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TWO-DIMENSIONAL MICRO-SEPARATION TECHNIQUE FOR PROTEINS AND PEPTIDES, COMBINING ISOELECTRIC FOCUSING AND GEL GRADIENT ELECTROPHORESIS*

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SUMMARY

The two-dimensional combination of gel electrofocusing and gel gradient electrophoresis yields separate information about differences in net charge and molecular weight of macro-ions. A micro-version of this technique is described. The first-dimension electrofocusing run is performed in cylindrical polyacrylamide gel of capillary size, using xylene cyanole FF as a reliable marker dye to indicate the final separation stage when proteins reach their isoelectric point. In the second-dimension run the focused proteins are separated in a continuous slab gel gradient of less than stamp size with total acrylamide concentration ranging from *ca.* 1% to more than 40%. Spreading of peaks in the gradient slab gel is shown to be related to the approach of protein to its pore limit in the gel. This spreading is a useful indicator of the final position of a protein in the gel if molecular weights are estimated according to the separation pattern in the gradient gel. The method described has been developed for the separation of proteins extracted from large single cells (*i.e.*, neurons of the mollusc *Aplysia californica*).

INTRODUCTION

Two-dimensional mapping techniques which employ different types of electrophoresis in polyacrylamide gel are considered to be the most powerful tools for the separation of macro-ions, *i.e.*, proteins, peptides, and nucleic acids. For the first-dimension run of this mapping technique, gel electrophoresis in cylindrical gels with a constant and low acrylamide concentration was employed. The second-dimension run was performed in polyacrylamide slabs of constant but higher acrylamide concentration. Additionally, buffer systems of different pH were used in both dimensions[1].

In order to achieve separation solely dependent on different charge properties

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of protein species, gel electrofocusing was adopted for the first-dimension run[2,3]. For the second dimension addition of the ionic detergent SDS has been recommended to obtain more reliable information about molecular weights of separated proteins[4]. Isoelectric focusing and gel gradient electrophoresis, which separates primarily according to molecular weight, were first combined in a two-dimensional technique by Kenrick and Margolis[5]. While avoiding the use of denaturing detergent the latter approach seems to be the method of choice if electrophoretic properties of macro-ions are investigated. An attempt has been made to scale this method down, so that proteins extracted from single cells can be traced by their optical density patterns.

MATERIALS

Acrylamide, N,N′-methylene-bisacrylamide (bisacrylamide), and N,N,N′,N′-tetramethylethylenediamine (Temed) were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.); ammonium persulfate (persulfate), tris(hydroxymethylaminomethane) (Tris), bromophenol blue, methyl green and xylene cyanole FF were purchased from Fisher Scientific (Fair Lawn, N.Y., U.S.A.); carrier ampholytes pH 3.5–10, from LKB (Bromma, Sweden) and carrier ampholytes pH 2–11 from Serva (Heidelberg, G.F.R.), were used; human serum albumin was also obtained from Serva; pepsin and bovine serum albumin were from Pentex (Kankakee, Ill., U.S.A.); Coomassie brilliant blue R, chymotrypsinogen A, cytochrome *c*, ferredoxin, ferritin, lactate dehydrogenase from rabbit muscle and from beef heart (EC 1.1.1.27), β-lactoglobulin, hemoglobin, ovalbumin, transferrin, glycine, β-nicotinamide adenine dinucleotide, nitro blue tetrazolium, phenazine methosulfate, and DL-lactic acid (sodium salt) were from Sigma (St. Louis, Mo., U.S.A.).

Glass capillaries (5 μl) were purchased from Brand (Wertheim, G.F.R.) and Drummond (Broomall, Pa., U.S.A.). Disposable sampling pipets (10 μl) (Corning, Corning, N.Y., U.S.A.) were also used; they have to be cut into pieces *ca.* 3 cm long and have to be cleaned with acetone.

For densitometry, a double-beam microdensitometer (Joyce Loebl, Gateshead-on-Tyne, Great Britain) was used.

Aplysia californica was purchased from Pacific Bio-Marine (Venice, Calif., U.S.A.).

METHODS

Isoelectric focusing

If not stated otherwise, polyacrylamide gels of T 5% and C 5%* were polymerized in 5-μl capillaries. The gel mixture was made of stock solutions, which were kept in the refrigerator, as follows: 20 μl of an acrylamide + bisacrylamide stock solution (T 50%, C 5%), 5 μl Temed stock solution (0.02 ml Temed made up to 1 ml with water), 20 μl persulfate stock solution (2.5 mg/ml), 15 μl carrier ampholytes 40% (to a final concentration of 3% in the gel), and 140 μl water.

As carrier ampholytes either Ampholine® pH 3.5–10 (LKB) or Servalyt®

* T = Comonomer concentration (acrylamide and bisacrylamide) in %(w/v) C = per cent of T which is bisacrylamide.

pH 2–11 (Serva) were used. The gel solution must polymerize at room temperature between 15 and 30 min after mixing. If necessary, corrections should be made by varying the persulfate concentration. The capillaries were dipped into the gel mixture and filled up to *ca.* four-fifths of their length by capillary force. For polymerization of the gel the filled capillaries are stored in an upright position in a vessel whose bottom has been covered with 60% sucrose solution. In a moist chamber the gels can be stored for several days in the refrigerator. Old gels show considerable loss of carrier ampholytes into the hold solution. Gels were only used after at least 12-h storage at room temperature. Such aging is required to reduce remaining oxygen radicals after polymerization.

The sample is mixed with sucrose or glycerol (to 15% final concentration) and is applied directly on top of the gel without removing the liquid supernatant that remains after polymerization of the gel. The supernatant contains carrier ampholytes which are mixed with the sample, thus providing some buffering capacity. If relatively large sample volumes are applied (*i.e.*, 2 μl and more), some additional carrier ampholyte should be added to the sample to a final concentration of about 0.1%. Loss of sample protein due to contamination with terminating electrolyte is thus decreased. Marker dye is added to the sample according to the chosen polarity of the run. If the anode is in the top position, methyl green is added. With the cathode in the top position, xylene cyanole or a mixture of bromophenol blue and xylene cyanole is added to the sample. Methyl green has the disadvantage of losing its color when approaching its isoelectric point. Rather xylene cyanole can be injected into the lower end of the gel if the anode is in the top position. The counter-migration of both marker dyes will take place without recognizable interference among the dyes itself or with proteins of the sample. Such an interaction between an acidic dye and a basic protein was observed when bromophenol blue was counter-migrating, resulting in an unstable precipitation peak in the middle of the gel.

Use of a mixture of bromophenol blue and xylene cyanole as anionic marker dyes offers a further advantage. The faster migrating bromophenol blue changes its color to yellow-brown and precipitates at its isoelectric point of pH 3.2 (ref. 6). At this stage of the run the voltage may be raised for better separation of protein peaks. When xylene cyanole has reached its isoelectric point at the precipitate of bromophenol blue, the run may be stopped or continued for some minutes, if sieving of large proteins must be overcome.

As terminating electrolytes 0.5 *N* NaOH at the cathode and 0.5 *N* H_2SO_4 at the anode were used. The arrangement for electrofocusing is shown in Fig. 1. The electrofocusing runs were performed at 70 V/cm. When very large sample volumes were applied, the electric field strength was raised to 100 V/cm. The runs take about 20 min, which is time enough to keep up with the following steps of the staining procedure.

The gels are pushed out of the capillaries with a fitting steel wire or by water pressure from a syringe. The wire will destroy a segment of the gel and the damaged end can be used for identification of the polarity. Pushing the gel out by water pressure rarely damages the gel if performed carefully, but the identification of polarity may be difficult in such gels. After removal from the capillary the electrofocusing gel is washed for 15 min at room temperature in 10% trichloroacetic acid (TCA) (ethanol up to *ca.* 33% may be added). Subsequently the gel is stained in Coomassie blue R

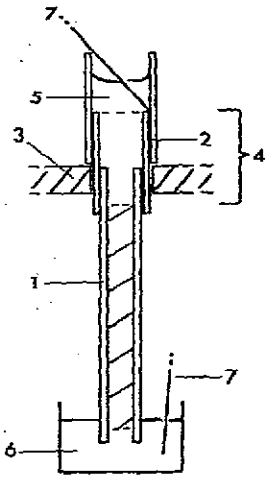


Fig. 1. Outfit for micro-electrofocusing. A glass capillary containing the gel (1) is fitted with two pieces of flexible tubing about 8 mm long (2) and is attached to a support (3). The maximum sample volume to be applied is defined by the length of the indicated segment (4). The sample is overlaid by the terminating electrolyte (5), and the lower end of the gel is immersed in the terminating electrolyte of the opposite type (6). Platinum wire electrodes (7) are introduced so that gas bubbles produced by electrolysis cannot clog the system.

(*ca.* 0.1% in 7% acetic acid and 33% ethanol) for 5 min at room temperature. For destaining 7% acetic acid and *ca.* 20% ethanol are used. The stained gels are stored in 7% acetic acid that contains a small amount of Coomassie blue.

Recording of capillary gels should be performed as soon as possible, either by photography or by densitometry (*i.e.*, with a Joyce Loebl microdensitometer). Large-pore gels as used for isoelectric focusing tend to pick up dye and dust particles very quickly, which can only be partially removed by washing the gel in absolute methanol for a few seconds.

Electrofocusing gels which were to be transferred to the second-dimension electrophoresis are neither washed in TCA nor stained but are placed immediately on top of the slab gel after having been rinsed for a moment in water.

Small-size slab gradient gels

Slab gradient gels are cast in chambers constructed of two pieces of slide glass. Double layers of cover slips are used as spacers. The chamber is kept together by pressure from a clothespin. The second opening of the clothspin is used to hold the mounted chamber on a rack (Fig. 2). Prior to use, the chamber is washed with water, methanol, and finally with acetone. Prior to filling of the chamber 10 μl of 60% sucrose solution may be poured into the lower end of the chamber. This drop of high-density liquid will produce a perfectly horizontal gel edge when overlayed with gel solution. For mixing of the gradient, two gel solutions are made from four stock solutions, three of which are identical to those given above for the electrofocusing gel. The gel mixture for small-size slab gradient gels (T max. 40%, C 5%) is as follows:

Solution 2 (first sucked into the pipet): 100 μl acrylamide + bisacrylamide stock solution (T 50%, C 5%), 10 μl Temed stock solution (0.02 ml Temed made up

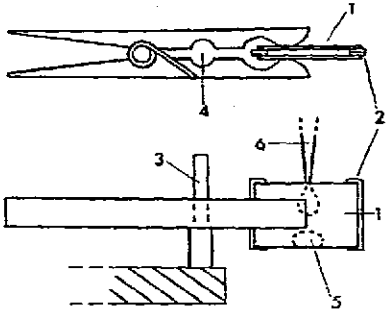


Fig. 2. Small-size slab gradient gels are cast in a chamber (1) made of two pieces of slide glass, which are held apart by spacers (2). Double-layered pieces of cover slips (for instance, thickness No. 2, Fisher Scientific) can be used as spacers, providing a distance of *ca.* 0.5 mm, which is sufficient to apply the cylindrical gel of the first-dimension run on top of the slab gel. The gel chamber is held together by a clothespin with a second orifice (4) by which the chamber can be mounted on a rack (3). Prior to filling the chamber with the gel solution in the tip of the pipet(6), a sucrose solution (10 μl, 60%) is placed in the chamber to seal its lower end (5).

to 1 ml with water), 10 μl gel buffer (3.3 *M* Tris–SO_4, pH 8.4), and 5 μl bromophenol blue solution (0.2% in water).

Solution 1: 100 μl persulfate stock solution (2.5 mg/ml in water), 100 μl water, and 10 μl sucrose solution (50% in water) (gel buffer may be added).

The acrylamide, Temed, and persulfate stock solutions are identical to those listed for electrofocusing. Gradient gels of lower maximum concentration can be produced by diluting the acrylamide solution. Any type of buffer may substitute the one given above. The acrylamide solution may be filtered prior to use to provide a cleaner background of the small-size slab gels.

In the discontinuous buffer system of pH 8.4 50 m*M* Tris–glycine pH 8.4 is used as running buffer.

Solution 1 contains persulfate and some sucrose, while solution 2 contains acrylamide, bisacrylamide, Temed, buffer, marker dye, and sucrose. The gradient is mixed by use of an automatic pipet (*i.e.*, Eppendorf). For filling of a chamber of the described dimensions, a 100-μl pipet is suitable. Different pipets work as well to fill chambers of other sizes and shapes, such as cylindrical tubes. First, about half of the pipet volume is filled with solution 2. Then the pipet is moved to solution 1, where the finger on the top button of the pipet is released quickly. Thus, solution 1 is forced to enter abruptly into the pipet. Due to its lower density, solution 1 passes through the volume of solution 2 and settles on top of solution 2. Due to the speed of suction, turbulences occur, mixing both solutions to an extent which creates a density gradient settling in the pipet tip. Therefore, the pipet must be held in vertical position for some 10 sec until a drop starts to develop at the orifice. The marker dye in solution 2 allows to control the development of the gradient in the pipet, as well as in the chamber. The content of the pipet is pushed out slowly into the upper slit of the gel chamber, as shown in Fig. 2. Only the last 5 μl are retained in the pipet because they contain a gel solution of higher density that comes from the inner surface of the pipet tip. This residual drop is mixed with water or with persulfate solution up to 100 μl and is used to overlay the gradient solution in the chamber. A chamber of larger volume

should be cooled prior to filling in order to delay polymerization of the gel. The filled chamber is left in a horizontal position on the rack at room temperature for 15 min until polymerization has started. This can be easily recognized by the appearance of a diffracting interface near the lower end of the chamber. The slope of such gel slabs has been tested by incorporation of dye into gel solution 2 and by densitometry of the polymerized gel (Figs. 3a and b).

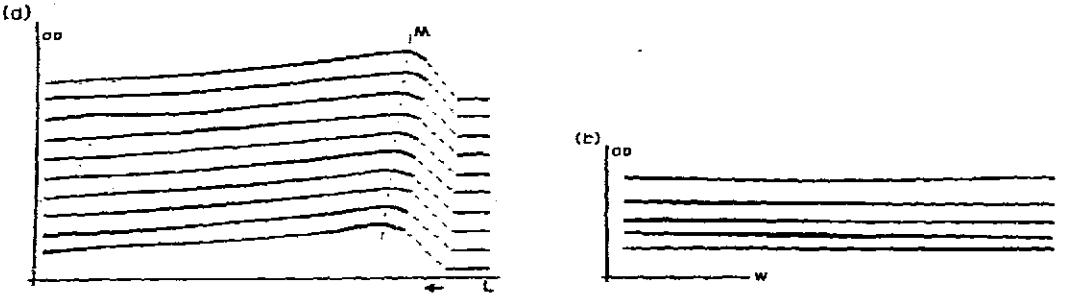


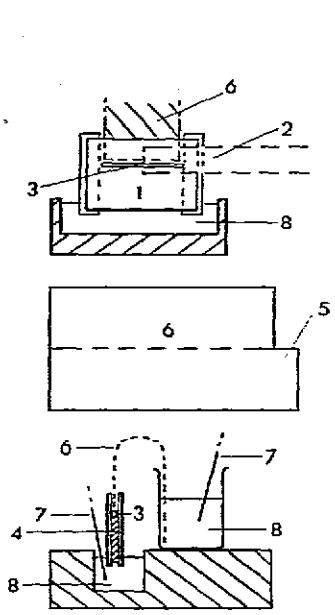
Fig. 3. (a) Optical density (OD) tracings of bromophenol blue distribution within the length (L) of a small-size slab gradient gel. The dye has been incorporated into the acrylamide solution only, thus representing the concentration gradient of the gel. The slab has been scanned in the gel chamber to avoid loss of dye by diffusion. Parallel scans were performed in the direction indicated (←) at a distance of 2 mm over the width of the slab gel, as indicated by the scan segments at the right-hand side. Irregular spikes caused by entry of the scanning beam into the gel chamber have been replaced by dashed connecting lines. The axis of maximum gel concentration is tilted, as indicated by the dashed line (M). (b) Optical density (OD) tracings of bromophenol blue distribution within the width (W) of a small slab gradient gel. The scanning conditions are the same as in (a). Parallel scans have been performed at a distance of 1 mm in the gel, thus the increasing distances of the scans reveal the exponential character of the gradient.

To enhance polymerization of low-concentrated acrylamide solution at the top of the gradient, the chamber may now be transferred for about 10 min into a 40° oven. The gel can be stored for about two days if it is kept in a covered vessel at 4°. The lower end of the gel chamber has to be immersed in gel buffer. In aged gels, clefts may develop between the glass surface and the gel which cause a bypass of current.

Before applying the first-dimension gel on top of the slab gel, the gel chamber must be totally filled with aqueous sucrose solution (*ca.* 50%) and some marker dye. The supernatant should be mixed slightly by turning the gel chamber several times. Then the cylindrical electrofocusing gel is placed on the upper slit of the gel chamber and pushed into the chamber with a thin tool (*i.e.*, forceps, steel wire).

Once immersed in the supernatant, the gel can be fully pushed down on top of the gel slab with the short edge of the filter paper, which is used as a buffer bridge (Fig. 4). With some experience, the gel transfer will take no more than 20 sec. Thus, severe losses due to diffusion of protein into the supernatant can be avoided. The area of high sucrose concentration on top of the gel slab provides an area of reduced conductivity in which the elution of proteins from the electrofocusing gel proceeds very quickly after voltage has been applied.

At 100 V/cm the running time in the second dimension will be about 30 min. At this stage the bromophenol blue marker has left the gel, while the xylene cyanole is still visible in the gel as a sharp line.



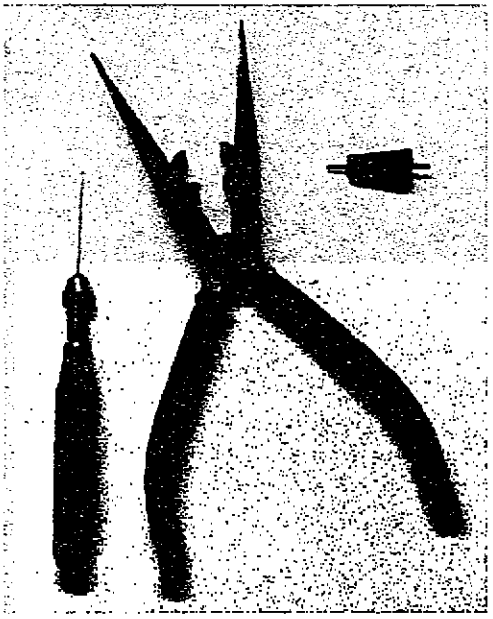


Fig. 4. Arrangement for the second-dimension electrophoresis in a slab gradient gel. The gel chamber (1) is mounted on a rack by a clothespin (2), as indicated in Fig. 2. The cylindrical gel of the first-dimension run (3) has been pushed down on top of the slab gel (4) and is held in place by the lid (5) of the filter paper that is used as a buffer bridge (6). Platinum wire electrodes (7) are dipped into the running buffer (8). The level of the running buffer in the beaker should not exceed the level of the upper edge of the gel chamber. The lower reservoir vessel is made by drilling a series of connected holes in a block of plexiglass.

Fig. 5. Extrusion of very steep, cylindrical gradient gels from capillaries can be reliably performed with a steel wire that is fixed into a holder (left) and with pliers that have been fitted with the corresponding halves of a brass tube of conic lumen (center). The capillary is held very tightly in the slit of a semi-split rubber stopper (right) by the pliers. The stopper fits into the orifice of the pliers. Since high pressure may be required, the wire must be used first at a length of 1 cm and then increased in length during the process.

If small peptides are under investigation, the run may be stopped at this stage. The approach of proteins to their pore limit will need at least twice the time. The gel chamber is carefully taken apart, and the gel is stained with Coomassie blue as mentioned above.

Steep cylindrical gradient gels

Instead of slab gels, in some instances very steep cylindrical gradient gels of capillary size have been used for the second-dimension run. They are produced by capillary force as previously described[7].

In order to obtain a T maximum value of 60% some changes in the general procedure have to be made: A stock solution containing acrylamide and bisacrylamide (T 71%, C 4%) is used. The required amount of bisacrylamide (3% w/v) will not dissolve in water separately, but will dissolve readily in the presence of acrylamide. This stock solution crystallizes at room temperature and has to be heated up to more

than 40° prior to use. All other stock solutions are identical to those given above. The two gel solutions for gradients with T max. 60% and C 4% are:

Solution 1: 30 μl persulfate stock solution, 10 μl buffer, and 60 μl water.

Solution 2: 85 μl acrylamide + bisacrylamide stock solution, 10 μl buffer, and 5 μl Temed stock solution.

Cleaned capillaries with strong glass walls and undamaged ends are first filled halfway with gel solution 1 and subsequently filled totally with gel solution 2; thus a gradient is created. After polymerization of the gel, the filled capillaries have to be stored for several days, being kept half immersed in gel buffer at 4°. Only after such aging can the gel be pushed out of the capillary without damage. Aged gels still sit very tightly in the capillary. Therefore, a tool to hold the capillary is helpful and prevents accidents (Fig. 5). The steel wire to push out the gel has to be fitted with a holder, and be used first at a short length to avoid bending of the wire and thus breaking of the capillary and gel (Fig. 5). Staining of such gradient gels takes much longer due to the restricted diffusion of dye into the small pore gel.

RESULTS

Isoelectric focusing

Isoelectric focusing has become a widely used analytical and preparative tool and there has been much development of the original technique[8,9]. Isoelectric focusing as a micro gel technique has been described by Quentin and Neuhoff[10], Gainer[11], and Grossbach[12]. To improve the results obtained by their methods, different variations have been tried, *i.e.*, carrier ampholytes may substitute Temed as the catalyst in the gel mixture[8]. Since considerably higher amounts of persulfate have to be used if Temed is omitted, the possibility of oxidation of sample proteins is increased. For the same reason, sample proteins should not be polymerized into the gel which has been recommended[12]. Gels were not overlayed with water or ampholyte solution prior to or after polymerization nor was the sample overlayed with ampholyte solution. The latter prevents contamination of the sample with terminating electrolyte, but it also causes an expansion of the pH gradient into the upper electrolyte reservoir. This may account for the problems with the choice of polarity which have been communicated[10,11]. Electrophoretic pre-runs intended to rid the gels of ionic impurities did not improve the separation. Coating of capillaries, with either Column Coat (Dow Chem., Midland, Mich., U.S.A.) or methyl cellulose, as recommended elsewhere[11,13], did not improve the results, indicating that electroendosmosis did not hamper the separation of proteins. Addition of sucrose, glycerol, or ethylene glycol to the gel mixture has been claimed to improve the separation of proteins (especially lipoproteins) in isoelectric focusing[14–16]. Incorporation of either 10% sucrose, glycerol, or ethylene glycol did not improve the separation of proteins investigated.

As often stated, contamination of the sample with terminating electrolyte must be avoided, for proteins may be altered by such contact. Since electrofocusing in its initial stage can be compared with an electrophoresis, contamination of the sample with high-mobility ions of the terminating electrolyte will raise the conductivity, and therefore interfere with the formation of a defined starting zone and lead to protein loss. When large sample volumes had to be applied, I tried to abolish the terminating electrolyte at the upper end of the capillary by immersing the electrode directly into

the sample that was made up to 0.2% with ampholytes. This caused a certain shift of the pH gradient to the upper electrode, but no alteration of the separation pattern was observed, which is in accordance with findings of other authors[17,18]. Thus the problem of sample contamination and related protein loss can possibly be avoided. Xylene cyanole has been used previously in sodium dodecyl sulfate (SDS) electrophoresis as a second anionic marker dye in addition to bromophenol blue[19]; it focuses at almost the same spot in the pH gradient, as bromophenol blue does. While xylene cyanole is migrating more slowly than bromophenol blue, the focusing of its peak indicates much better the moment when invisible proteins are focused in the gel. Even with chromoproteins like cytochrome *c*, ferritin, or hemoglobin, one has to wait additional time after focusing of such markers before focusing of all proteins is completed[8]. This is getting crucial if running conditions are changed more often or if the applied voltage is raised at the end of the run in order to obtain additional sharpening of the focused peaks. Xylene cyanole and proteins have always focused at the same time, provided that there was no considerable sieving of protein in the gel. Molecular sieving of macro-ions is a feature of the supporting polyacrylamide gel matrix that may interfere with the isoelectric focusing, as has been stated by various authors[6,8,9].

The electrofocusing of horse ferritin and its dimer in T 5%, C 5% gels provides an example of such an incompatibility, where the molecular size of one species (the dimer) is too large for the pore size of the gel. Tailing and precipitation of protein in wrong places of the gel are to be observed under such conditions. To reduce sieving, either concentration T or C has to be lowered. When only peptides are to be focused, the gel concentration may be raised to T 7% or more. Provided that there is no interfering sieving, such gel with smaller pore size reduces diffusion and thus enhances the separation power of the technique.

From a theoretical point of view, the polarity in an electrofocusing system should not influence the separation of proteins, but preference to one or the other position has been expressed without further explanation[10,11]. The choice of polarity has to be considered in relationship to charge properties of the proteins that are to be separated, for there is a more or less expressed shift of the pH gradient in the capillary to one side or the other, depending on the chosen polarity. Placing the anode at the top will improve the development of the pH gradient at its basic side in the lower end of the gel and vice versa. Thus, focusing of very basic proteins should be performed with the anode in the top position, while focusing of very acidic proteins will be improved with the cathode at the top (Fig. 6). The dependence of focusing properties upon the polarity is related to the fact that proteins are focusing better when applied at the far distant end of the gel, providing maximum mobility of the protein at the beginning of the run[20]. The shift of the pH gradient may be related to the ratio of volumes of both terminating electrolytes, to the loss of ampholytes into the hold solution during storage of gel, and it may be due to extension of the pH gradient into the sample segment at the top, if large sample volumes are applied. The polarity-dependent shift of the pH gradient does not seem to be identical with the "cathodic shift" or "plateau phenomenon" which has been described by various authors, and which occurred at prolonged running times and at lower ampholyte concentrations independently of the chosen polarity[9,21].

A major problem of isoelectric focusing is the interference of carrier ampho-

lytes with the staining procedure for proteins. Removal of ampholytes prior to staining is performed in the presence of TCA. It has long been known that TCA can render certain proteins soluble in alcohol[22]. This may be the reason of protein loss in the staining process, which has not yet been totally overcome. For a small gel with a relatively large surface, and thus good diffusion properties, the TCA treatment should not be performed at elevated temperatures, which has been recommended[8,23], since the timing of such treatment is critical with respect to the protein loss. The TCA

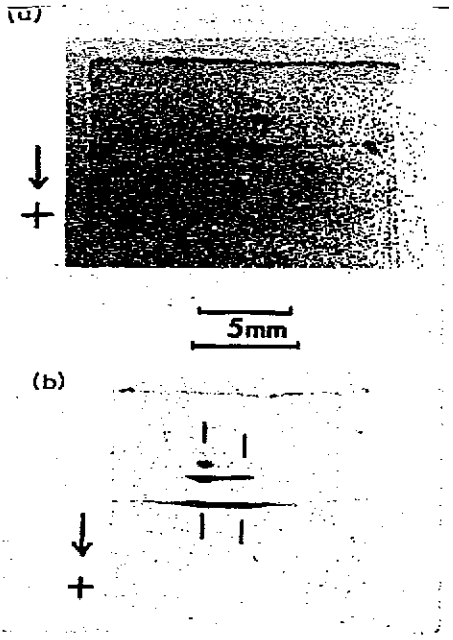


Fig. 6. Isoelectric focusing of standard proteins in a large pore gel (T 5%, C 5%) of capillary size, after Coomassie blue staining. The runs were performed at 150 V in the presence of 3% carrier ampholytes (pH 2–11). 1 = Cytochrome *c*; 2 = chymotrypsinogen A; 3 = hemoglobin; 4 = transferrin; 5 = β-lactoglobulin; 6 = ovalbumin; 7 = ferredoxin; 8 = pepsin. The pH gradient has shifted depending on polarity, allowing separation of basic proteins, preferentially when the sample has been applied at the anode (left), and separation of acidic proteins if the sample has been applied at the cathode (right). To allow comparison, the gels have been adjusted to the position of hemoglobin, which indicates the neutral range of the pH gradient. The shift of the pH gradient is apparent from early stages of the electrofocusing run and is enhanced if large sample volumes are applied, causing extension and thus shift of the pH gradient to the top.

Fig. 7. (a) Two-dimensional electrophoresis of human serum albumin (HSA) after Coomassie blue staining. Following isoelectric focusing, the HSA was eluted into a continuous slab gradient gel (T max. 40%, C 5%) by means of the discontinuous buffer system of pH 8.4. The pattern of HSA oligomers reveals a vertical line of discs, typical for size-isomeric particles that have focused in the same place in the first-dimension run. (b) Two-dimensional electrophoresis of bovine serum albumin (BSA) after Coomassie blue staining. After isoelectric focusing, the albumin was eluted into a continuous slab gradient gel (T max. 30%, C 5%) by means of the discontinuous buffer system of pH 8.4. The pattern shows not only the vertical array of BSA oligomers, but also the charge-isomers of BSA monomer and dimer. The difference in isoelectric points is about 0.3 pH units. The apparent spreading of the protein discs indicates the approach to their pore limit in the gel.

concentration should not exceed 10%, for this enhances the protein loss severely. Staining and destaining should also be performed at room temperature since the present alcohol may solubilize converted protein.

Removal of the carrier ampholytes prior to the second-dimension run has been tried, employing 10% TCA at room temperature and subsequently removing the TCA by diethyl ether[11] or acetone[24]. The TCA ethanol treatment as described, but extended over 12 h, rendered most of the water-soluble protein of a cell (*Aplysia californica* neuron R_2) water insoluble, thus prohibiting a second-dimension run without use of a detergent. Therefore, the amount of carrier ampholytes contained in the electrofocusing gel has been taken into account in the second-dimension gradient electrophoresis, even though this caused some interference with the staining of the slab gel, as has been shown elsewhere[25].

Slab gradient gels

Small-size slab gradient gels can be produced at any value of T maximum up to *ca.* 40%. At higher concentrations, the gel chamber may burst due to swelling of the gel, and thus draw air bubbles between the glass wall and the gel. The experimental possibilities offered by these gels are generally the same as those described for cylindrical gradient gels in capillaries[7,25,26]. If separated proteins have to be identified

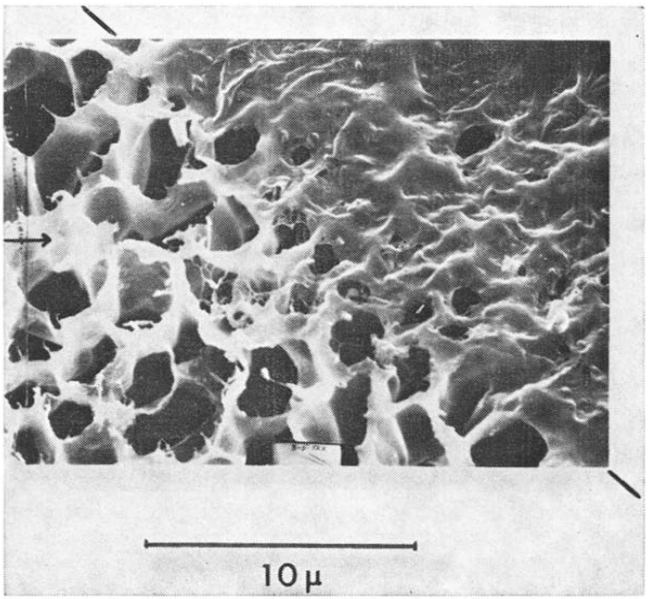


Fig. 8. Scanning electron microscopic (SEM) image of the surface area of a continuous slab gradient gel that contains α_2-macroglobulin of sheep serum after electrophoresis in the discontinuous buffer system of pH 8.4 has taken place. The macroscopic impression of a very sharp protein disc that is approaching its pore limit is confirmed by the SEM image. The SEM image reveals a defined edge between the gel matrix that is totally clogged with protein and an area that shows some protein at the gel membranes (→) only. The gel was prepared for SEM by freeze drying and rotary coating, as described previously[31].

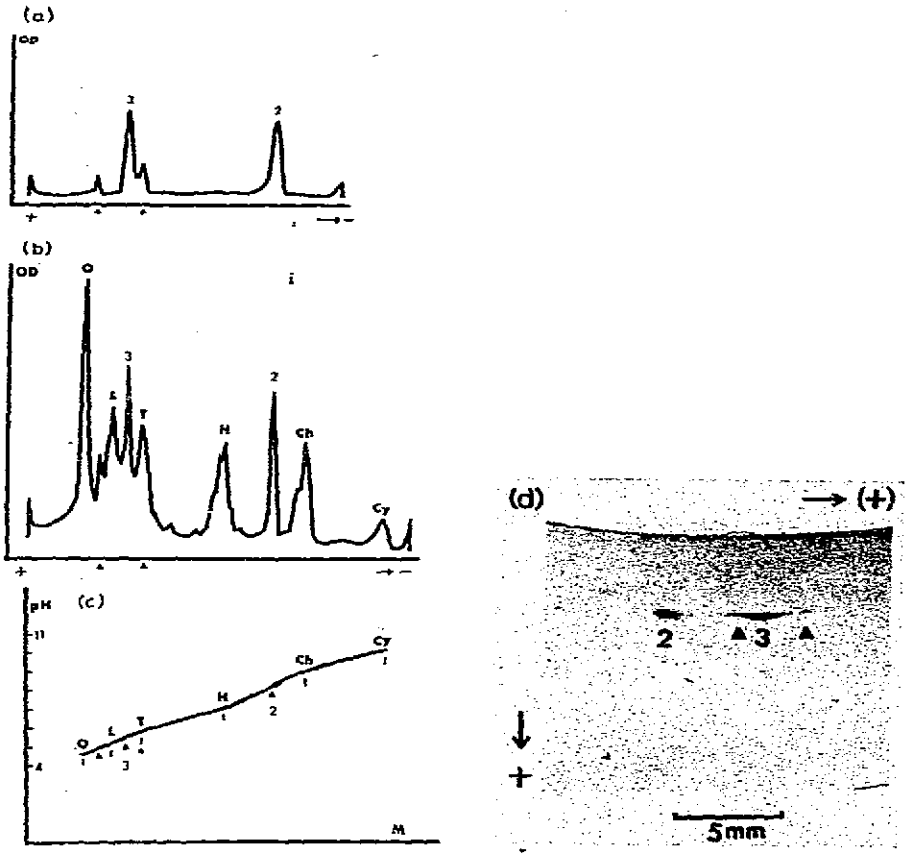


Fig. 9. (a) Optical density (OD) scan of electrofocused lactic dehydrogenase (LDH) from rabbit muscle [Sigma Type 2 (2)] with subunits M_4 and from beef heart [Sigma Type 3 (3)] with subunit patterns H_4 (left), H_3M (middle), and H_2M_2 (right). The enzymes have been detected by the tetrazolium system (see, *e.g.*, ref. 12). (b) Optical density (OD) pattern of electrofocused lactic dehydrogenases (2,3) and marker proteins of known isoelectric points: ovalbumin (O), p*I* = 4.6; *β*-lactoglobulin (L), p*I* = 5.2; transferrin (T), p*I* = 5.8; hemoglobin (H), p*I* = 7.0; chymotrypsinogen A (Ch), p*I* = 8.9; cytochrome *c* (Cy), p*I* = 10.0. The enzymes have been localized by the tetrazolium essay prior to staining with Coomassie blue. (c) Plot of the isoelectric points of the proteins, separated as shown in (b), against their linear migration distance (M). According to repeated experiments, the isoelectric points of LDH Type 2 have been determined at 8.1, while the species of LDH Type 3 have isoelectric points of 4.9, 5.5, and 5.8, respectively. (d) Two-dimensional, "dead run" electrophoresis of LDH Types 2 (left) and 3 (right). The enzymes have been detected by the tetrazolium essay. In the first dimension, the enzymes were separated according to their charge properties, as shown in (a). The second-dimension run has been performed in a continuous gradient gel (T max. 40%, C 5%). Tris–borate buffer (pH 9.5) was used throughout the system. The buffer had a maximum concentration of 160 m*M* Tris and was distributed in the gel in a continuous concentration gradient, as well as in the acrylamide. The buffer gradient is provided by the omission of buffer from the persulfate solution (see Methods). This buffer gradient provides low conductivity in the gel top and the supernatant, thus allowing rapid elution of protein from the first-dimension gel into the slab gel. The terminal stage of electrophoresis shown here has only been attained after a 10-h run at 100 V/cm gel. In earlier stages of the run, the basic LDH Type 2 trails, as a blurred spot, behind the favorably migrating acidic LDH Type 3. At the terminal stage, all proteins have closely approached their pore limit in the gel and form an almost horizontal line in the gel. Such migration behavior is typical for charge isomers of proteins in a steep gradient gel. Since the difference between the p*I* of the basic LDH Type 2 (8.1) and the separation pH in the gel gradient (9.5) is smaller than the difference between the p*I* of the separated LDH species, this experiment outlines the power of pore limit (dead run) electrophoresis that is used to identify charge-isomeric proteins.

without staining of the whole gel, a pilot strip can be cut from the edge of the slab for staining and thus localization of proteins. Slab gradient gels tend to swell into a wedgelike shape when removed from the chamber[27]. Thus, they have to be photographed under cover of a glass slide.

An unexpected feature demonstrated in Fig. 7 is the severe spreading of proteins over the width of the gel when they are forced against their pore limit in the slab gradient gel. This effect, though at lesser extent, has been observed by Margolis and Wrigley[28]. The spreading goes to both sides; thus, it cannot be due to tailing of protein in the electrofocusing run and is not caused by diffusion parallel to the direction of migration[29,30]. Artefactual loss of protein into the supernatant

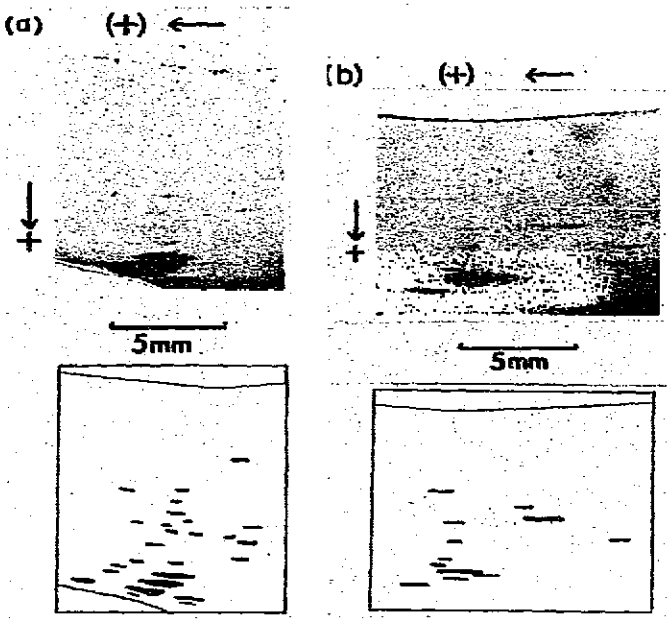


Fig. 10. (a) Two-dimensional electrophoresis of water-soluble proteins stained with Coomassie blue that have been extracted from the Bag cells and the Bag sheath of the abdominal ganglion of *Aplysia californica* by means of ethylene glycol passage and osmotic shock[55]. After isoelectric focusing in the indicated direction, the proteins were eluted into a continuous slab gradient gel (T max. 30%, C 5%) by means of the discontinuous buffer system of pH 8.4. The run was stopped when bromophenol blue had just reached the lower end of the gel. The area of the slab gel shown here covers the pH range of 4–5 and demonstrates the variety of proteins with low molecular weights that are contained in such neurosecretory cells. Using radioactive-labeled protein and autoradiography of the gel, the sensitivity of such separation may further be increased. (b) Two-dimensional electrophoresis of water-soluble proteins stained with Coomassie blue that have been extracted from the giant neuron R_2 of the abdominal ganglion of *Aplysia californica* by means of ethylene glycol passage and osmotic shock. This treatment especially mobilizes smaller peptides, while large proteins are retained in the cell carcass[55]. The running conditions are identical to those of (a). The area of the slab gel shown here covers a slightly more basic pH range than in (a). The differences in both patterns are evident if the peptide discs on the lower left side are matched for comparison. The remaining carrier ampholytes are stained in the right lower corner. They may be removed either by washing the gel in TCA prior to staining or by extending the running time. Both of these possibilities jeopardize small peptides, which may either pass through the gel or be washed out of the gel prior to staining. The residual carrier ampholytes also cause most of the small, grainy dye precipitations in the gel surface.

during the transfer to the top of the slab gel may contribute to the effect, but is not the only cause. The spreading may be due to increasing resistance and decreasing conductivity in gel areas which are heavily laden with protein under very restricted migration conditions. Thus, the current distribution within the cross-section of such a gradient gel may show a minimum in the area of high protein concentration, and particles traveling at the rear end of the peak will tend to bypass the obstacle on both sides according to current density. These particles run into the same area of restricting pores and thus make up such broad fronts as those seen developing in Fig. 7. The protein concentration across such a disc no longer shows a bell-shaped (Gaussian) distribution curve, but one that rises abruptly at the front of the disc (from nearly zero) to the maximum value, while the rear end of the disc ends in a tail. Such a distribution has been demonstrated by scanning electron microscopic (SEM) examination in gradient gels (Fig. 8)[31]. The peak spreading of proteins in the slab gradient gel may be compared to the results of two-dimensional immunoelectrophoretic runs where the deceleration of migrating antigenic particles is not caused by narrowing pores, but by immobilization that is caused by reaction with the antibody (see, *e.g.*, ref. 32).

Two-dimensional electrophoresis

There are two experiments that outline the framework in which results can be obtained by a two-dimensional technique as described: one includes the separation of particles which are identical in size but different in charge, and the other is the separation of particles identical in charge, but different in size. Macro-ions of different size but with identical net charge are provided by a set of oligomers of the same protein. In the first-dimension electrofocusing, they will settle in one peak, while in the second dimension in the slab gradient, they will form a vertical line of spots (Fig. 7). Macro-ions of almost identical size but with different charge are provided by isoenzymes of lactate dehydrogenase (Fig. 9). These proteins settle in different places in the first-dimension electrofocusing run and will migrate in an almost horizontal front as they approach their pore limit in the gradient gel of the second dimension. This approach is an asymptotical function of time[29]; thus, a residual distance will always remain at finite electrophoresis times, provided the proteins are not precipitating under very restricted migration conditions in narrow gel pores. Such a result will not be ideal, but will be useful for practical purposes, if some precautions are observed[7,25]. Spot patterns of the "finger print" type are not to be gained by the technique described here, but disc patterns of proteins that are typical for various extracts are to be obtained, for instance from a large single neuron (Fig. 10)[25], or of serum proteins (Fig. 11).

To achieve higher sensitivity, the cylindrical gel of the first-dimension run can be sliced, and proteins from those slices can be eluted individually into cylindrical gel gradients (Fig. 12). To facilitate the location of the major protein peaks in the electrofocusing gel, the gel can be immersed in 10% TCA, which will render the proteins white. Prior to the elution run, the TCA can be washed out of the gel with diethyl ether or acetone[11,24]. In order to get reliable size-dependent separation of small peptides that are contained in the pattern of the electrofocusing run, cylindrical pore gradients with T maximum concentrations of 60% have been used in the second-dimension run. The production and handling of such steep gradient gels have been described above.

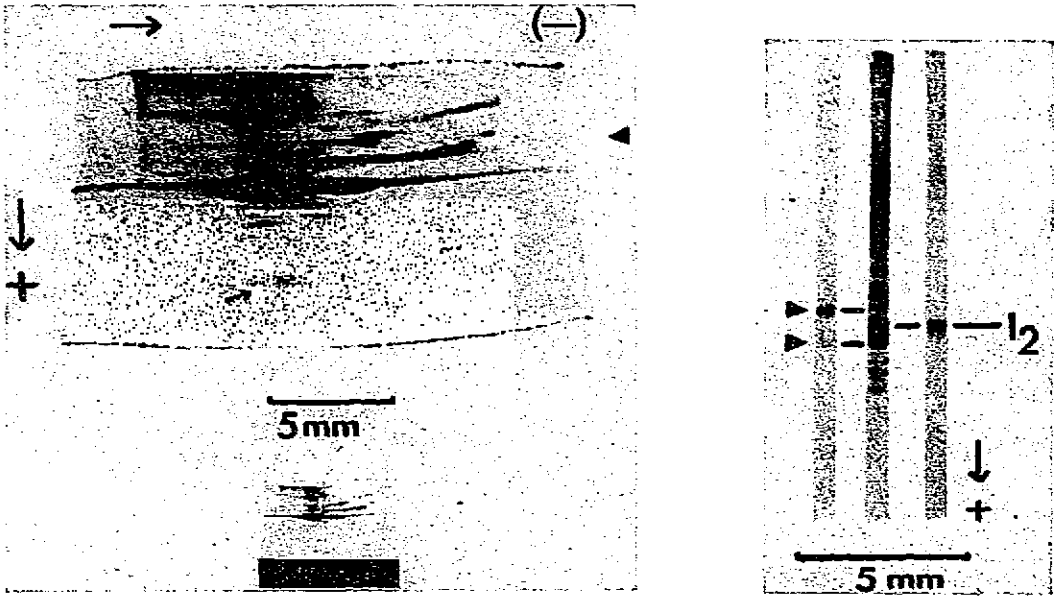


Fig. 11. Two-dimensional electrophoresis of rat serum proteins after staining with Coomassie blue. In the first dimension, electrofocusing was performed in the indicated direction. The section of the cylindrical electrofocusing gel, covering *ca.* pH 3–8, was cut out and applied on top of a continuous gradient gel (T max. 40%, C 5%). The discontinuous buffer system of pH 8.4 was used at 100 V/cm of gel for 30 min (see Methods). The run was stopped when bromophenol blue had just passed the gel, and xylene cyanole was still short of the gel edge by about one-fifth of the gel length. The pattern shows two charge-isomeric albumins that have spread over the whole width of the gel. Such spreading is partly due to the elution of protein into the supernatant during the transfer of the cylindrical gel on top of the slab gel. Spreading also indicates the approach of a protein to its pore limit in the gradient gel. An indicated series of protein spots (▶) that stretches from the acidic range to the neutral range and forms a slightly bent curve can be seen. Depending on their apparent molecular weight (>100,000) and charge properties, these proteins may be immunoglobulins of the A and G types. At prolonged running times, these proteins form an almost horizontal line in the gel that indicates their closely related molecular weights. Peptides such as those in front of the albumin (→), however, have passed through the gel under such conditions. A picture of the slab gel in its original size has been added for the sake of comparison.

Fig. 12. Selective, two-dimensional electrophoresis of proteins from neurons of *Aplysia californica* after they have been stained with Coomassie blue. Proteins extracted from the Bag cells and the sheath of the abdominal ganglion (compare Fig. 10a) were submitted to electrofocusing, and a peak identified by its white appearance in 10% TCA was cut out of the pH 5.5 area. The gel segment was washed for 2 min in water prior to elution of the protein into a steep, cylindrical gel gradient (T max. 60%, C 4%). The discontinuous buffer system of pH 8.4 was used. The run was performed at 120 V/cm of gel until bromophenol blue reached the lower fifth of the gel and xylene cyanole migrated only two-thirds of the gel length. After staining, two peaks can be identified (▶) that correspond with the pattern of all water-soluble proteins of the same sample run under comparable conditions (middle gel). Since insulin dimer has been added to the heterogeneous sample, an estimation of the molecular weight range is possible. The position of insulin dimer (I_2) alone is shown in the right gel. Such a selective, two-dimensional technique, though more elaborate, is also more sensitive to faint protein peaks, since no peak spreading hampers the pattern as in the slab gels.

DISCUSSION

The behavior of a protein in an electric field is dependent upon its electrophoretic mobility[33]. The two main parameters of mobility —the charge and the size of the particle— can be investigated separately by two-dimensional electrophoretic techniques. Therefore, isoelectric focusing as a separation technique that is strictly dependent on charge is mostly performed in the first dimension[2,3,5,34–39]. Other electrophoretic variations of the first-dimension run include cationic electrophoresis in acidic buffers[4,40–42] and SDS-electrophoresis[43–45].

The second-dimension run is often an electrophoresis in a sieving polyacrylamide gel of constant pore size to achieve separation due to size differences[1–4,34,40,42,43]. Without the use of detergent, the integrity of protein is widely unaffected, and charge differences still contribute to the electrophoretic mobility of migrating proteins. When the bulk of protein is eluted from a isoelectric focusing gel, it will always migrate in a diagonal array in the slab gel that has a constant pore size[3]. This pattern illustrates the persistent influence of charge on protein mobility even in the second-dimension run. The separation of protein spots within such a "milky way" pattern is due to size and shape differences only, if positions are compared on lines parallel to the axis of migration. Diagonal patterns are also typical of two-dimensional combinations of electrophoresis at acidic pH and in buffers containing SDS[4,40–42,44]. This may be due to the separation at acidic running pH being predominantly dependent on molecular weight[46,47]. Thus the combination of electrofocusing and electrophoresis in acidic buffer yielded good separation[35]. Recognizing the same parameter, SDS-electrophoresis in both dimensions produces pronounced diagonal patterns[43] and thus yields only limited information. In the presence of SDS, the influence of charge is widely overcome and migration of proteins according to molecular weight is assumed[48–50]. In combination with electrofocusing the SDS method yields beautiful results[37,39]; however, several inconsistencies of the SDS method have to be considered: Abnormal migration has been reported for glycoproteins and for very basic and very acidic proteins and for peptides with a molecular weight smaller than 15,000[51,52]. Since most of the proteins or protein subunits present after SDS treatment and disulfide reduction are of intermediate size, slab gels of constant pore size and of 10–15% acrylamide concentration have been used in the second-dimension run[36,37,39]. To improve the separation power of the second-dimension run, slab gradient gels have been used, even in the presence of SDS[37]. The use of such gels has been introduced in the two-dimensional electrophoresis by Kenrick and Margolis to obtain separation according to size without the presence of detergent[5]. The combination of electrofocusing and gradient gel electrophoresis in detergent-free buffer systems has been used successfully to separate immunoglobulins and other serum proteins[38]. In gradient gels, proteins will approach their pore limit only on an asymptotical function of time[29]. At finite running times, reasonable results can be obtained if the gradient gels are steep enough[7,53]. The difference between the separation pH and the isoelectric point of the most basic protein (in basic buffer systems) or the most acidic protein (in acidic buffer systems) should be larger than the pH differences of the isoelectric points of all proteins involved in the run. In slab gradient gels, the results are hampered by spreading of the protein peaks when they approach their pore limit[28] (see Figs. 8–11). When the estimations of molecular weights are to be drawn from the separation

patterns, the spreading can serve to indicate the pore limit. The lack of separation that is dependent on the molecular weight of immunoglobulins in slab gradient gels at basic running pH has been reported by Leaback[47]. Such proteins appear blurred and form discs only in an acidic buffer system when they reach their pore limit (*cf.* Fig. 11).

The microversion of the electrofocusing gel gradient technique described here has been designed for the separation of peptides and proteins that have been extracted sequentially from large single neurons or cell clusters of the abdominal ganglion of the sea slug *Aplysia californica*. Therefore, isoelectric focusing in polyacrylamide gels of capillary size, as reported by other authors[10–12], has been improved. The anionic marker dye xylene cyanole FF has been used in place of or in addition to bromophenol blue to determine the end of the focusing run. Xylene cyanole focuses at the same time as proteins do, provided there is no significant sieving of the proteins in the gel. Thus the crucial minimum focusing time can be determined easily and problems (such as the plateau phenomenon) can be avoided[6,8,9,21]. Protein extracts from identified neurons display specific differences at the peptide level. They also contain large proteins with molecular weights up to 10 [56,54]. The use of gel with a constant pore size in the second-dimension run would confine the investigation to a fairly narrow range of molecular weights, since proteins that are too large are excluded from the gel and peptides that are too small to be sieved are migrating unseparated with the buffer front in the discontinuous system used. Steep gradient gels, on the contrary, allow the separation of protein mixtures covering several orders of magnitude of molecular weights in one run[7,26]. Since all the protein of one extract in most of the neurons investigated has to be expended for one run in the two-dimensional system, the slab gradient gel is the carrier of choice for the second-dimension run in a detergent-free buffer.

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