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PREPARATIVE PURIFICATION OF HUMAN MONOCLONAL ANTIBODY ISOFORMS IN A MULTI-COMPARTMENT ELECTROLYSER WITH IMMOBILINE MEMBRANES

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SUMMARY

The performance of a multi-compartment electrolyser with isoelectric Immobiline membranes for large-scale protein purification is evaluated. Owing to the presence of isoelectric membranes possessing a high buffering capacity and ionic strength, isoelectric protein precipitation inside the membranes, one of the major drawbacks of present membrane uses, is fully avoided. In addition, owing to this novel membrane technology, pH gradient decay, typical of isoelectric focusing in carrier ampholytes, is fully eliminated and pH and conductivity constancy is guaranteed in all flow chambers for running periods of more than 11 days (160 000 V h). The membranes described possess a unique selectivity, in that they act by modulating the surface charge (*i.e.*, the mobility) of macroions crossing or tangential to them. The concept of isoelectric Immobiline membranes acting like a pH-stat unit is introduced. Protein homogeneity in each chamber of the electrolyser can be achieved even when purifying human monoclonal antibodies against HIV-1, which possess high p*I* values (9.0–9.6), are large molecules (M_r 150 000) and are fractionated in the presence of large micelles of neutral detergents.

INTRODUCTION

There is a serious problem with the preparative electrophoretic purification of proteins, namely that the gel matrix is incompatible even with moderate protein loads. Except perhaps for preparative isoelectric focusing (IEF) in granulated gel layers, where the protein is believed to focus also in the liquid layer on the surface of the Sephadex beads[1], in polyacrylamide gels, where the protein is physically trapped

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inside the matrix network, the "invading" macromolecule finds little free water available for hydration[2]. This phenomenon, in combination with the possibility of co-eluting large amounts of unreacted monomers and ungrafted oligomers from the gel support[3], has led to the abandonment of the use of polyacrylamide gels for preparative purification, especially when applied to recombinant-DNA proteins for human consumption. However, one of the most powerful techniques available today is IEF in immobilized pH gradients (IPG), which is compatible with both an extremely high resolving power and a very high protein load ability[4]. In order to exploit these advantages fully, we have previously described a novel approach to preparative IPGs, called segmented IPGs, by which a single component of interest in a protein mixture is forced to focus and is kept isoelectric in a "window" opened in an IPG gel, the impurities collecting in the anodic and/or cathodic gel segments or simply being lost in the electrodic compartments[5–8]. For this to occur, the IPG gel was cast in two segments having the gel extremities facing the "window" satisfying the inequality $pI_a < pI_p < pI_c$, where the subscripts denote anodic, protein and cathodic, respectively, and p*I* is the isoelectric point. Hence the gel extremities tangential to the flow chamber would keep the protein of interest isoelectric by a continuous titration process. It was soon realized that for this to occur, one in fact only needed just membranes with well defined isoelectric points and good buffering capacity and not segments of immobilized pH gradient gels. A one-compartment electrolyser based on the principle of Immobiline membranes was therefore built, consisting only of an anodic and a cathodic reservoir flanking the central flow chamber where the protein of interest would eventually remain as the sole isoelectric species[9]. In a subsequent version, a multi-compartment electrolyser based on multiple isoelectric membranes was described[10] for purifying several components in a mixture or isoforms in a family of related isoproteins.

Here we report the performance of this instrument and its application to the purification of isoforms of a human monoclonal antibody against the transmembrane gp41 protein of the immunodeficiency virus HIV-1 (AIDS disease). The production and purification to homogeneity of such an antibody has recently been described[11]. However, the pure antibody consists of a family of isoproteins, three major (with p*I* 9.15, 9.28 and 9.37) and three minor (with p*I* 9.07, 9.54 and 9.66), whose further purification into single species proved to be difficult by all conventional techniques (ion-exchange chromatography, chromatofocusing and fast protein liquid chromatography (FPLC)-based material, *i.e.*, mono-P from Pharmacia)[12].

EXPERIMENTAL

Materials

Repel-Silane, Gel Bond PAG, the Multiphor 2 chamber, Multitemp thermostat and the Macrodrive power supply were obtained from LKB (Bromma, Sweden) and Pharmalyte and the protein p*I* marker kit were purchased from Pharmacia (Uppsala, Sweden). The multi-channel peristaltic pump was from Ismatec (Zürich, Switzerland). The glass microfibre filters (GF/D) were purchased from Whatman (Maidstone, U.K.), light paraffin oil from Merck (Darmstadt, F.R.G.) and acrylamide, N,N′-methylenebisacrylamide (Bis), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulphate and Coomassie Brilliant Blue R-250 from Bio-

Rad Labs. (Richmond, CA, U.S.A.). Monoclonal antibodies against the gp41 from AIDS virus were prepared and purified by Jungbauer *et al.*[11].

pH and conductivity measurements

At the times indicated in Figs. 2, 3, 6A and B, 2-ml aliquots were collected from each chamber and the conductivity was measured at 25°C with an Orion conductivity meter fitted with a 1-cm cell. The pH was then assessed (under anaerobic conditions) at 25°C with a Radiometer Model PHM-64 pH meter fitted with a GK2421C combination microelectrode.

Analytical IPGs

For following the progress of purification of monoclonal antibodies, analytical IPG gels, in the pH range 8.5–10, were made according to Gelfi *et al.*[13]. The samples were loaded in pockets precast at the anodic side, in a pH 8.0 plateau gel segment (2 cm long). About 20 μl, containing up to 50 μg of protein, were loaded and focusing was continued for 30 000 V h as described by Sinha and Righetti[14]. The gels were stained with Coomassie Brilliant Blue R-250 in Cu^{2+}, according to Righetti and Drysdale[15].

Description of the apparatus

Basically, the multi-compartment electrolyser consists of a stack of chambers sandwiched between an anodic and a cathodic reservoir. The apparatus has been already extensively described in Righetti *et al.*[10] and here we just show a drawing of the instrument. Fig. 1 is an exploded view, with a stack of three chambers already assembled to the left. a central compartment and a thinner chamber to the right for connection to the other electrode. All flow chambers are provided with inlet and outlet for sample or electrolyte recycling, an O-ring for ensuring flow-tight connections and four holes for threading four long metal rods which can be tightened by manually adjusted butterfly nuts for assembling the apparatus. We have built several versions of these cells, capable of housing Immobiline membranes from 4.7 cm (the present apparatus) up to 9 cm diameter. The pH-controlling membranes are housed in the central depression between two 1-cm-wide rings of rubber. 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 electrosmosis). The platinum electrodes are housed in two rectangular Perspex mountings, which also act as legs on which the electrolyser stands. The distance between adjacent cells is only 10 mm, so that each chamber holds *ca.* 5 ml of liquid. Note that, owing to the way in which the apparatus has to be assembled, one of the two terminal chambers has half the normal thickness; we used it as the catholyte compartment in the present study. For a more detailed description, see ref. 10.

Pre-running the apparatus

After assembling the apparatus, a 2-l volume of stock, supporting solution is prepared containing 25% glycerol and 1% Nonidet P-40 (pH 5.0 and conductivity 13.2 μmho). This solution is sterilized by filtration through a Millipore Millex-GV filter of 0.22 μm porosity. The apparatus is filled with this solution and a first cleaning run is performed in presence of 0.3% of added Pharmalytes (pH 8–10.5), whereas

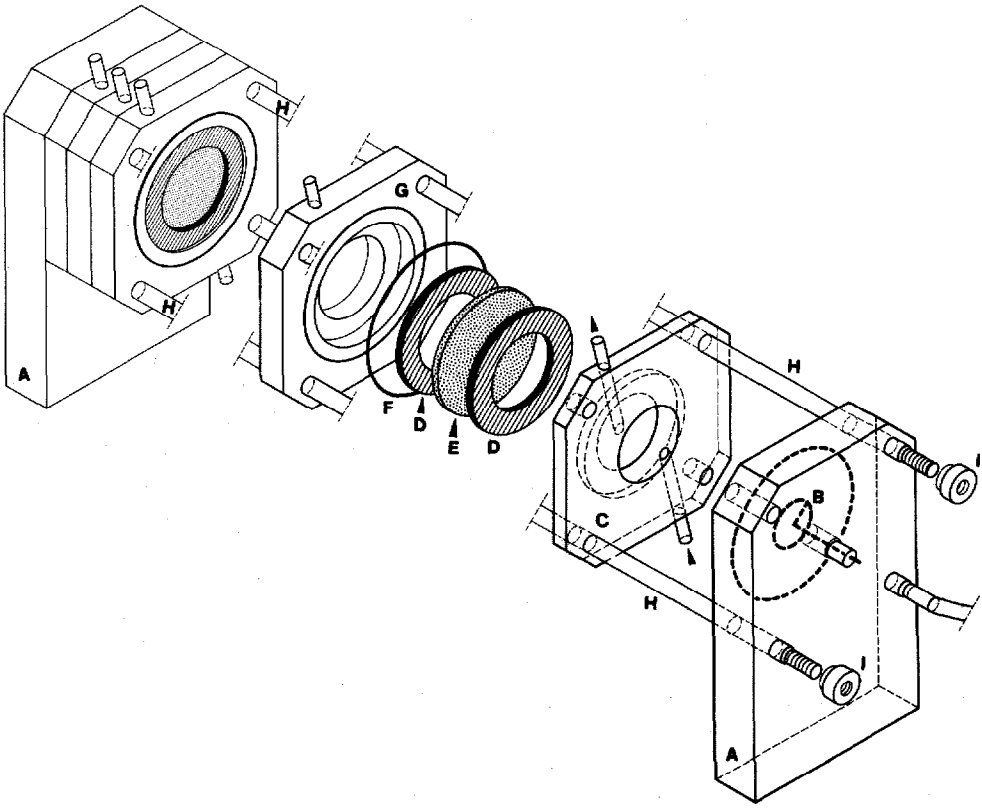

Fig. 1. Exploded view of the multi-compartment electrolyser. A = Rectangular supporting legs; B = Pt electrode; C = thin terminal flow chamber; D = rubber rings for supporting the membrane; E = isoelectric Immobiline membrane cast onto the glass-fibre filter; F = O-ring; G = one of the sample flow chambers; H = four threaded metal rods for assembling the apparatus; I = nuts to fasten the metal bolts.

1 m*M* N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) is added to the anolyte (pH 5.1, 19.0 μmho) and 1 m*M* sodium hydroxide to the catholyte (pH 10, 69.3 μmho). This is run for 1 day (step 1). After this, the solutions are discarded, and the reservoirs are filled with the supporting solution in the absence of carrier ampholytes (CAs). This is run for 1 day and the solutions are kept as a reservoir to be added to vessels in which decremental liquid flows occur (see Figs. 4 and 5) (step 2). Subsequent to this, a run is performed in supporting solution with 3% Pharmalyte (pH 8–10.5) added; this focused material will then be left in the flow chambers as pH-controlling starting liquid for focusing the protein sample (step 3).

RESULTS

Establishment and stability of the pH gradient

For purifying the anti-gp41 monoclonal antibody isoforms, five IPG membranes were prepared of p*I* 9.11, 9.25, 9.36, 9.49 and 9.64 (see Fig. 7). The membranes facing the cathodic and anodic reservoirs were made more robust (10%T, 4%C),

whereas the membranes between the sample flow chambers were more porous (5%T, 8%C) so as to allow passage of large protein molecules (IgGs, M_r 150 000 Da) and diffusion of large detergent micelles (Nonidet P-40). The molarity of the buffering and titrant Immobilines in each membrane needed to define a desired pH value are simply interpolated from an analytical IPG run (as an example, see Fig. 7) assuming a linear pH course between the two limiting solutions. Membranes 1 mm thick are cast enmeshed in a glass-fibre filter (GF/D glass microfibre from Whatman), to which they firmly adhere, giving mechanical strength and support to the otherwise pliable polyacrylamide matrix. After polymerization, the membranes are extensively washed (6 × 30 min) in excess of distilled water, followed by one equilibration step in 25% glycerol and 1% Nonidet P-40 (*i.e.*, in the same solvent in which the IgGs are dissolved). After assembling the apparatus and pre-running it as described above (steps 1 and 2), the four sample reservoirs are filled with 50 ml each of 3% Pharmalyte (pH 8–10.5), while the anodic and cathodic reservoirs are replenished with 250 ml of 1 m*M* HEPES (pH 5.1) and 1 m*M* sodium hydroxide (pH 10), respectively. Each solution is overlayed with a layer of paraffin oil, so as to seal it from the atmosphere and thus prevent adsorption of carbon dioxide. All solutions are then recycled (2 ml/min) under the electric field.

As shown in Fig. 2, the system is seen to attain a steady state after 4000 V h (corresponding to 1 day of focusing at *ca.* 150 V) and to maintain pH constancy in

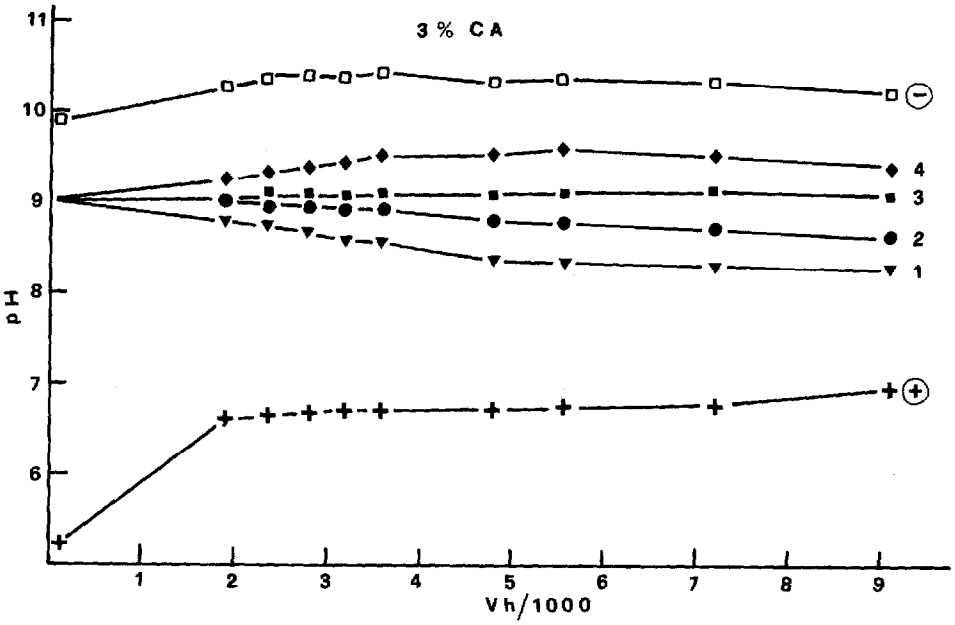

Fig. 2. pH profiles in the multi-compartment electrolyser. A solution of 3% Pharmalyte (pH 8–10.5) was focused for 10 000 V h (corresponding to a time of 4 days). In this and all subsequent graphs, the membranes dividing the four sample flow chambers had p*I*s of 9.11, 9.25, 9.36, 9.49 and 9.64. At the times indicated, 2 ml of solution were collected from each chamber and the pH was measured at 25°C. The numbers on each curve refer to the flow-chambers (1 being the most anodic) and the symbols + and − indicate anodic and cathodic reservoirs, respectively.

each chamber thereafter (up to 4 days in the experiment in Fig. 2). Analogous results are obtained when checking the conductivity in all chambers (Fig. 3). Here too after 5000 V h all conductivity values reach a plateau. Judging from these two figures, and at least within the sensitivity limits of both techniques, it would appear that the multi-membrane system is capable of blocking the cathodic drift, *i.e.*, the pH gradient decay which ensues on prolonged focusing and results in an overall movement of all carrier ampholytes towards the cathode, with a progressive acidification of the anodic extreme[16] (see Discussion).

One of the major problems when running IEF experiments, especially at alkaline pH, is the strong electrosmotic flow which results in progressive dehydration of the gel (when run in a gel system) and in slow destruction of the pH gradient. Although in our system we have stability of the pH gradient (see Fig. 2), there is, however, a small but appreciable liquid flow. As shown in Fig. 4, it appears that there is a progressive loss of water from all the sample flow chambers with a net transport in both anodic and cathodic directions, although the net flux to the cathode at any given time is predominant. In contrast to what happens in conventional IEF, though, this phenomenon is not deleterious to the separation process, as it is not accompanied by escape of protein from the chambers or destruction of the pH gradient. In fact, if at all, it could be favourable to the separation as it results in an increase in protein concentration in each sample chamber. At the steady state, it appears that the water

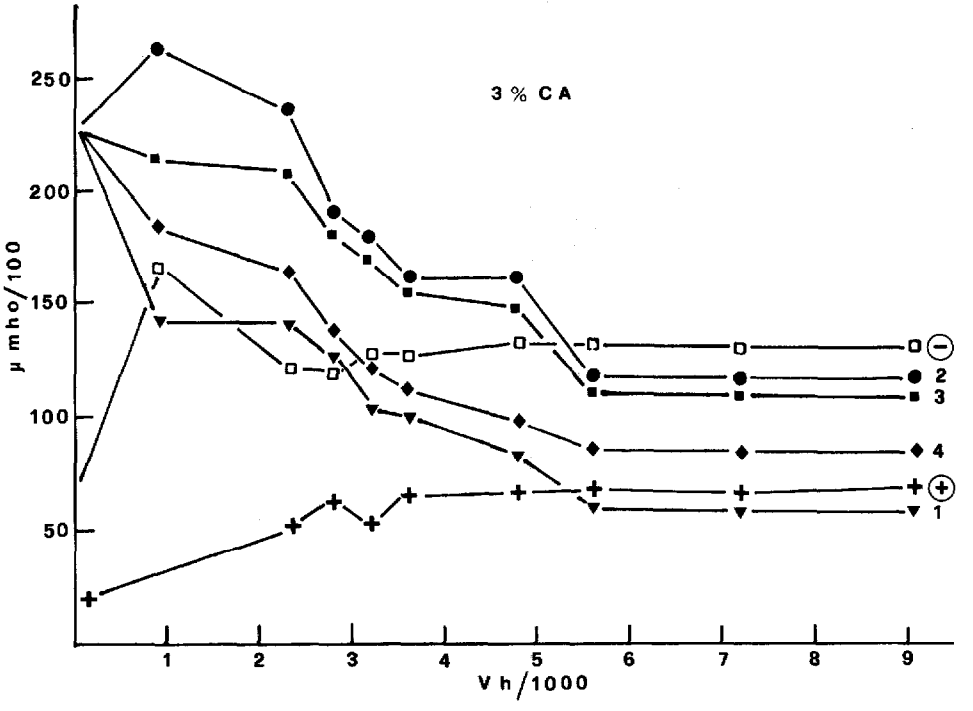

Fig. 3. Conductivity profiles in the multi-compartment electrolyser. All conditions and symbols as in Fig. 2, except that the 2-ml aliquot collected was subjected to conductivity measurements at 25°C with an Orion conductivity meter.

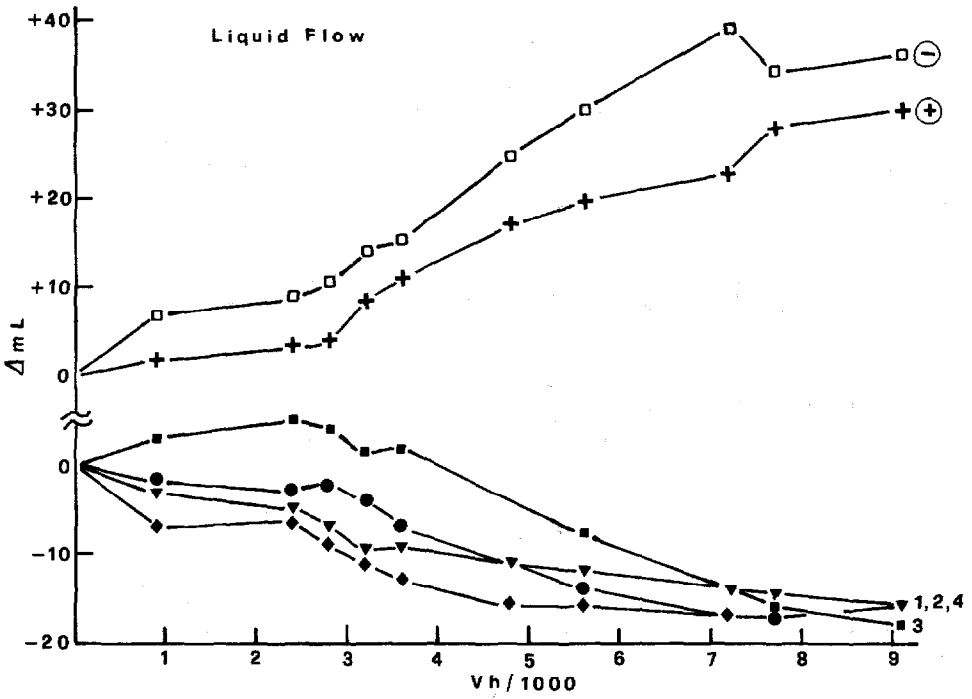

Fig. 4. Liquid flows in the multi-compartment electrolyser. All other conditions and symbols as in Fig. 2, except that here the total liquid level in each reservoir was measured at the times indicated. Note that the flux out of the sample flow chambers is bidirectional, *i.e.*, it occurs simultaneously towards the anode and cathode. The incremental liquid levels (Δml) are plotted, the inital volumes being 250 ml in the electrode reservoirs and 50 ml in each sample flow chamber.

loss from the sample flow chambers is similar in all compartments and it amounts to a total of *ca.* 15 ml over a 4-day period (the initial liquid level in each reservoir being 50 ml). As there is no water evaporation in the system (all liquids are under a layer of paraffin oil), the total fluid balance in all compartments is constant. What happens in the presence of proteins is shown in Fig. 5: here the sample flow chambers contained a pre-focused CA solution to which a total of 235 mg of protein was added. The experiment was continued for 11 days. Under these conditions, water transport towards the anode and cathode becomes negligible, whereas a new phenomenon becomes prevalent, *i.e.*, massive transport of water into chamber 1, with a concomitant loss largely from the adjacent chamber 2 and to a minor extent from chambers 3 and 4. Curiously, chamber 3 contains the highest amount of protein, whereas very little protein accumulates in chamber 1; hence, if this phenomenon were purely osmotic in nature, solvent should gather in chamber 3 and be lost from chamber 1.

Fig. 6A and B gives the corresponding pH and conductivity profiles, respectively, for the long-duration experiment in Fig. 5. The remarkable constancy of these parameters should be appreciated: the 160 000 V h for this experiment correspond to 11 days of continuous operation. The fact that during such a long time period both the pH–conductivity profiles and the protein patterns remained stable suggests that there was no (or minimal) degradation of the chemicals (the neutral acrylamide and

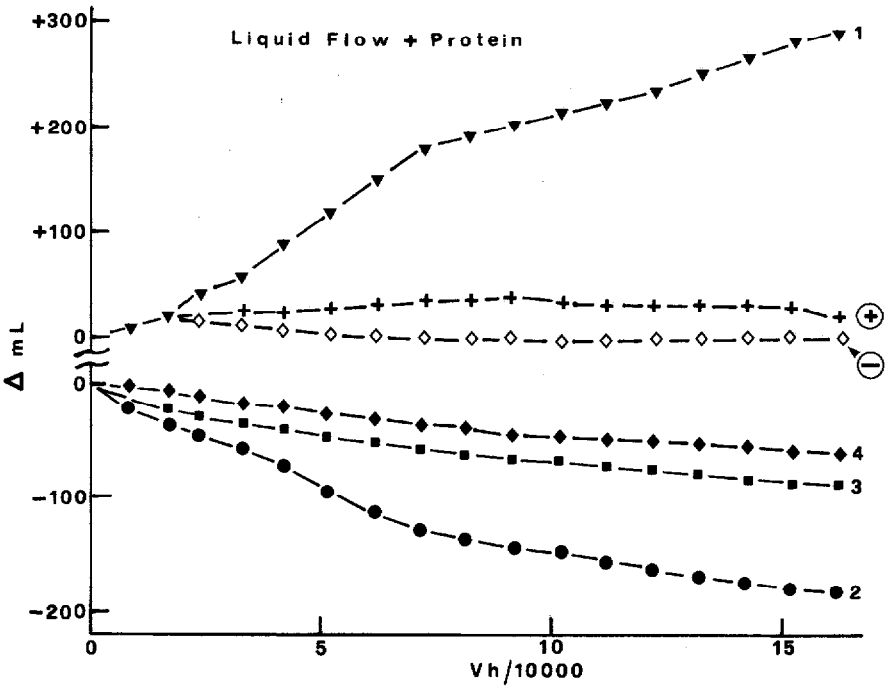

Fig. 5. Liquid flow profile in a protein run. The same experiment as in Fig. 2 was performed, except that the electrolyser was loaded with 235 mg of monoclonal antibodies and the run continued for 160 000 V h (corresponding to 11 days). At the V h values indicated, the incremental (Δml) liquid flows were measured. Note the constancy of volume in the electrode reservoirs and the marked volume increment in chamber 1 at the expenses of the neighbouring chambers (particularly chamber 2).

charged acrylamido–buffer monomers) even at these mildly alkaline pH values (see Discussion).

Purification of human anti-gp41 antibodies

Fig. 7 shows the initial stage of the purification process of human anti-gp41 monoclonal antibodies in the multi-compartment electrolyser. A 235-mg amount was loaded in the four sample chambers (58.5 mg per chamber) of an apparatus in which a pH gradient had been pre-established by focusing Pharmalytes for 10 000 V h. Samples were taken from each chamber after only 2500 V h and analysed in a pH 8.5–10 IPG gel. The left panel shows the starting material with the p*I*s of the six major IgG fractions resolved, whereas in the adjacent set of four tracks are marked the isoelectric points of the five Immobiline membranes used to delimit each flow chamber. As shown, the membrane p*I* values are selected in between the p*I*s of the IgG fractions of interest for the purification process. The progress of purification can be appreciated at a glance: even after such a short focusing time, it is seen that the most anodic chamber (No. 1) is enriched in the most acidic fractions and has already lost the most alkaline species; the opposite phenomenon occurs simultaneously in the most cathodic chamber (No. 4).

Fig. 8 compares the purification achieved in the multi-compartment electrolyser

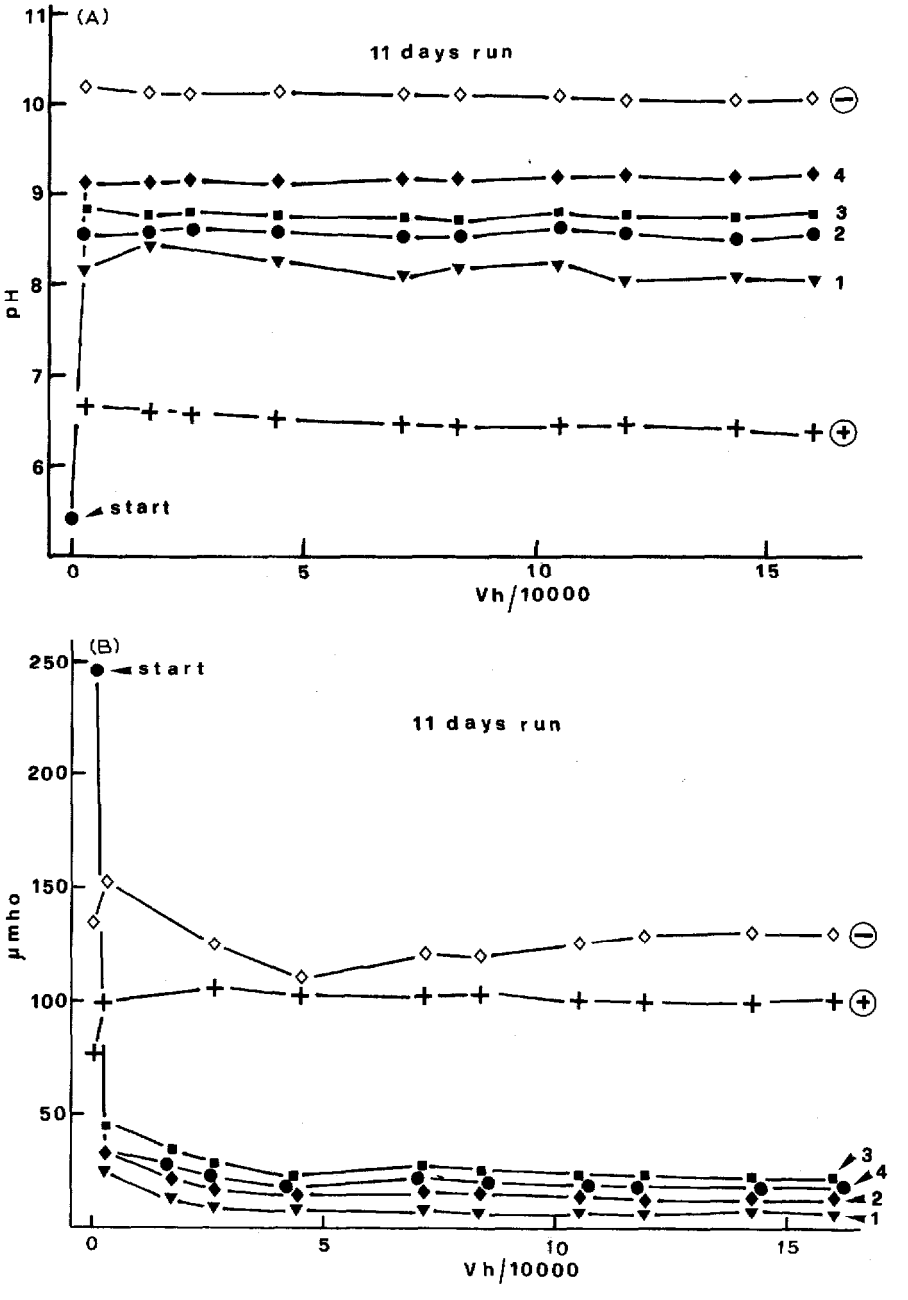

Fig. 6. pH and conductivity profiles in a protein run. The same experiment as in Fig. 2 was performed, except that the electrolyser was loaded with 235 mg of monoclonal antibodies and the run continued for 160 000 V h (corresponding to 11 days). At the V h values indicated, 2-ml aliquots were collected and the pH (A) and conductivity (B) were measured in all chambers at 25°C. Note the remarkable constancy of both values for the entire duration of the run. In A and B "start" refers to the initial pH and conductivity values, respectively, of the protein sample injected into the electrolyser (prefocused through steps 1–3 as described under Experimental).

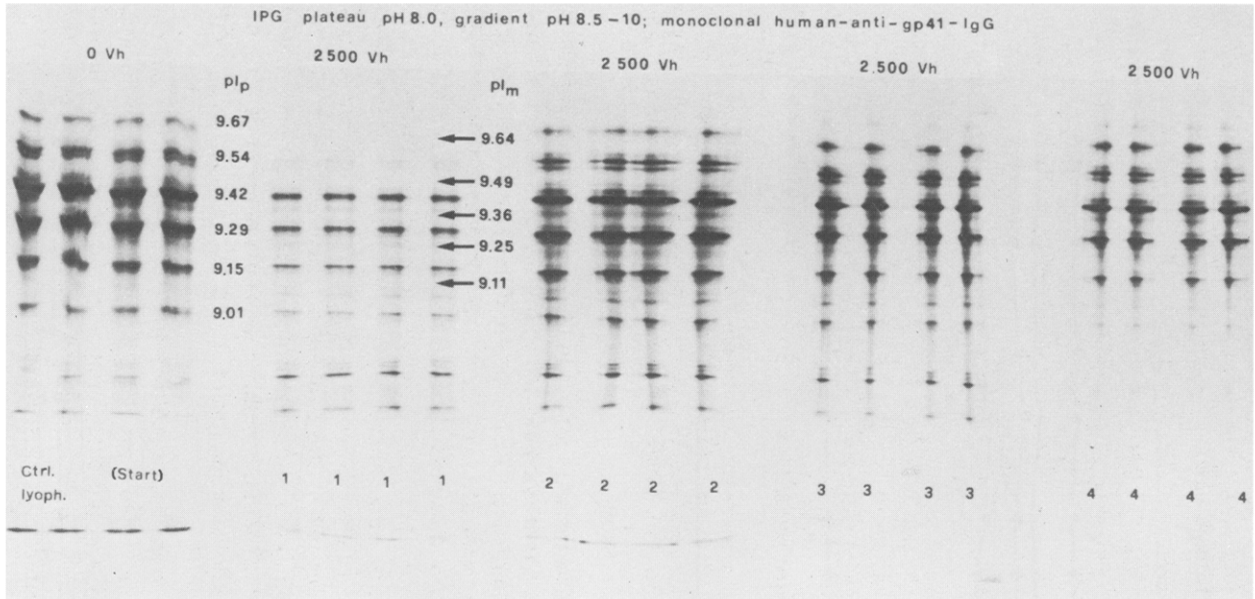

Fig. 7. Initial protein patterns in the purification of monoclonal antibodies. During the experiments in Figs. 5 and 6, at an early stage of purification (2500 V h), small aliquots were collected and 20 μl loaded in an anodic, pH 8 plateau of an analytical IPG gel, in pre-cast pockets. Conditions: 5%T, 2.6%C matrix, pH 8.5–10 IPG gel, run for 30 000 V h. Staining: Coomassie Brilliant Blue R-250 in Cu^{2+}. Total sample load per track: *ca.* 50 μg protein. The panel 0 V h represents the control, unfractionated mixture; the numbers to its right are the isoelectric points of the major protein fractions (pI_p). The numbers to the right of the adjacent panel are the isoelectric points of the membranes delimiting the sample flow chambers (pI_m).

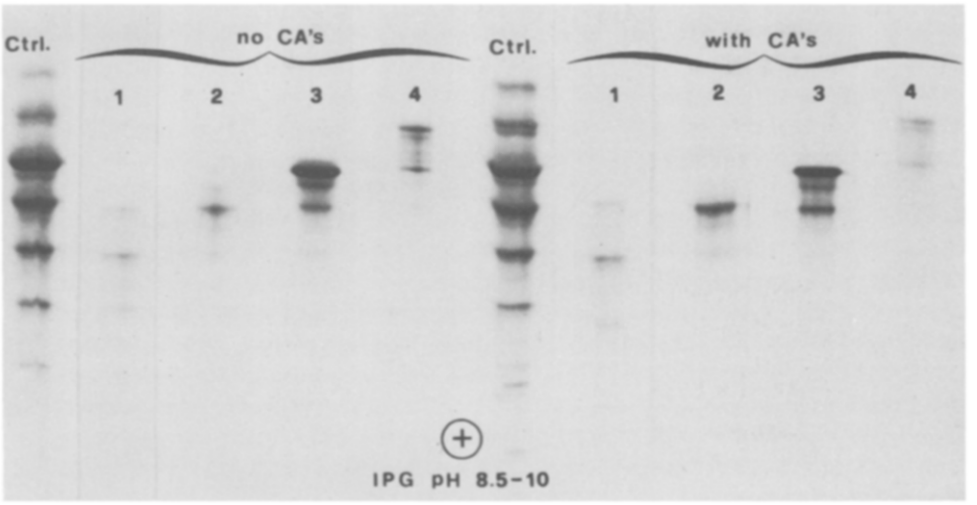

Fig. 8. Progress of purification in the multi-compartment electrolyser in the presence and absence of CAs. Two different preparative runs were made with a load of 235 mg of monoclonal antibodies, either in the presence (right panel) or absence (left panel) of Pharmalytes. As seen, the protein patterns in each sample chamber (1–4) are identical in the two cases. Ctrl.: control, unfractionated starting material. Analytical IPG gel as in Fig. 7.

in presence and absence of added carrier ampholytes in the sample flow chambers. It is seen that, as stated by Righetti *et al.*[10], the presence of soluble CA chemicals is not really necessary for the purification process: the two sets of data are fully comparable. It is clear that, if the protein concentration in the sample flow chambers is high enough (*e.g.*, 10 mg/ml), the macroion can act effectively as a buffering species at its own p*I* value[10]. It was not possible, perhaps owing to the slow movement of the macroions in the proximity of the p*I* value, complicated by the presence of detergent and the incremented viscosity due to glycerol, to obtain a single, homogeneous fraction during a single run. However, when the content of chamber 3 (see Fig. 8), representing the main IgG fraction in our population of monoclonal antibodies, was re-run in a cascade fashion (utilizing the same membranes as in the previous run), we would obtain essentially a homogenous, single species in chamber 3 in the second run (Fig. 9).

One of the main objections to the use of multi-compartment electrolysers is the possibility of membrane fouling, *i.e.*, protein precipitation, unspecific adsorption and trapping inside the membrane. This would completely alter the original electric and flow characteristics of the membrane, *e.g.*, by altering its net charge and clogging the pores. In order to check this possibility, we performed the experiment shown in Fig. 10; at the end of a first purification cycle, three membranes, delimiting chambers 1 and 2 ($M_{1,2}$), chambers 2 and 3 ($M_{2,3}$) and chambers 3 and 4 ($M_{3,4}$) were crushed and allowed to elute in 25% glycerol–1% Nonidet P-40 overnight at 4°C. The membrane eluate (with some pieces of membrane) and the protein content in each chamber were analysed side by side. As is clearly seen in Fig. 10, little if any protein is trapped inside the membranes, even after prolonged focusing times (11 days in this particular experiment).

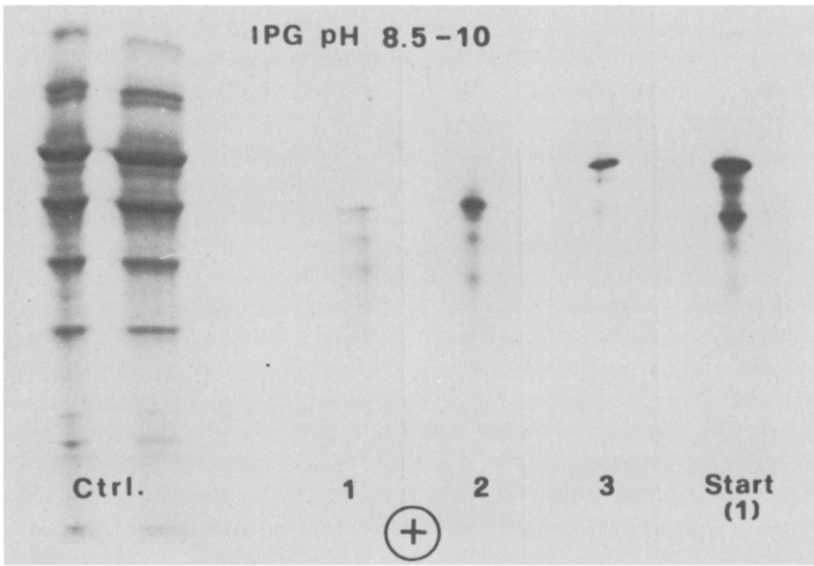

Fig. 9. Cascade preparative run. The content of chamber 3 in Fig. 8 was used as total sample material (50 mg of monoclonals) applied to the first chamber of the electrolyser equipped with only three sample flow chambers and allowed to migrate to equilibrium. All conditions for the analytical gel as in Fig. 7. Ctrl.: control, unfractionated starting material. Start: protein profile of the starting material for this experiment, corresponding to the content of chamber 3 in Fig. 8.

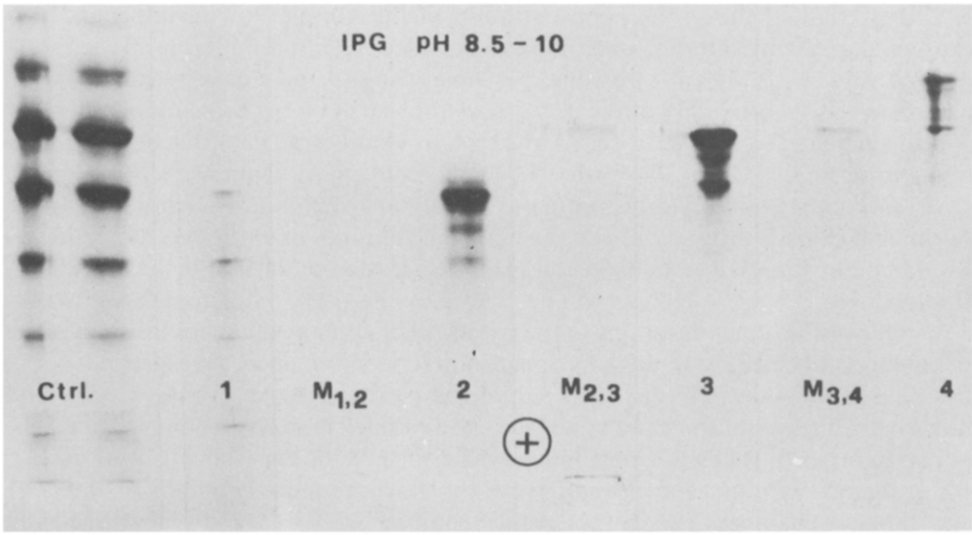

Fig. 10. Control of potential protein trapping in the membranes. At the end of the experiment described in Fig. 8, the membranes dividing chambers 1 and 2 ($M_{1,2}$), chambers 2 and 3 ($M_{2,3}$) and chambers 3 and 4 ($M_{3,4}$) were crushed, eluted in a small volume (1 ml) of 25% glycerol–1% Nonidet P-40 overnight at 4°C and 100 μl supernatant loaded in an analytical gel, side by side with the content of each neighbouring chamber. All conditions for the analytical gel as in Fig. 7. Note that essentially no protein is detectable inside the membranes.

The extreme flexibility of operation of the apparatus is shown in Fig. 11. It might often be necessary not to isolate single isoforms in a biological sample, but just to remove some degradation products present in it. We have recently found (unpublished work) that, on prolonged storage, two undesirable phenomena occur with the monoclonal antobodies: if the sample is stored at 4°C, a strongly alkaline band (p*I* 10.1) develops (right panel in Fig. 11), whereas, if the sample is lyophilized, more acidic forms are produced (left panel in Fig. 11). The nature of both phenomena is not understood and is being investigated. For human consumption, it might not be necessary to inject just a single p*I* band of the monoclonals, but certainly degradation products are undesirable as they could cause side-effects (*e.g.*, be immunogenic and the like). In this last instance, it might not be necessary to achieve such a fine purification as shown in Fig. 9; in fact, with only two membranes (p*I* 9.11 and 9.49) delimiting a single central chamber, it is possible to use the electrolyser to eliminate only the unwanted degradation products (Fig. 11, central track).

DISCUSSION

Isoelectric membranes as a pH-stat

The concept of isoelectric Immobiline membranes is revolutionary and deserves further comment. We should like to introduce here the idea of isoelectric Immobiline

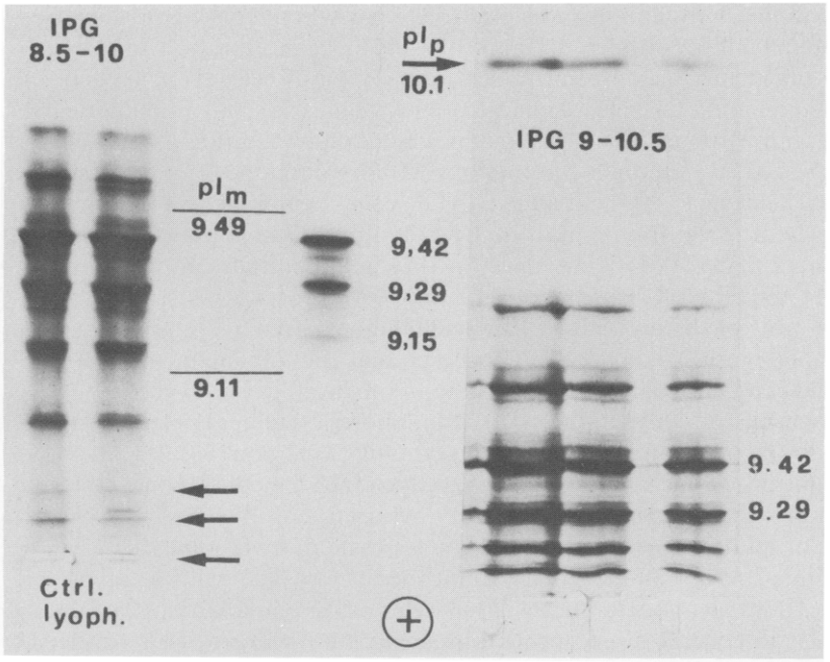

Fig. 11. Purification of total monoclonals from degradation products. In the left-hand gel (IPG, pH 8.5–10) are visible acidic components formed during lyophilization (three arrows). In the right-hand gel (IPG, pH 9–10.5) corresponding degradation products formed on storage at 4°C are seen (p*I* 10.1 band). If the electrolyser is run with only two membranes of p*I* 9.11 and 9.49, all the degradation products are eliminated (central panel). All conditions for the analytical gel as in Fig. 7.

membranes as pH-dictating assemblies in an IEF separation, much like a pH-stat unit is set up for controlling, *e.g.*, the pH during a biochemical reaction or during *in vitro* tissue growth. Each species which is tangential to or crosses such isoelectric membranes is titrated to the pH of the membrane (provided it does not overcome its intrinsic buffering power, β). For amphoteric compounds, this results in a drastic change in mobility, which could reach zero if the two membranes delimiting a single flow chamber have p*I*s just slightly lower (on the anodic side) and slightly higher (on the cathodic side) of a given macroion present therein. Any other amphoteric species with lower or higher p*I* value will be forced to exit from such a chamber either towards the anode or towards the cathode, respectively. Hence it is clear that, with a proper set of membranes, it is possible to define in a given chamber isoelectric conditions for just a given component of a protein mixture, which ultimately will be arrested as the sole isoelectric species in such a chamber. Moreover, if the protein concentration is high enough (at least 10 mg/ml), the macroion present in the liquid stream will possess enough buffering power to control the pH, in the absence of exogenous ions migrating through the system. We have in fact already discussed[10] that, in the case of haemoglobin, such concentration will ensure $\beta = 1$ μequiv. l^{-1} pH^{-1}, which, although low, is adequate to ensure pH control at the steady state.

This is a revolution in the field of membranology. So far, in biochemistry, most membranes have been used with the following selectivity bases: (a) size exclusion (*e.g.*, dialysis sacs, hollow fibres); (b) charge selectivity (*e.g.*, cationic and anionic membranes used in electrodialysis or as electrolyte barriers in continuous-flow electrophoresis equipment according to Hannig[17]) and (c) concentration by osmosis. With the present membranes, we introduce a new type of selectivity, namely the possibility of modulating the surface charge of macroions (or small amphoteric ions as well as ions with weak protolytic groups) by a continuous titration process. Given the highly sophisticated Immobiline technology we have developed (for a review, see ref. 18), we can achieve a very high selectivity, as each membrane can be produced with an accuracy of isoelectric point as high as the third decimal place. We have in fact demonstrated that the resolving power in IPG fractionations can be as high as $\Delta pI = 0.001$ pH unit (in surface charge difference between two just resolved proteins)[19] and in principle this resolution can be maintained even in a preparative run.

There is one more striking feature of the present Immobiline membrane technology, *viz.*, the capability of acting as "salting-in" milieus. It is known that one of the major drawbacks of conventional IEF in amphoteric buffers is protein precipitation at and in the proximity of the isoelectric point of macromolecules. Owing to the very small intrinsic ionic strength of focused CAs, such a medium behaves, for all practical purposes, like a "salting-out" milieu, favouring precipitation of isoelectric macroions out of solution. In contrast, inside an Immobiline membrane, there will be a fairly high ionic strength (in general > 10 mequiv. l^{-1}) which will favour protein solubilization. Thus, such membranes will have the unique capability of preventing fouling, which is in general due to protein precipitation (or adsorption) inside the membrane network. Isoelectric precipitation, if at all, will thus occur outside, never inside our Immobiline membranes. Consistent with this hypothesis is the fact that indeed no protein can be found trapped inside the membranes (see Fig. 10) and that, in conventional preparative runs in IPG gels, the IPG matrix is found to have solubilizing properties towards proteins focusing in it[20].

Long-term membrane operation

The membranes described here, in addition to the property of not adsorbing or trapping proteins, appear to be compatible with long-term use. As shown in Fig. 6A and B, the membranes used in the protein fractionation experiment guarantee stable pH and conductivity profiles for 11 days (corresponding to 160 000 V h). Considering that the electrolyser had been operating with the same membranes for 4 days prior to adding the protein sample, this amounts to a total of 15 days of continuous operation. After this period, on dismantling the apparatus, the membranes were inspected under a microscope and found to be still integral (*i.e.*, still firmly bound to the glass filter, unperforated and minimally or not swollen). Such a membrane stability is remarkable, considering that the separations are conducted at alkaline pH (pH 9.0–9.8) and that polyacrylamide (as well as Immobilines) are known to be prone to hydrolysis of the amido bond in basic media[21]. For long-term storage of pre-cast membranes, however, it would be advisable to keep them equilibrated in dilute acetate buffer at pH 5.0; this is the pH of maximum stability for both neutral and charged monomers[21]. For additional data on membrane preparation and handling, see ref. 10.

Liquid flows

The phenomena of liquid transport in and out of the different chambers are not fully understood. Judging from Fig. 5, it would appear that, in presence of CAs, there is a two-directional water transport to both the anode and cathode (substantially greater in the latter compartment), compatible with an electrosmotic phenomenon. For this to occur, the liquid should be transported out of the central chambers as water of hydration of the carrier ampholytes. The latter are in fact known to participate in the pH gradient decay and electrosmotic pump in IEF[16]. This phenomenon is clearly prevalent during the transient state, but as the steady state is reached, the constancy of pH and conductivity in each chamber (see Fig. 6A and B) should require constant molarities of CAs in the same vessels, which conflicts with the notion of transport of CAs out of the different chambers. The other possibility is that the CA:solvent ratio in the electrosmotically transported liquid is the same as in the reservoirs, so that the CA molarity in the latter vessels remains constant. As another alternative, the amount of CAs transported out could be so minute as not to alter the pH and conductivity of the solutions remaining in the reservoirs. By definition, the isoionic point of an ampholyte is that pH value which does not change on addition (or subtraction) of more ampholyte. Superimposed to this, there is another phenomenon that occurs in presence of proteins: as protein accumulates in a chamber, water is "pumped" out and collects in the chambers having the least (or no) protein (see the large increment in water in chamber 1 and loss from chamber 3 in Fig. 5). This "inverse osmosis" effect is puzzling and not readily understood. Although both phenomena are currently under study, they do not seem to represent a serious threat to the technique; we are building a semi-automated version with level sensors in each reservoir, so that the chambers can be replenished in case of liquid loss. In fact, in some instances, this phenomenon could be beneficial as it would result in protein concentration in some of the chambers of the electrolyser.

Seeding the electrolyser with pure protein

This is an interesting concept that could deserve further exploitation. We have seen in Fig. 8 that there is no difference in the final purified product between experiments run in the presence or in the absence of carrier ampholytes. Hence, in principle, it would be highly preferable to run the electrolyser in the absence of CAs, especially because the FDA would forbid human consumption of recombinant DNA proteins if contaminated by CAs. Moreover, CAs seem to induce a strong electrosmotic pump (see Fig. 5). However, in practice, at least during the transient state, with salt-rich protein samples or with highly dilute proteins, CAs are needed for controlling the pH in the liquid stream of the flow chambers, where the Immobiline membranes cannot extend their buffering action. How can we solve this dilemma? With repeated use of the instrument for purification of the same protein, one could save the protein fractions purified in a previous run, and use the content of each chamber to seed the chambers for subsequent purification steps. The purified protein sample will act as a buffering macroion in the liquid stream.

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