *Ann. N.Y. Acad. Sci.*, 209 (1973) 80–93.
13 H. Rilbe, A. Forcheimer, S. Pettersson and M. Jonsson, in P. G. Righetti (Editor), *Progress in Isoelectric Focusing and Isotachophoresis*, Elsevier, Amsterdam, 1975, pp. 51–63.
14 M. Jonsson and H. Rilbe, *Electrophoresis*, 1 (1980) 3–14.

15 M. Bier and N.B. Egen, in H. Haglund, J. C. Westerfield and J. T. Ball, Jr. (Editors), *Electrofocus:78*, Elsevier, Amsterdam, 1979, pp. 35–48.
16 M. Bier, N. B. Egen, T. T. Allgyer, G. E. Twitty and R. A. Mosher, in E. Gross and J. Meienhofer (Editors), *Peptides: Structure and Biological Function*, Pierce, Rockford Il, 1979, pp. 79–89.
17 N. B. Egen, W. Thormann, G. E. Twitty and M. Bier, in H. Hirai (Editor), *Electrophoresis '83*, Walter de Gruyter, Berlin, 1984, pp. 547–550.
18 K. Hannig, in J. R. Norris and D. W. Ribbons (Editors), *Methods in Microbiology*, Vol. 5B, Academic Press, London, 1971, pp. 513–520.
19 A. R. Thompson, in C. F. Simpson and M. Whittaker (Editors), *Electrophoretic Techniques*, Academic Press, London, 1983, pp. 253–274.
20 S. Hjertèn, *Chromatogr. Rev.*, 9 (1967) 122–219.
21 M. Faupel, B. Barzaghi, C. Gelfi and P. G. Righetti, *J. Biochem. Biophys. Methods*, 15 (1987) 147–162.
22 P. G. Righetti, B. Barzaghi and M. Faupel, *J. Biochem. Biophys. Methods*, 15 (1987) 163–176.
23 B. Barzaghi, P. G. Righetti and M. Faupel, *J. Biochem. Biophys. Methods*, 15 (1987) 177–188.
24 P. G. Righetti, B. Barzaghi, M. Luzzana, G. Manfredi and M. Faupel, *J. Biochem. Biophys. Methods*, 15 (1987) 189–198.
25 P. G. Righetti, B. Barzaghi and M. Faupel, *Trends Biotech.*, 6 (1988) 121–125.
26 P. Wenger, M. de Zuanni, P. Javet, C. Gelfi and P. G. Righetti, *J. Biochem. Biophys. Methods*, 14 (1987) 29–43.
27 A. Jungbauer, C. Tauer, F. Steindl, E. Wenisch, M. Purtscher, M. Reiter, F. Unterluggauer, A. Buchacher, K. Uhl and H. Katinger, *J. Biochem. Biophys. Methods*, (1989) in press.
28 C. Gelfi, M.L. Bossi, B. Bjellqvist and P. G. Righetti, *J. Biochem. Biophys. Methods*, 15 (1987) 41–48.
29 P. K. Sinha and P. G. Righetti, *J. Biochem. Biophys. Methods*, 15 (1987) 199–206.
30 C. Gelfi and P. G. Righetti, *Electrophoresis*, 5 (1984) 257–262.
31 M. Chiari, E. Casale, E. Santaniello and P. G. Righetti, *Theor. Appl. Electrophoresis*, 1 (1989) 99–102.
32 M. Chiari, E. Casale, E. Santaniello and P. G. Righetti, *Theor. Appl. Electrophoresis*, 1 (1989) 103–107.
33 P. G. Righetti, *J. Chromatogr.*, 300 (1984) 165–223.
34 P. Pietta, E. Pocaterra, A. Fiorino, E. Gianazza and P. G. Righetti, *Electrophoresis*, 6 (1985) 162–170.
35 P. G. Righetti, M. Chiari and C. Gelfi, *Electrophoresis*, 9 (1988) 65–73.
36 H. Rink, M. Liersch, P. Sieber, F. Meyer, *Nucleic Acids Res.*, 12 (1984) 63–69.