Two-dimensional electrophoresis

Review

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The current state of two-dimensional electrophoresis with immobilized pH gradients

Contents

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Abbreviations: **2-D**, two-dimensional; **Bis**, *N,N'*-methylenebisacrylamide; **CA**, carrier ampholytes; **DTT**, dithiothreitol; **IEF**, isoelectric focusing; **IPG**, immobilized pH gradients; **IPG-CA**, immobilized pH gradient with added CA; **IPG-Dalt**, 2-D electrophoresis with immobilized pH gradients in the first dimension; **NEPHGE**, non-equilibrium pH gradient electrophoresis; **NP-40**, Nonidet P-40; **PMSF**, phenylmethylsulfonyl fluoride; **SDS**, sodium dodecyl sulfate; **TEMED**, *N,N,N',N'*-tetramethylethylenediamine

1 Introduction

Immobilized pH gradients (IPG) were introduced in 1982 [1]. An immediate advance of isoelectric focusing (IEF) with IPG was the practical feasibility of setting up very narrow pH gradients (0.05 pH/cm or less) in most regions of the pH scale, with a resultant dramatic improvement in resolution for some applications [1–6]. The possibility of forming pH gradients from extremely shallow to very broad (being linear, non-linear, step-wise or partially flattened in regions of special interest along the separation axis) does not really exist using the synthetic carrier ampholyte (CA) system. Whereas this feature renders IPG potentially able to solve any charge fractionating problem, the central promise inherent to IPG is a pH gradient stable with time, generated by the covalent anchoring of the pH gradient to a polymer matrix, that will consistently improve the reproducibility of IEF patterns. Moreover, in view of the complexity of the polypeptides and numerous sources of variability in two-dimensional (2-D) electrophoresis, attempts to develop 2-D patterns with absolute spot positions by coupling two steady-state separations (charge and mass) is an appealing approach with respect to spot identification, pattern matching and inter-laboratory comparison. Constant zone positions, even over several days, have been demonstrated with soybean proteins in an IPG of pH 4–6 [7]. Pattern constancy with time along the IEF axis in 2-D separations of myeloblast proteins with an IPG of pH 4–7 [8] and of yeast cell proteins with an IPG of pH 7–10 [9] in the first dimension has also been verified. However, in order to achieve high resolution 2-D patterns with IPG in the first dimension, the classical protocols for 2-D electrophoresis [10–14] based on O'Farrell's method [10] had to be modified, taking into ac-

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count that IPGs behave differently compared to CA-generated pH gradients. Critical parameters inherent to IEF with IPG and a number of experimental conditions which are not part of the classical 2-D electrophoresis repertory are described. 2-D patterns obtained with first-dimensional narrow or wide IPGs for the separation of acidic, neutral or alkaline yeast or blood cell proteins, or plant proteins from seeds or tissue are demonstrated and CA 2-D maps are compared with IPG 2-D maps.

2 Materials and methods

2.1 Apparatus and chemicals

IEF was performed in IPG gel strips with the Ultrophor, and sodium dodecyl sulfate (SDS)-electrophoresis was run horizontally in the Multiphor II connected to the constant power supply Macrodrive 5 from Pharmacia-LKB (Uppsala, Sweden). For cooling, the Pharmacia-LKB MultiTemp was used. Small-scale IPG-Dalt was carried out with Phast-System, Pharmacia-LKB (Uppsala, Sweden). For IEF, laboratory-made IPG gels or commercially available IPG DryPlates (Pharmacia-LKB) were used. Horizontal SDS electrophoresis was carried out in 0.5 × 190 × 250 mm³ gels (laboratory-made) or in 0.45 × 50 × 43 mm³ gels (commercially available PhastGel Gradient 10–15). All gels were cast on GelBond PAG film using the gradient gel kit (Pharmacia-LKB). Immobilines, Ampholines, acrylamide, *N,N'*-methylenebisacrylamide (Bis), ammonium persulfate, *N,N,N',N'*-tetramethylethylenediamine (TEMED), Repel-Silane, Bromophenol Blue and Ultrodex were from Pharmacia-LKB. SDS (2 × crystallized, analytical grade), glycine (Gly) and β-mercaptoethanol were from Serva (Heidelberg, FRG). Trizma Base, Nonidet P-40 (NP-40), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT) and iodoacetamide were from Sigma (St. Louis, MO, USA). Silver nitrate and all other chemicals were from Merck (Darmstadt, FRG). Electrode wicks for SDS electrophoresis were from Bio-Rad (Ultra wicks 170-4127, Richmond, CA, USA) and were used repeatedly.

2.2 Current method of horizontal IPG-Dalt

IEF was performed in individual IPG gel strips. For the second-dimensional run, the equilibrated IPG gel strip was placed onto the surface of the horizontal SDS gel (Fig. 1) without agarose sealing or embedding gels.

2.2.1 Sample preparation

In general, samples (*e.g.* yeast, blood cells, seed or tissue proteins from plants) were dissolved in a lysis solution containing 9M urea, 2 % v/v NP-40, 2 % v/v β-mercaptoethanol, 0.8 % w/v Ampholines, pH 3.5–10, and 8mM PMSF. *Saccharomyces uvarum* (120 mg) or ground seeds from legumes (20 mg) or myeloblasts (5 × 10^8 cell equivalents, isolated from peripheral blood [15]), were suspended in 1 mL lysis solution, disrupted by sonification in an ice bath for 10 min and centrifuged (10 min, 42 000 *g*). Plant tissue proteins, *e.g.* leaf proteins, were treated with acetone to remove phenolic compounds. Leaves were crushed in a liquid nitrogen-cooled mortar, and the powder was resuspended in a solution of 10 % w/v trichloroacetic acid (TCA) in acetone with 0.07 % v/v β-mercaptoethanol. Proteins were allowed to precipitate for 45 min at −18 °C. After centrifugation the pellet was washed with acetone containing 0.07 % v/v β-mercaptoethanol at −18 °C. The supernatant was discarded and the pellet dried under vacuum [16] prior to solubilization in the lysis buffer (20 mg/mL); described above. An Ultrodex suspension was prepared by swelling 40 mg Ultrodex in 1 mL lysis solution. Ten µL sample solution was mixed with 10 µL Ultrodex suspension prior to IEF [9]. Twenty µL were applied into 1 mm thick silicone rubber frames (10 × 5 mm² outer diameter, 8 × 3 mm² inner diameter) placed on a 5 mm wide IPG gel strip.

2.2.2 Gel casting

All IPG and SDS gels were 0.5 mm thick and cast on GelBond PAG film. GelBond PAG films were washed 6 × 10 min with deionized water prior to use [17]. The mold (Fig. 1, A–C), consisting of two glass plates, one covered with the GelBond PAG film, the other bearing the U-frame (0.5 mm thick), was loaded in a vertical position and filled from the top according to the casting procedure of Görg *et al.* [18] for ultrathin pore gradient gels.

2.2.2.1 IPG gels

IPG gels with linear gradients, pH 4–10, 4–7 and 7–10 (4 % T, 4 % C; 0.5 × 180 × 250 mm³, 0.5 × 110 × 250 mm³ or 0.5 × 50 × 250 mm³) were cast. The two starter solutions were prepared according to published protocols [19]. For better polymerization the acidic and basic solutions were adjusted to pH 7 with 1 N sodium hydroxide and 1.5 N acetic acid, respectively. The acidic, dense solution was pipetted into the mixing chamber, the basic, light solution into the reservoir of the gradient mixer. After pouring the gradient into the precooled mold (refrigerator), the mold was kept for 10 min at room temperature to allow adequate levelling of the density gradient prior to polymerization for 1 h at 50 °C. When a pH plateau for the sample application area was desired, an extra portion of dense solution was prepared and pipetted into the mold prior to pouring of the gradient (Fig. 1C). Gradient gels with pH plateaus were cast without an intermediate polymerization step.

2.2.2.2 SDS pore-gradient gels

The SDS gels on plastic backing (0.5 × 190 × 250 mm³) contained a linear gradient from 12–15 % T, 4 % C constant, 0.1 % SDS and 0.375 M Tris-HCl, pH 8.8, together with a stacking gel, 3–5 cm wide, with 6 % T, 4 % C, 0.1 % SDS and 0.125 M Tris-HCl, pH 6.8. The solutions for gel casting are described in Table 1. The buffer stock solutions contained 0.01 % NaN_3 for preservation. Immediately before casting, 5 µL TEMED and 10 µL ammonium persulfate (40 % w/v) were pipetted to the stacking gel solution, and 10 µL TEMED and 20 µL ammonium persulfate (40 % w/v) were added to both the dense and the light solutions. For casting an SDS gel with a stacking gel length of 35 mm, 5.5 mL stacking gel solution was pipetted into the precooled mold (4 °C). The pore gradient was cast on top of the stacking gel solution by mixing 12.2 mL each of the heavy and light solution with the help of the gradient maker (Fig. 1C). The high glycerol concentration of the stacking gel solution allows the overlaying of the pore gradient mixture without an intermediate polymerization step.

Table 1. Solutions for preparing horizontal SDS pore gradient gels

	Stacking gel 0.125 M Tris-HCl pH 6.8; 0.1 % SDS T = 6 %; C = 4 % 37.5 % glycerol	Running gel 0.375 M Tris-HCl pH 8.8; 0.1 % SDS Heavy solution T = 12 %; C = 4 % 25 % glycerol	Running gel 0.375 M Tris-HCl pH 8.8; 0.1 % SDS Light solution T = 15 %; C = 4 % 0 % glycerol
Acrylamide/Bis (28.8 g + 1.2 g made up to 100 mL)	2.0 mL	8.0 mL	10.0 mL
0.5 M Tris-HCl pH 6.8; 0.4 % SDS	2.5 mL	–	–
1.5 M Tris-HCl pH 8.8; 0.4 % SDS	–	5.0 mL	5.0 mL
Glycerol	3.75 g	5.0 g	–
Distilled water	2.5 mL	3.0 mL	5.0 mL
Final volumes	10.0 mL	20.0 mL	20.0 mL
TEMED	5 µL	10 µL	10 µL
Ammonuium persulfate (40 % w/v)	10 µL	20 µL	20 µL
Volumes used for an 0.5 × 190 × 250 mm^3 gel (stacking gel 35 mm running gel 155 mm)	5.5 mL	12.2 mL	12.2 mL

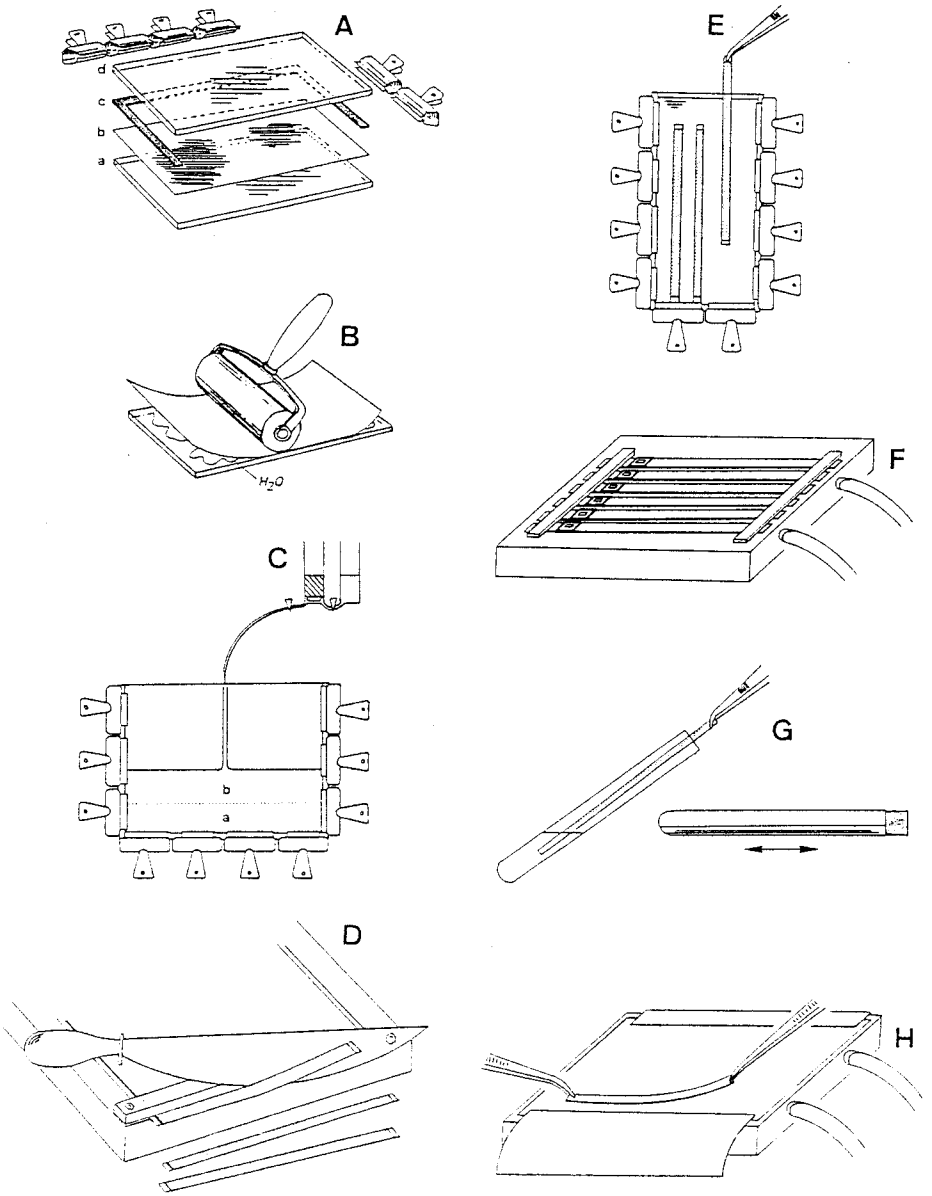


Figure 1. Procedure of horizontal IPG-Dalt. (A) Assembly of the polymerization cassette for the preparation of IEF and SDS gels cast on plastic backings. Glass plate (a), GelBond PAG film (b), U-frame (c) and cover glass plate (d). (B) GelBond PAG film is rolled onto the glass plate with a few drops of water. (C) The polymerization cassette is filled from the top in a vertical position. Stacking gel solution (SDS gel) or pH plateau solution (IPG gel) is pipetted into the mold (a) followed by the solution from the gradient mixer for casting a pore gradient or pH gradient (b) without intermediate polymerization step. (D) Dried and washed IPG slab gels or IPG DryPlates are cut into individual strips prior to rehydration and IEF. (E) IPG gel strips on plastic backing are rehydrated in the mold containing the desired additives needed for IEF. (F) Rehydrated IPG gel strips are blotted and placed on the cooling block side by side, 2 mm apart. Silicone rubber frames for sample application are applied to the IPG gel strips, 5 mm apart from the anode. (G) After IEF, IPG gel strips are stored at −80 °C or used immediately for the second-dimensional run. IPG gel strips are transferred into a test tube filled with 10 mL equilibration solution and placed on a shaker. (H) The equilibrated and blotted IPG gel strip is placed on the surface of the horizontal SDS gel along the cathodal electrode wick, 1 mm apart. One (18 cm) or two (each 11 cm) IPG gel strips are applied. The IPG gel strips are removed after 75 min (200 V max) of electrophoresis time, and the cathodal electrode wick moved forward so that it overlaps the former application area of the IPG gel strips. Electrophoresis is continued with 30 mA max until the Bromophenol Blue dye front has reached the anodic end of the SDS gel.

After pouring, the mold was left for 10 min at room temperature to allow adequate levelling and was then placed in a heating cabinet at 50 °C for 30 min for polymerization. Alternatively, an SDS pore gradient gel with stacking gel (6 % T, 4 % C) but continuous buffer system was used. For this purpose the Tris-HCl buffer, pH 6.8, of the stacking gel solution was replaced by 2.5 mL 1.5 M Tris-HCl, pH 8.8, with 0.4 % SDS and 0.01 % NaN_3 (see Table 1).

2.2.3 Preparation of IPG gel strips

After polymerization, the IPG gel was washed 6 × 10 min with deionized water and 30 min with 2 % w/v glycerol, dried at room temperature in a dust-free cabinet with a ventilator, and, if not used immediately, wrapped with a plastic film for storage at –20 °C. For IEF in individual strips, dried IPG gels or commercially available IPG DryPlates were cut into 5 mm (or less) wide strips with the help of a paper cutter (Fig. 1D) and rehydrated overnight in the mold (Fig. 1E) to the original gel thickness (0.5 mm). The mold, with a 0.7 mm thick U-frame (0.5 mm gel plus 0.2 mm GelBond PAG film), was filled with a solution of 8 M urea, 0.5 % v/v NP-40 and 10 mM DTT. For IEF in IPG in the presence of CA, 0.5 % (w/v) CA were added to the rehydration solution, overlapping the pH range of the IPG.

2.2.4 IEF in IPG gel strips

The rehydrated IPG gel strips were lightly blotted between two sheets of water-saturated filter paper to remove excess rehydration solution and to prevent urea crystallization on the surface of the gel during focusing. IPG gel strips were placed side by side, 2 mm apart, on the kerosene-wetted flat-bed cooling plate, 15 °C (Fig. 1F). The electrode strips were soaked with 10 mM glutamic acid (anode) and 10 mM lysine (cathode). Silicone rubber frames, described above, were applied onto the dry gel surface, 5 mm apart from the anode for sample application. 20 µL of sample (10 µL sample solution plus 10 µL Ultrodex suspension) were applied. For improved sample entry, voltage was limited for 1 h (300 V/10 cm). IEF was continued with the maximum settings of 5000 V, 1 mA and 5 W until constant focusing patterns were obtained. For IEF in IPGs with 3–6 pH units, steady state IEF was achieved within 75 min for 5 cm [20], 6–7 h for 11 cm [9], and within 16–18 h for 18 cm [9] gel length. IPG gels containing 0.5 % w/v Ampholines were run with the same time course, but with lowered voltage (3000 V max, 2 mA max, 5 W max) in order to diminish liquid exudation and zone distortions. After IEF, IPG gel strips, if not used immediately, were stored in a freezer at –80 °C or in liquid nitrogen prior to equilibration.

2.2.5 Equilibration

The IPG gel strips were equilibrated with gentle shaking for 2 × 15 min in 2 × 10 mL equilibration solution (0.05 M Tris-HCl buffer, pH 6.8, containing 6 M urea, 30 % w/v glycerol, 2 % w/v SDS, 1 % w/v DTT (65 mM) and a trace of Bromophenol Blue pipetted into a test tube (Fig. 1G). Iodoacetamide, 260 mM was added to the second portion of equilibration solution [20, 21].

2.2.6 Horizontal SDS-electrophoresis

The SDS gel was placed on the kerosene-wetted cooling block of the Multiphor II; the electrode wicks, soaked with electrode buffer (0.1 % SDS in 0.025 M Tris/0.192 M glycine, pH 8.3), were applied so that they overlapped the gel by 15 mm on each side. Minimum background staining in the silver-stained 2-D patterns was obtained by using Ultra wicks (170-4127) from BioRad. For this purpose one layer (200 × 250 mm^2) was cut into two halves (100 × 250 mm^2) and used as anodic or cathodic electrode wick, respectively. The electrode buffer was replaced after each run, whereas the electrode wicks were used repeatedly, increasing the purity of the Ultra wick. The equilibrated IPG strips were blotted on filter paper to remove excess equilibration buffer and were transferred, gel-side-down, onto the surface of the horizontal SDS gel along the cathodic electrode wick, 1 mm apart (Fig. 1H); air bubbles were squeezed out. In order to keep the electrode wicks in place, they were weighted with a heavy glass plate (3 × 200 × 260 mm^3). Electrophoresis was carried out at a maximum voltage of 200 V for 75 min. After this time, when the Bromophenol Blue tracking dye had migrated out of the IPG gel strips, the IPG gel strips were removed and, simultaneously, the cathodic electrode wick moved forward so that it overlapped the former application area of the IEF strips. Electrophoresis was continued for 5 h at 10 °C with maximum settings of 30 mA, 800 V and 30 W, until the Bromophenol Blue front reached the end of the gel.

2.3 Current method of horizontal 2-D electrophoresis with carrier ampholytes

Horizontal 2-D electrophoresis with CA in the first dimension, using first and second-dimensional slab gels cast on plastic films and run horizontally according to Görg *et al.* [18, 22] was updated and performed as described here.

2.3.1 Casting of CA gels

The mold (Figs. 1, A, B), consisting of two glass plates, one covered with the GelBond PAG film, the other bearing the U-frame (0.5 mm thick), was filled from the top with a pipette. The acrylamide solution (4 % T, 4 % C) containing 8 M urea, 0.5 % v/v Nonidet P-40, 2.5 % w/v carrier ampholytes (1 % Ampholine pH 4–6, 1 % Ampholine pH 5–7 and 0.5 % Ampholine pH 3.5–9.5), was deaerated; the catalysts (0.025 % v/v TEMED, 0.04 % w/v ammonium persulfate) were added immediately before use. After polymerization (at room temperature for 1 h) the gel was removed from the cassette. The gel adhered firmly to the GelBond PAG film.

2.3.2 IEF in CA slab gels

After prefocusing (700 V max, 20 mA max, 20 min), 20 µL samples were applied with pieces of paper (10 × 5 mm^2) onto the slab gel, 1.5 cm apart from the anode. The electrode strips were soaked with 10 mM glutamic acid (anode) and 10 mM lysine (cathode). IEF was performed with 400 V max for 30 min, and, after removal of the pieces of paper, continued with 800 V (30 min), 1200 V (30 min), 1500 V (60 min) and 2000 V (10 min).

2.3.3 Excision of the sample lanes

After IEF, the individual sample lanes (5 mm wide) were cut off the slab gel with the help of fine scissors.

2.3.4 Equilibration

IEF strips were equilibrated 2 times for only 2 min in the buffer solutions described for IPG gel strips (see Section 2.2.5).

2.3.5 Horizontal SDS-electrophoresis

The equilibrated and blotted IEF strip was transferred, gel-side-down, onto the surface (no gel trough!) of the horizontal SDS gel (see Section 2.2.2.2) along the cathodic electrode wick, 1 mm apart (Fig. 1H). Electrophoresis was carried out as described for horizontal IPG-Dalt (see Section 2.2.6).

2.4 Silver staining

SDS gels on GelBond PAG film were fixed in 20 % w/v TCA for 20 min or in 50 % v/v methanol/12 % v/v acetic acid for at least 3 h or, generally, overnight. The gels were washed with: a) 25 % methanol/5 % acetic acid, v/v, b) 5 % acetic acid, c) 1 % acetic acid, and d) deionized water for 30 to 60 min for each wash. After treating with 1 g/L $K_2Cr_2O_7$ and 4.6 mL/L 65 % HNO_3 in deionized water for 5 min, the gels were washed with deionized water for 4 min. Then they were immersed in 0.2 % aqueous silver nitrate for 30 min, briefly rinsed (2 × 10 s) with deionized water and developed in a solution containing 30 g/L Na_2CO_3 and 2.5 mL/L 37 % formaldehyde; the solution was replaced with a fresh solution within a few seconds to avoid silver coating on the gel surface and GelBond PAG film. Developing is stopped after staining to equilibrium (about 10 min) with 1 % acetic acid for 5 min, and the gels were washed with several changes of deionized water [23].

2.5 Storage

For long-term storage, the washed gels were sealed in a plastic bag, a few drops of water included. Residual acetic acid should be avoided; otherwise the silver stained gel will bleach upon storage. Alternatively, the gels were dried at room temperature. For this purpose the washed gel was soaked in preserving solution containing 3–4 % w/v glycerol and 70 % v/v methanol for 5 min (until the transparent gel became opaque), and wrapped with a sheet of cellophane, wetted with the preserving solution. After drying, the gel adhered to the cellophane and was peeled off from the GelBond PAG film. However, with the latter procedure, there is a risk that the dry gel on the cellophane will crack after a couple of months, because silver-stained gels are in general more brittle than Coomassie Brilliant Blue stained gels.

3 Results and discussion

3.1 Methodology and critical parameters

Methods of 2-D electrophoresis based on the original O'Farrell [10] procedure involve the use of cylindrical first-dimensional IEF gels containing 2 % w/v carrier ampholytes, 9 M urea and 2 % v/v NP-40 with the vertical SDS polyacrylamide gradient gel slab system of Laemmli [24]. Detailed and elaborate descriptions of 2-D electrophoresis for multiple runs are given by Anderson *et al.* [12] for the Iso-Dalt system. However, in order to achieve high resolution 2-D patterns with IPGs in the first dimension, the classical protocols had to be modified, taking into account that IPGs behave differently compared with carrier ampholyte generated pH gradients.

3.1.1 Electroendosmosis

One of the first problems we were confronted with using first-dimensional IPG gels was the poor protein transfer from first to second-dimensional gels, revealing only a reduced amount of the expected protein spots, accompanied by heavy horizontal and vertical streaking in the silver stained 2-D patterns. The observation that the IPG gel dug into the second-dimensional SDS gel upon electrophoresis with resultant gel gluing helped us to realize that the IPG being grafted in the gel matrix caused some problems related to electroendosmotic effects [17, 25]. The immobilized buffering groups in the IPG gel matrix become ionized (*e. g.* $-COO^-$) upon electrophoresis in the alkaline surrounding, with a resultant flow of hydration water in the contact area of the two gels towards the cathode of the SDS gel. During SDS-electrophoresis the IPG gel shrinks at the anodic and swells at the cathodic edge (Fig. 2). Moreover, the negatively charged IPG gel tends to migrate to the anode upon electrophoresis and digs into the SDS gel with consequent gel gluing and hindered protein transfer, which is also indicated by decreasing current and increasing field strength. On removal of the IPG gel, remaining parts are observed on the surface of the horizontal SDS gel or on top of the vertical SDS gel. To overcome the shrinkage of the IPG gel at the anodic edge, as well as the formation of a depression in the SDS gel due to active transport of hydration water to the cathode and migration of the negatively charged IPG gel strip to the anode, we added 6 M urea and 30 % w/v glycerol to O'Farrell's [10] equilibration buffer, which proved to minimize electroendosmotic effects [17]. Alternatively, diluting the Immobiline concentrations in the first-dimensional IPG gels also reduces the electroendosmotic effects, but with the risk of pH gradient instability [9].

3.1.2 Geometry of the first-dimensional gel

Classical 2-D electrophoresis with CA is carried out with first-dimensional tube gels and second-dimensional slab gels [10–14]. Whereas horizontal flat-bed gels are almost always the method of choice for one-dimensional IEF separations, tube gels are still favored for 2-D electrophoresis. Iborra and

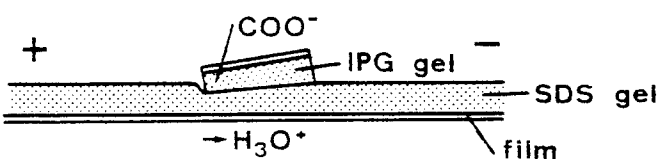


Figure 2. Transverse section of the contact area of the first- and second-dimensional gels in horizontal IPG-Dalt. The fixed negative charges in the IPG gel matrix cause a flow of hydration water towards the cathode whereas the negatively charged IPG gel tries to migrate to the anode of the SDS gel with resultant gel gluing and poor protein transfer from the first to the second dimension, which is also observed in vertical set-ups. The addition of 30 % w/v glycerol and 6 M urea to the equilibration buffer minimizes the electroendosmotic effects [17].

Buhler [26] and Goldsmith *et al.* [27] described first-dimensional IEF slab-gels followed by SDS electrophoresis on vertical slab-gels. Multiple samples were applied to the horizontal IEF slab gel, and after IEF, the gel plate was rapidly frozen on dry ice. The strip containing the sample was cut with a razor blade during unfreezing. The gel strip ($90 \times 10 \times 1$ mm^3) was carefully removed from the glass plate and transferred on top of the vertical SDS slab gel. This is a tedious procedure because cutting and handling of the fragile polyacrylamide strip is not easy. With the introduction of ultrathin IEF gels on cellophane [28], handling and gel slicing of the IEF slab gels were drastically facilitated, especially when Mylar polyester films [29, 30] or reliable binding polyester films, *e. g.* GelBond PAG film, as gel supports were used. After IEF, each sample track is easily cut out with fine scissors and, after equilibration, transferred to the second-dimensional gel. Plastic film-supported IEF gel strips with CA were first described for horizontal 2-D electrophoresis [22, 31] but can also be used for vertical second-dimensional gels. The simplest method is to apply the IEF strip onto the surface of a horizontal SDS gel because no agarose sealing or embedding gels are needed [31].

However, the use of flat-bed focusing systems for 2-D electrophoresis only became standard with the introduction of IPG for the first dimension [8, 9, 15, 17, 32]. IEF with IPG [1] is usually performed in slab gels because of the gradient casting procedure and the need for gel washing prior to IEF. Our first attempts to cut out the sample tracks after IEF, according to the procedure we described for horizontal 2-D electrophoresis with CA in the first dimension, failed because samples with different concentrations and different salt contents showed lateral band spreading upon IEF in slab gels with IPG. In order to avoid fusion of neighboring sample zones, gel troughs between the sample tracks were suggested [33]. However, the easiest way to have defined sample amounts on a sample track is to run first-dimensional IEF with IPG in individual gel strips [34]. This is only feasible with IPG gels because in contrast to IEF patterns with CA, IEF patterns with IPG show no edge effects. Straight protein zones are obtained. In Fig. 3A, bean proteins were focused in an individual IPG gel strip. The geometry of the IPG gel strips used for first-dimensional IEF runs is given in Fig. 3B.

The width of the strip should be as small as possible. We reduced the gel width from 10 to 5 mm (3 mm is even better), thereby minimizing electroendosmotic effects (see Section 3.1.2) and the amount of detergent being transferred from the first to the second dimension [34]. First attempts were to slice the washed, dried and rehydrated slab gel into individual strips with the help of a scalpel [17]. Although the slab gel is stabilized by the plastic film, it is difficult to cut through a soft gel and a rigid support without tearing the gel. Therefore, we prefer

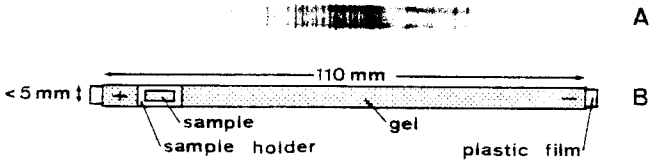


Figure 3. (A) IEF pattern of bean proteins focused in an individual IPG gel strip. Straight protein zones without edge effects are obtained. (B) Geometry of the first dimension. For first-dimensional IEF, IPG gel strips are cut from the dry IPG slab gel, rehydrated in the mold and placed onto the cooling plate of the flat-bed apparatus. Sample solution is pipetted into a silicone rubber frame, applied to the blotted surface of the IPG gel strip.

to cut the washed and dried IPG gel into 5 mm or 3 mm wide strips with a paper cutter prior to rehydration (Fig. 1D). This procedure is not only most convenient and precise but also the method of choice for commercially available IPG gels (Immobiline DryPlates). Only those strips needed for the next experiments are rehydrated in the reassembled polymerization cassette filled with the reswelling solution containing the desired additives (urea, detergent, *etc.*) needed for the first-dimensional IEF (Fig. 1E). In order to reswell the IPG gel strips to the original gel thickness (0.5 mm), the U-frame of the mold has to be 0.7 mm instead of 0.5 mm, taking into account that the gel is supported by a 0.2 mm plastic film (GelBond PAG film). Reswelling times are for several hours, and, in the presence of NP-40, overnight [34].

Because it is no problem to cast IPG gels with different lengths (the mold is loaded in a vertical position and filled from the top), any separation distance for large- and small-scale 2-D electrophoresis [9, 20, 31] can be used. Convenient gel thickness is 0.5 mm. However, there is no problem in casting *e. g.* 0.3 mm thick gradient gels on plastic backing with the procedure described for ultrathin pore gradient gels [18, 22].

In general, compared to tube gels, IPG gel strips (Fig. 3) are easily prepared by cutting off the required number of strips from the dry IPG slab gel (laboratory-made or premanufactured). The preparation of tube gels with identical pH gradients and gel lengths is a more critical step, including extrusion of the gels from the glass tubes without stretching and breakage. In order to run IEF in IPG tube gels, CA has to be added and the concentration of Immobilines has to be decreased [35, 36, 37], which impairs the stability of the pH gradient [9], the most important feature of IPG for 2-D electrophoresis. Owing to the rigid plastic film support (Fig. 3B), handling of IPG gel strips is not only facilitated but the reprodubibility of the spot positions along the IEF axis in the resultant 2-D patterns is also improved because IPG gel strips do not stretch when transferred to the second dimension.

3.1.3 Sample application

Compared with IEF in tube gels, the sample volume that can be applied is limited when IEF is performed in horizontal slab gels. However, when IEF is performed in IPG, large sample volumes can be applied, portion by portion, during IEF, without disturbing the pH gradient or the final IEF patterns. Alternatively, sample volumes up to 50 µL were easily accomodated by using containment collars cut from disposable pipette tips placed on the surface of the IPG gel [15]. We currently prefer silicone rubber frames cut out from 1 mm thick silicone rubber plates. For applying 20 µL sample solution on 5 mm wide IPG gel strip, 10×5 mm^2 silicone rubber frames with an inner diameter of 8×3 mm^2 are used (Fig. 3B). The silicone rubber frames, cut out from 1 mm thick plates or cut off from IEF applicator strips (Pharmacia-LKB, 2117-215), are placed on the blotted IPG gel strip, 5 mm from the anodal electrode strip. In most cases, cathodic application was less successful when complex protein mixtures were to be analyzed [8, 9]. Usually, we prepare an Ultrodex (Pharmacia-LKB, Sweden) suspension by swelling 40 mg Ultrodex in 1 mL of O'Farrell's lysis solution. Ten µL of the centrifuged sample solution is mixed with 10 µL Ultrodex suspension immediately before use. Adding Ultrodex suspensions to protein solutions has two beneficial effects: (i) it prevents proteins, which may precipitate at the beginning of IEF, from clogging the gel

surface [38] and (ii) it prevents leakage of sample solution on application into slots of silicone rubber frames [9]. Addition of Ultrodex powder instead of Ultrodex suspension to the sample solution is not recommended because of the risk of protein adsorption.

3.1.4 Sample entry

For first-dimensional IEF in IPG in the presence of urea and detergent, the most beneficial effects for improved sample entry were to apply diluted sample solutions [4] (*e. g.* 60–100 µg protein/20 µL) and to limit the voltage to 10–30 V/cm for the initial 1–2 h of focusing [9]. Sample application at the anode proved to be superior to cathodic application [8, 9]. However, this depends on the sample to be analyzed, the pH gradient used, as well as the additives utilized. The presence of CA (0.8 % w/v) in the sample solution (*e. g.* lysis buffer) proved to be beneficial, whereas reducing the Immobiline concentrations in the IPG gel strips to one third of the manufacturer's recommended amount did not improve the final 2-D patterns [9], which is in contrast to the findings of Hochstrasser [35], who used first-dimensional IPG tube gels. The addition of 0.5 % w/v CA [39, 40, 41] to first-dimensional IPG gel strips resulted in improved 2-D patterns of yeast cell lysates, whereas there was no visible difference in the 2-D patterns of legume seed proteins, or plant proteins from leaf and stem [42]. However, with the introduction of the stabilized Immobilines (Immobiline II) the addition of CA to IPG gels became meaningless even for the separation of yeast cell proteins.

3.1.5 Running conditions – first dimension

Horizontal and vertical streaking in the silver-stained 2-D patterns were also observed due to insufficient focusing times [9]. IEF to equilibrium does not only consistently improve reproducibility and inter-gel comparison but also the quality of the final 2-D patterns, which was demonstrated with basic yeast cell proteins [9]. Focusing time is prolonged in the presence of detergents and increases exponentially with increasing separation distance. In order to find out the proper focusing time, depending on the amount of sample and presence of detergent, the separation distance and the pH interval used, it is worthwhile prior to a series of experiments, to check the focusing time required to obtain steady state IEF patterns. This is the minimum focusing time to be used. A prolonged focusing time does not shift the IEF patterns to the cathode because of the stability of the IPG. However, a loss of protein spots was observed after excessively long focusing [9]. Steady state conditions for first-dimensional IEF in IPG with a pH interval of 3–6 pH units were obtained within 75 min for 5 cm [20], 6–7 h for 11 cm [9] and 16–18 h for 18 cm [9] gel length. Under those conditions IEF with IPG in the presence of 0.5 % CA was limited to 2 mA max and 3000 V max in order to avoid excess water exudation on the IPG gel surface leading to protein zone distortions. IEF with IPG in absence of CA was performed with the maximum settings of 5000 V and 1 mA.

3.1.6 Equilibration of the IPG gel strip

Compared with CA gel strips, equilibration time of IPG gel strips had to be prolonged from 2 min to 20–30 min in order to ensure proper protein transfer from the first to the second dimension [17]. However, when the width of the IPG gel strip was reduced from 10 to 5 or 3 mm (or even 1 mm for the Phast-System [20]), it was found that the equilibration time could be reduced. Omitting the equilibration step totally did not yield more protein spots in the resulting 2-D pattern; however, more background staining in the silver stained 2-D map was observed, partially hiding spots. The addition of urea and glycerol to the equilibration buffer [17] proved to be beneficial, as well as a second equilibration step, where iodoacetamide (in a 4-fold concentration of DTT) was added to the second portion of the equilibration buffer [21] in order to eliminate non-protein-related point streaking (see also Section 3.1.10).

3.1.7 Geometry of the second dimension

3.1.7.1 Vertical set-up

For multiple runs (*e. g.* 20 SDS gels), the Iso-Dalt System (Electro-Nucleonics, Oak Ridge, TN) according to Anderson *et al.* [12] was used. Myeloblast proteins were separated in alkaline [9, 15] and acidic [8] pH gradients. For this purpose, first-dimensional IEF was carried out in IPG gel strips as described in Section 2.2.4. The equilibrated IPG gel strips (see Section 2.2.5) were blotted and embedded at the top of the vertical slab gels with 0.5 % agarose containing electrode buffer (25 mM Tris, 192 mM glycine and 0.1 % SDS; pH 8.3). No stacking gel was used. Slab gels (1.5 × 160 × 160 mm^3) contained an 11.5 – 14 % T (2.6 % C) acrylamide gradient. The geometry of the Iso-Dalt system has been thoroughly described [12].

3.1.7.2 Horizontal set-up

Horizontal 2-D electrophoresis with CA using ultrathin first- and second-dimensional gels on plastic films was introduced by Görg *et al.* [18, 22]. The actual procedure, taking into account the modifications we have worked out in the last few years, is described in Section 2.3. Horizontal 2-D electrophoresis with IPG in the first dimension is based on the same principle. After focusing, the equilibrated IEF gel strip was placed on the surface of the horizontal SDS gel, within the stacking gel area, along the cathodic electrode wick. All SDS gels were 0.3–0.5 mm thick and cast on prewashed GelBond PAG film. The geometry of a horizontal SDS gel is shown in Fig. 4. The gel size of 190 × 250 mm^2 fits the horizontal electrophoresis unit of Pharmacia-LKB (Multiphor II). One IPG gel strip with a long separation distance or several IPG gel strips with shorter separation distances can be applied. Using IPG DryPlates, two IPG gel strips (each 110 mm long) are applied along the cathodic wick so that they are separated simultaneously under identical electrophoresis conditions and, finally, two 2-D maps on one SDS gel are obtained for optimal comparison. Horizontal systems are flexible in size. The separation distances can be varied by moving the electrodes (first dimension) and the electrode wicks (second dimension). Different gel sizes are easily cast [31]. Automated 2-D electrophoresis with IPG was performed with the Phast-System (Pharmacia-LKB) using premanufactured 0.45 × 50 × 43 mm^3 SDS gels [20].

In general, the horizontal SDS gel contained a running gel with a 12–15 % T (4 % C) acrylamide gradient together with a

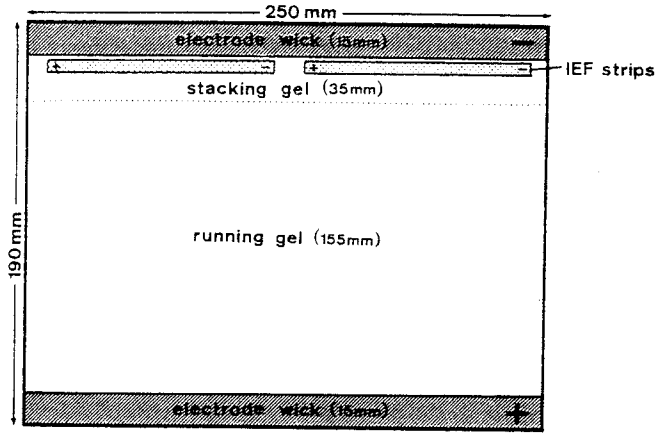


Figure 4. Geometry of the second dimension. A horizontal SDS gel (0.5 × 190 × 250 mm³) consisting of a pore gradient gel and stacking gel cast on GelBond PAG film (0.2 × 200 × 260 mm³) is placed on the cooling plate of the flat-bed apparatus. Wicks, soaked with SDS electrode buffer solution, overlap the gel by 15 mm on each side. Two IPG gel strips (0.5 × 5 × 110 mm³) on GelBond PAG film are placed, gel-side-down, onto the surface of the SDS gel along the cathodic electrode wick, 1 mm apart. Alternatively, one long IEF gel strip can be applied.

stacking gel of 4 or 6 % T, and 4 % C (Table 1). In order to obtain sufficient stacking of the proteins migrating out of the IPG gel strip, a stacking gel length of 50 mm was recommended when 5–10 mm wide IPG gel strips were used [34]. It has to be considered that, when 50 mm long stacking gels are used, only 35 mm remain functional because the electrode wick covers 15 mm. However, the length of the stacking gel can be reduced with decreasing width of the IPG gel strip, *e. g.* 35 mm proved to be sufficient for 3–5 mm wide IPG gel strips (Fig. 4). An insufficient stacking gel length is indicated by elongated or distorted protein spots.

3.1.8 Running conditions – second dimension

In order to prevent an indentation of the horizontal SDS gel by the IPG gel strip through electroendosmotic effects (see Section 3.1.1), electrophoresis was started at the maximum voltage of 200 V/19 cm for 75 min. Additionally, after this time, when the Bromophenol Blue tracking dye had migrated out of the IPG gel strip by a few millimeters the strips were removed and the cathodic wick was moved into its place (Fig. 5). SDS electrophoresis was completed at 600 V – 800 V/19 cm with a limit of 30 mA. Running conditions for 0.5 × 190 × 250 mm³ gels, exceeding the maximum setting of 30 mA, in order to shorten the electrophoresis time, resulted in distorted protein spots.

3.1.9 Detergent smear

By adopting protocols of high resolution 2-D electrophoresis with CA, urea and nonionic detergent in the first dimension for the analysis of complex protein mixtures, *e. g.* myeloblast proteins [43], to horizontal 2-D electrophoresis with IPGs, heavily distorted patterns with proteins partially hidden under clouds of detergents were obtained [34]. In order to reduce the excess detergent transferred from the first-dimensional IPG gel to the SDS gel we investigated three possibilities: (i) reduction of the IPG gel volume, containing 2 % NP-40, by minimizing the width of the IPG gel strips, (ii) washing out the detergent from the IPG gel strip after IEF and prior to equilibration [44], and (iii) reducing the concentration of detergent in the IPG gel [34]. Washing procedures or prestaining of the IEF gel strip prior to equilibration resulted in a severe loss of protein spots in the second dimension. Optimizing the ratio of the gel volumes for the first- and second-dimensional separations by decreasing the width of the IPG gel strip from 10 to 5 mm or even less, and by the reduction of NP-40 incorporated into the IPG gel from 2 to 0.5 %, excellent 2-D patterns of complex protein mixtures were obtained [34, 42].

3.1.10 Point streaking

Silver staining artifacts, unrelated to protein, such as horizontal lines and vertical micro or point streaking on 2-D gels were observed when thiol-reducing agents for first-dimensional IEF and/or equilibration of the IEF gel prior to SDS-electrophoresis were used. Vertical point streaking has been ascribed to particulates entrapped within the polyacrylamide gel. During SDS electrophoresis, particulates are partially solubilized by thiol-reducing agents and form streaks which begin at their original position within the gel and extend from a few millimeters to several centimeters in length (Fig. 6A). Point streaking can be minimized by adding an appropriate amount of iodoacetamide (260 mM) to the DTT (65 mM) containing equilibration buffer [21]. Under these conditions iodoacetamide reacts with the excess thiol-reducing agents without alkylating proteins (Fig. 6B).

3.1.11 Spot streaking

GelBond PAG films proved to be an excellent gel support. Handling of the first- and second-dimensional slab gels is facilitated, the dimensional stability of the gel or gel strips is guaranteed and the gels are protected from rupture during visualization steps. However, spot streaking in the 2-D patterns was occassionally observed [17]. This kind of spot streaking was eliminated by washing GelBond PAG films in distilled water (6 times for 10 min) prior to use [17].

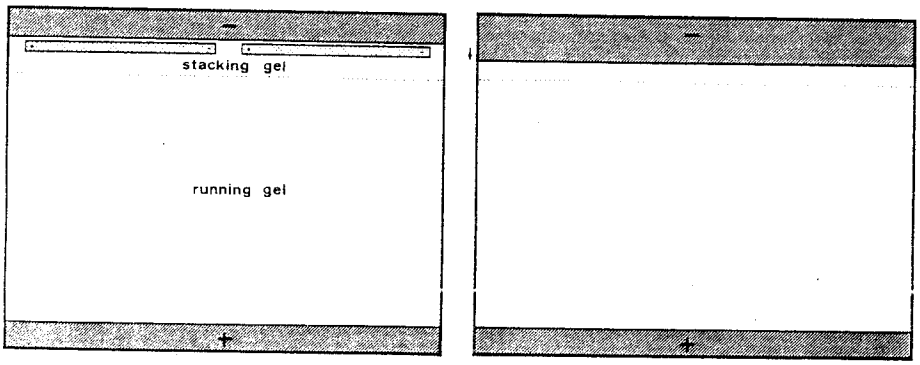


Figure 5. Running conditions. (A) For improved protein transfer from the first to the second dimension, electrophoresis is started with 200 V max for 75 min, after which time the Bromophenol Blue tracking dye has migrated out of the IPG gel strip. (B) The IPG gel strips are then removed from the SDS gel and the cathodic electrode wick is placed so that it overlaps the application area of the IEF gel. Electrophoresis is continued at 600 V (30 mA, 30 W limiting) until the Bromophenol Blue tracking dye reaches the anodic end of the SDS gel.

3.1.12 Stacking gel

In contrast to vertical second-dimensional SDS-Electrophoresis, where usually only a running gel is utilized [13], the presence of a stacking gel in the horizontal SDS gel was found to be essential (see section 3.1.7). However, in our experience, only the length of the stacking gel (see Section 3.1.7.2) and a low acrylamide concentration (*e. g.* 4 or 6 % T, 4 % C) are important, whereas the stacking and running gel do not necessarily have to differ in pH values and buffer molarities. The resulting 2-D patterns, obtained with a discontinuous buffer system (Fig. 7A) or continuous buffer system (Fig. 7B), do

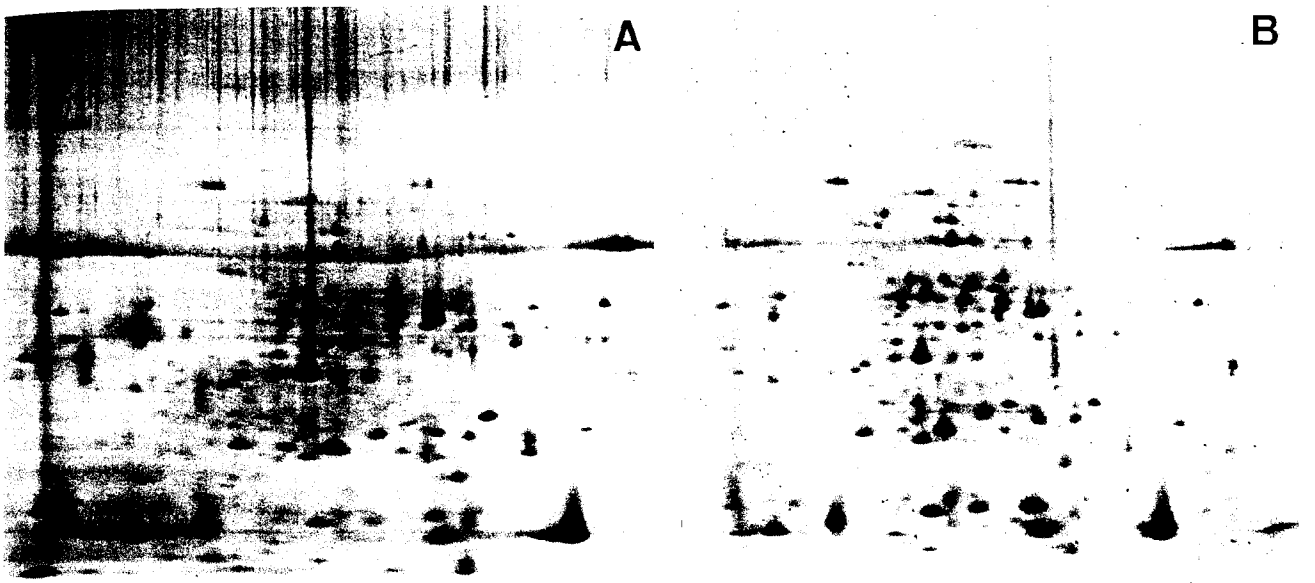


Figure 6. Point streaking in (A), due to presence of DTT in the equilibration solution, is eliminated in (B) by adding iodoacetamide to the second equilibration solution. IPG-Dalt of leaf proteins from barley using PhastSystem [20].

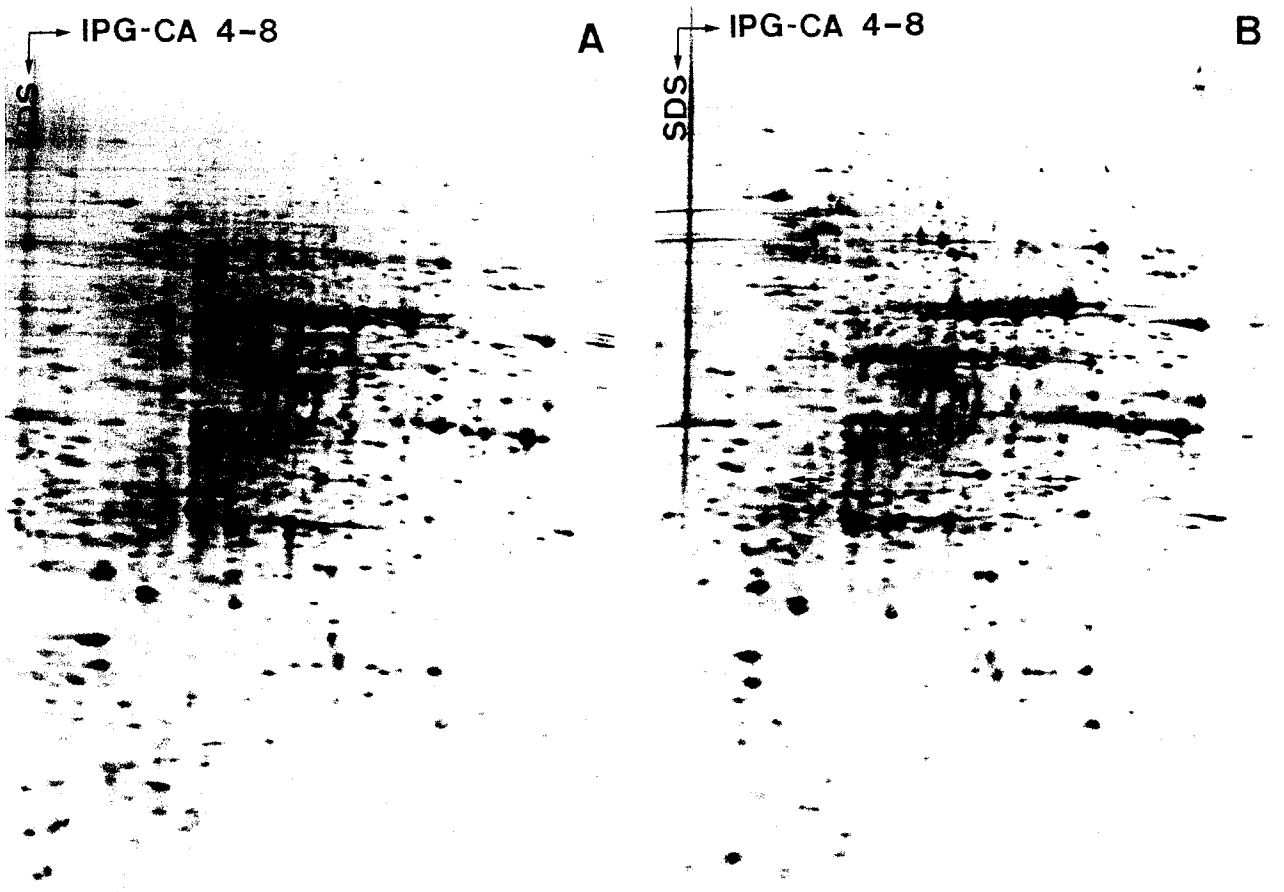


Figure 7. Horizontal IPG Dalt of yeast cell proteins (Saccharomyces uvarum). First dimension: IPG-CA 4–8, 8 M urea, 0.5 % NP-40. Second dimension was performed with (A) discontinuous buffer system: 125 mM Tris-HCl, pH 6.8, (stacking gel) and 375 mM Tris-HCl, pH 8.8, (running gel) or (B) continuous buffer system: 375 mM Tris-HCl, pH 8.8, (stacking and running gel). 2-D maps do not differ consistently. Slightly distorted protein spots are indicated by arrows.

Figure 8. Horizontal IPG-Dalt of yeast cell proteins (*Saccharomyces uvarum*) in the absence of CA. Compared to the 2-D patterns obtained with IPG-CA (Fig. 7), there is no evident difference, indicating an impaired sample entry or protein transfer from first- to second-dimensional gel or protein adsorption to the IPG gel in the absence of CA.

not differ consistently. Occassionally, when continuous buffer systems are used, partially wavy protein spots are obtained (indicated by arrows in Fig. 7B).

3.1.13 Addition of CA to first-dimensional IPG gels

Adding CA to the IPG gel has some disadvantages with respect to equilibrium IEF. Compared with IEF in IPG gels, there is less tolerance of maximum voltage and running time. Voltage has to be decreased and with prolonged focusing time the focused IEF patterns deteriorate due to liquid exudation. However, it is essential to conduct electrofocusing to its steady state, not only in order to improve the reproducibility of 2-D patterns but also to eliminate horizontal and vertical streaking in the 2-D patterns, which is obtained when IEF is terminated during its transient state [9]. Additionally, when CA is added to IPG gels, spot variations from gel to gel within a run were described [15]. Recommendations to decrease Immobiline concentrations (down to 1/16) and to add more CA instead (up to 4 % [35]) create new problems, so that, depending on the Immobiline/CA ratios, IEF running conditions have to be individually optimized for optimum resolution without equilibrium [45]. This adversely effects the approach of the IEF with IPG to obtain stationary 2-D patterns for improved reproducibility and spot matching in inter-laboratory comparison.

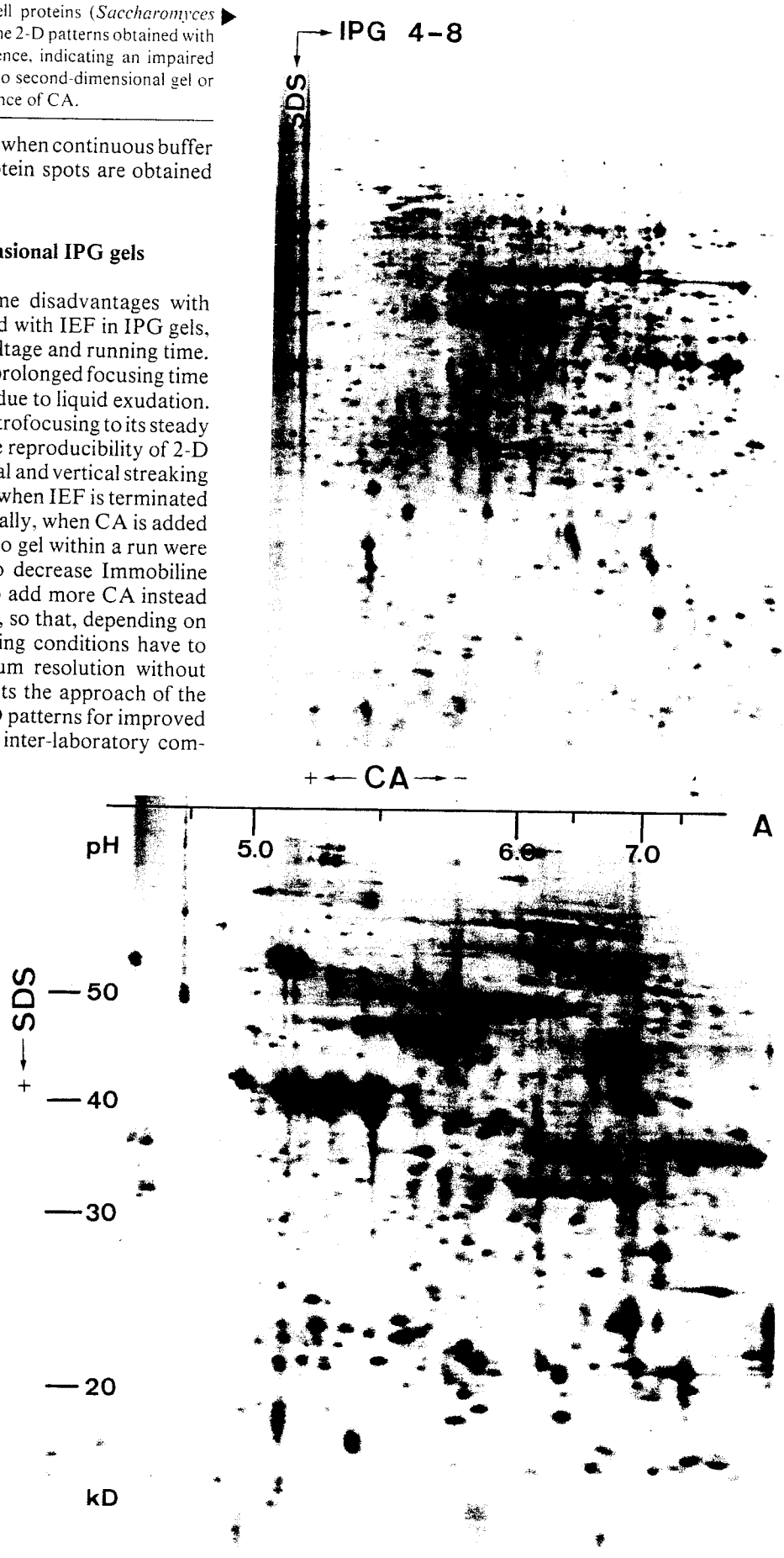


Figure 9. IPG-Dalt *versus* Iso-Dalt. Comparison of 2-D patterns of bean proteins (Vicia faba, Kristall) obtained after first-dimensional IEF with (A) CA or (B) Immobilines without CA. The resulting 2-D maps indicate that there is no visible protein adsorption or poor protein transfer from the first to the second dimension when IPG-Dalt is performed according to Section 2.2.

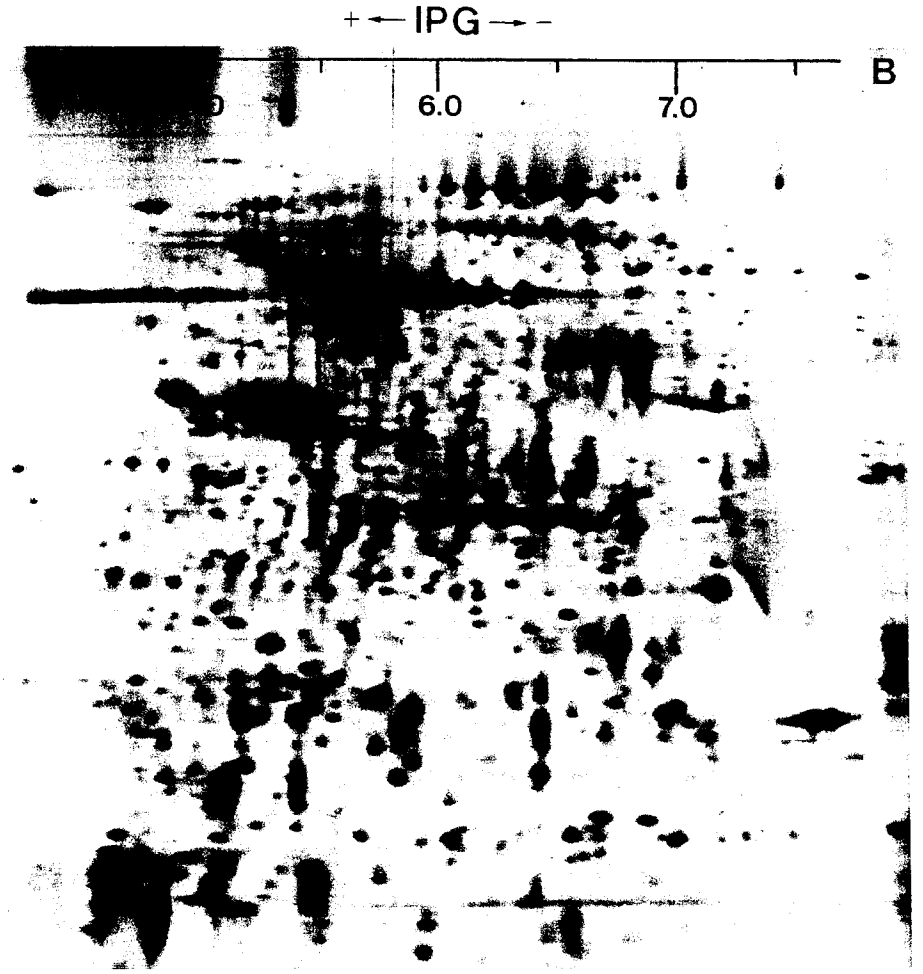
However, by adding only 0.5 % w/v CA to IPG gels with standard Immobiline concentrations, 2-D patterns with constant spot positions along the IEF axis (pH 7–10) were obtained [9], so that the decision whether CA should be added or not, does not have to be made at the expense of stationary 2-D patterns. In our experience, excellent 2-D patterns of plant proteins from leaf, stem or seeds solubilized in lysis buffer (8 M urea, 0.5 % NP-40 and 0.8 % CA) were obtained without adding CA to the IPG gel. In contrast, consistently clearer 2-D patterns of lyophilyzed yeast cells (*Saccharomyces cerevisiae*) or myeloblast proteins [9] were obtained when 0.5 % CA were added to the IPG gel. This effect is not any longer obvious when stabilized Immobilines, preferably dissolved in propanol [47] were used for pH gradient casting (Figs. 7 and 8). There is no striking difference in the 2-D patterns obtained with IPG-CA 4–8 (Figs. 7A and B) or IPG 4–8 (Fig. 8). There are a number of parameters which might explain the occassional advantages of adding CA to first-dimensional IEF with IPG. However, as long as this mechanism is not fully understood, the recommendation for the addition of CA to the IPG gel depends on the type of sample to be analyzed. This is only true for IEF in washed IPG gels; when IEF is performed in IPG tube gels, these gels must always contain CA.

3.2 IPG *versus* CA focusing

Protein adsorption to IPG gels and/or insufficient protein transfer from first- to second-dimensional gels has been described [35, 45, 46]. Comparing the 2-D patterns of legume seed proteins obtained with first-dimensional IEF in pH gradients generated with CA (Fig. 9A) or Immobilines (Fig. 9B), there is no evident difference with respect to the amount of proteins detected in the resulting 2-D patterns. In both cases large amounts of α-legumin and vicilin (storage proteins of broad beans) dominate the 2-D patterns. The resolution along the IEF axis varies because a non-linear CA pH gradient (approximately pH 4–7, by mixing 1 % Ampholines pH 4–6 and 1 % Ampholines 5–7 with 0.5 % Ampholines 3.5–9.5) or a linear IPG 4–8 without added CA were used, respectively. Comparing these two 2-D patterns, there is apparently no need to dilute Immobiline concentrations or to add increased amounts of CA to the IPG gel when IPG-Dalt is performed according to the procedure described here (see Section 2.2) and fresh Immobiline solutions, preferably dissolved in propanol [47], are used.

3.3 IPG-Dalt *versus* non-equilibrium pH gradient electrophoresis

The main purpose of using IPG for 2-D electrophoresis is to overcome the irreproducibility of IEF patterns due to batch variations of CA and pH gradient instability. Because CA generated pH gradients are not stable with time, stationary focusing patterns are not obtained in practice. A maximum of equilibrium and resolution in IEF patterns is achieved with long focusing times, described in the classical 2-D protocols [10–14] based on O'Farrell's method [10]. Under these conditions acidic and neutral proteins have enough time to reach their isoelectric points, at the expense of basic proteins, however. Due to the severe cathodic drift with prolonged focusing times, the resultant pH gradient does not extend significantly above pH 7.5, so that basic proteins migrate off or appear as streaks in 2-D patterns. In order to overcome these problems, a method of non-equilibrium pH gradient electrophoresis (NEPHGE) has been devised [48] in which the sample is loaded at the anode and focusing times are reduced,



so that the basic proteins are retained in the gel. The non-equilibrium conditions result in 2-D patterns with distinct spots; reproducibility, however, has proven sensitive to experimental conditions, focusing time, gel length, composition of the CA and the sample used. With the introduction of IPG for the first time, basic proteins have been separated under equilibrium conditions. Stationary 2-D patterns with distinct protein spots were obtained [9]. In Fig. 10 horizontal IPG-Dalt of basic myeloblast proteins is demonstrated.

3.4 Blow-up experiments

One of the most interesting features of IPG for 2-D electrophoresis is not only pH gradient stability but also the practical feasibility of setting up narrow or ultranarrow pH gradients. The ideal 2-D pattern with protein spots equally distributed over the entire gel often exists only in the theory. Most of the time, the evaluation of 2-D patterns of complex protein mixtures is limited by severe crowding of protein spots in different areas of the 2-D map. Improved resolution of myeloblast proteins or yeast cell proteins has been demonstrated by using separate pH 4–7 and 7–10 first-dimensional IPG gels instead of one pH 4–10 gel [34]. Resolution of 2-D maps is increased further when narrow pH gradients and/or longer separation distances are used. An example of improved resolution is given in Fig. 11, where pea proteins were separated in IPG 5–8 and IPG 5.5–6.5; alternatively, the gel length of the IPG gel 5.5–6.5 was increased from 10 cm (ΔpH = 0.1/cm) to 16 cm (ΔpH = 0.06/cm). Resolution of crowded protein spots is consistently improved in the latter case (Fig. 11). However, when narrow pH gradients and/or longer separation distances are used, running times have to be checked carefully. Focusing time has to be essentially prolonged, otherwise horizontal streaks instead of distinct spots are obtained in the resulting 2-D patterns, due to insufficient focusing of the proteins.

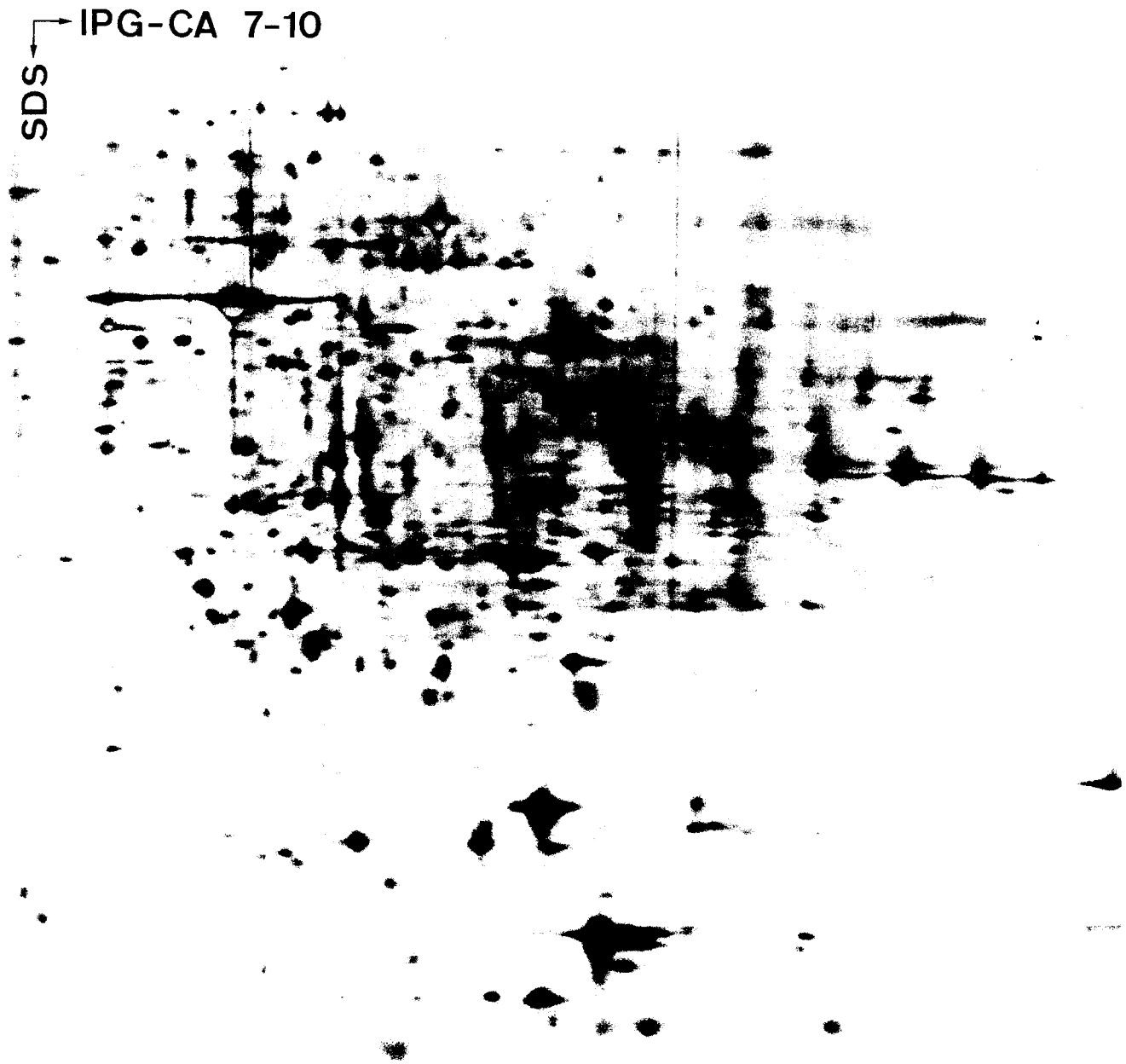


Figure 10. IPG-Dalt *versus* NEPHGE. Horizontal IPG-Dalt of alkaline myeloblast proteins. First-dimensional IEF was in an IPG 7–10 at the steady state. Distinct protein spots with constant spot positions along the IEF axis are obtained [9].

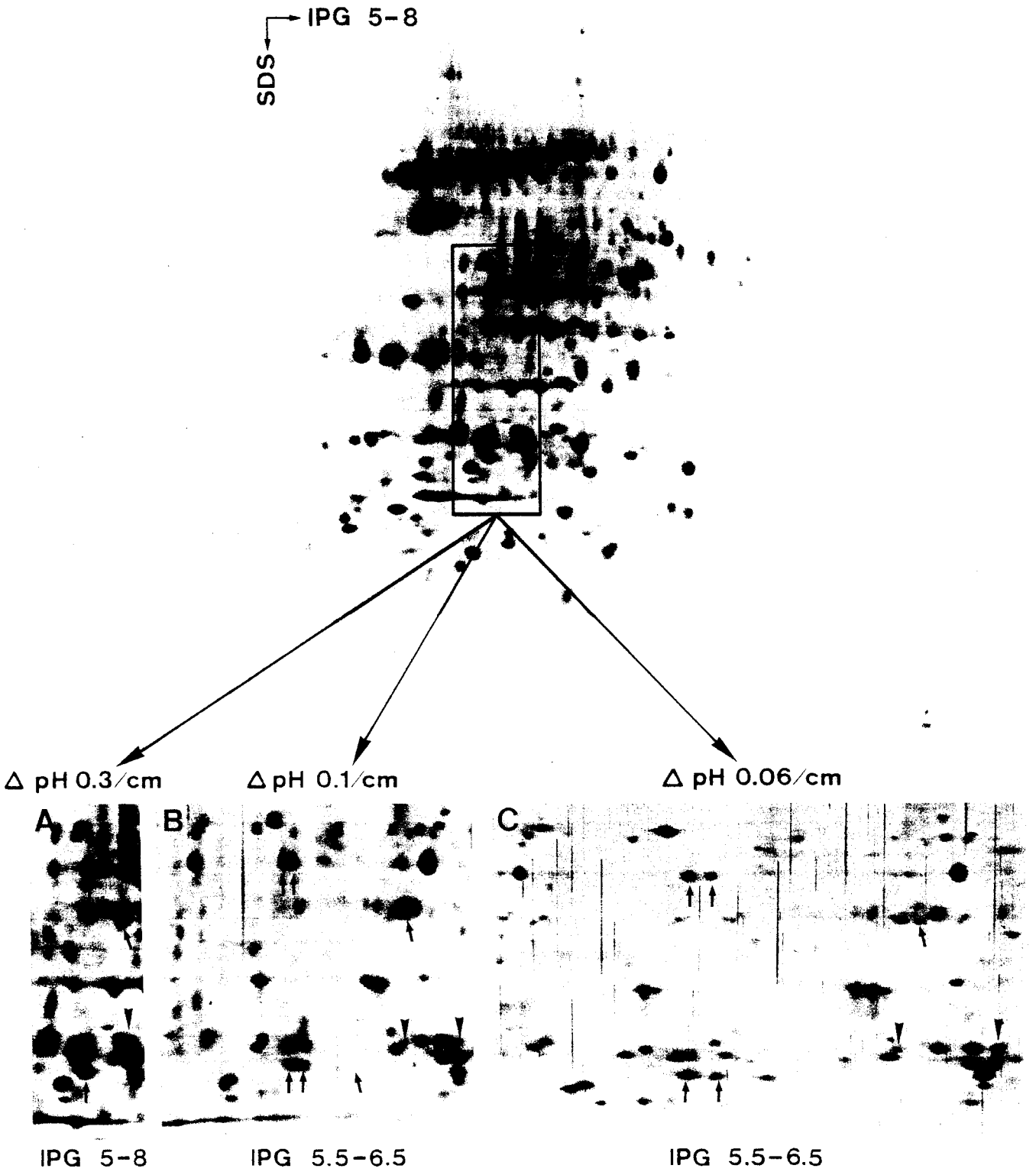


Figure 11.. Blow-up experiments. Improved resolution of clustered protein spot areas by using narrow or/and long separation distances in the first dimension. 2-D map of pea proteins with an IPG 5–8, 110 mm long, as first dimension on top. Close-up of the window marked in the 2-D map (A). Blow-ups of (A) by using a narrow IPG 5.5–6.5, 11 cm long (B) or by using an IPG 5.5–6.5 over a gel length of 16 cm (C).

3.5 Micro-scale versions and automated procedures

Compared to the blow-up experiments described in section 3.4, micro-scale versions of IPG-Dalt are consistently time saving [31]. Steady-state IEF patterns in an IPG of 4–7 over a gel length of 50 mm was achieved within 75 min. By using small gels (50 × 43 mm^2) and a horizontal electrophoresis system with programmed running conditions and automated staining procedures (PhastSystem [49]), silver-stained 2-D patterns were obtained within 3.5 h [20].

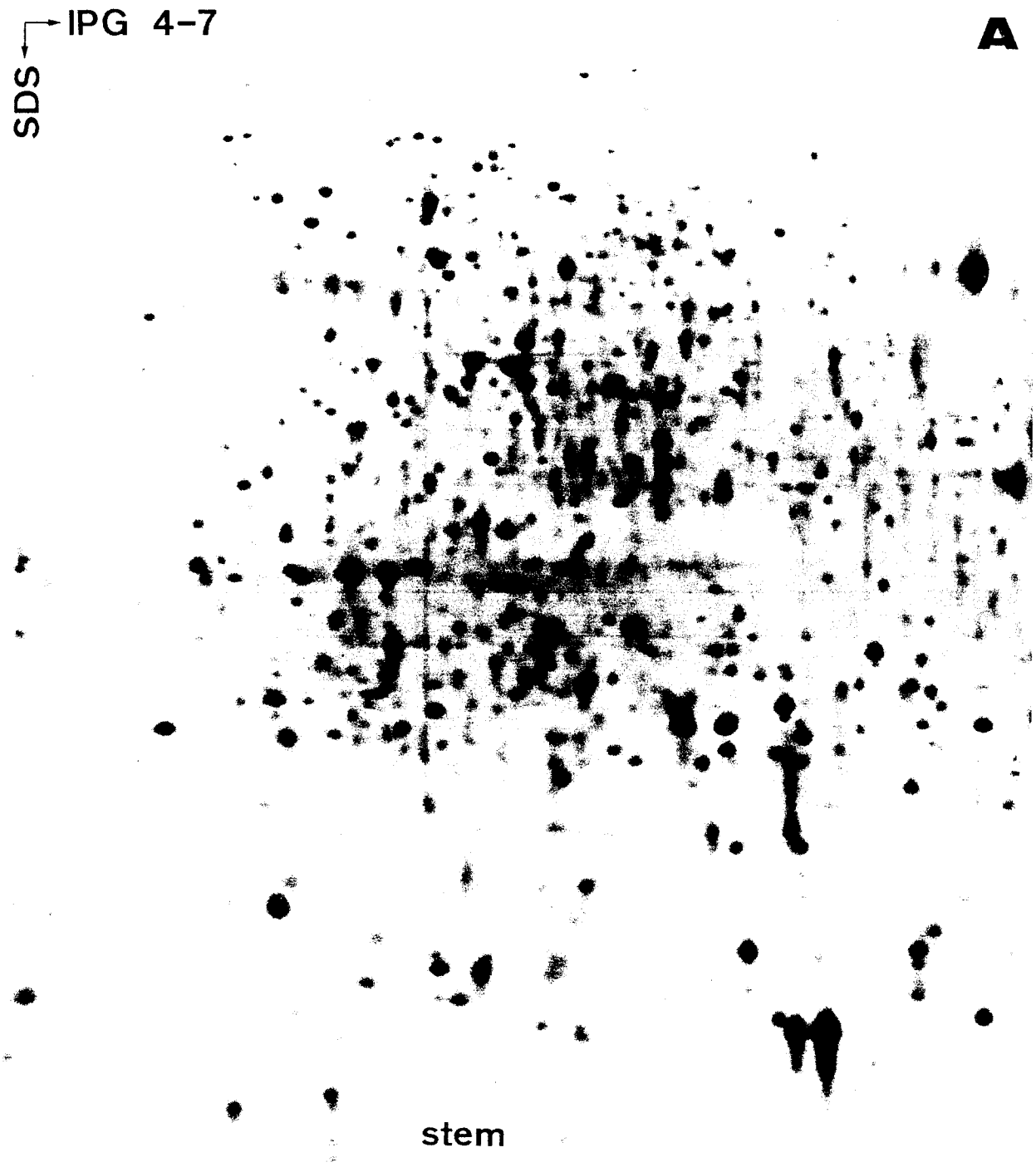


Figure 12. Horizontal IPG-Dalt of stem (A) and leaf (B, on opposite page) proteins from barley (*Hordeum vulgare*). Procedure: see Section 2.2. First dimension: IPG DryPlate 4–7, with 8 M urea and 0.5 % NP-40, no CA were added to the IPG gel. Second dimension: Horizontal SDS gel cast on washed GelBond PAG film.

3.6 IPG-Dalt for routine analysis

Routine analysis is ideally performed with prefabricated first- and second-dimensional gels, leaving the production and quality control to manufacturers. Prefabricated IEF and SDS gels, cast on plastic backings, are easily combined for horizontal 2-D electrophoresis [31]. In Fig. 12, horizontal 2-D electrophoresis of barley proteins from leaf and stem with premanufactured Immobiline DryPlates pH 4–7 (Pharmacia-LKB) are demonstrated. The reproducibility of the 2-D patterns was successfully tested by analyzing more than 30 varieties of barley (to be published). Strips, cut off from the IPG DryPlate, were rehydrated and used for IEF. After equilibration, the IPG gel strip on plastic backing was transferred to the second dimension without stretching and placed onto the surface of a horizontal SDS gel. Prefabricated IPG gels with well defined, time-stable pH gradients in combination with prefabricated second-dimensional SDS gels on plastic backings will certainly facilitate routine analysis and inter-laboratory comparison of 2-D patterns.

4 Concluding remarks

Sumarizing the critical parameters and remedies described here to obtain excellent 2-D patterns with IPG of complex protein mixtures, the most important points seem to be that sample entry should be slow and only diluted protein samples should be used, and applied to individual IPG gel strips, which should be as narrow as possible (< 5 mm), minimizing the problems of electroendosmosis, excess detergent and insufficient stacking in the second-dimensional gel. IEF conducted to equilibrium not only consistently improves the reproducibility of 2-D patterns but also minimizes horizontal and vertical streaking in the 2-D map due to insufficient focusing times. The presence of glycerol and urea in the equilibration buffer additionally prevents the IPG gel strips from digging into the SDS gel with resultant poor protein transfer from the first to the second dimension. The addition of 0.5 % CA to IPG gels can be beneficial; however, it is not essential for 2-D electrophoresis. Comparison of the 2-D patterns obtained with CA or IPG in the first-dimensional gel demonstrates

that under the conditions described here, protein adsorption to IPG gels or poor protein transfer from the first- to the second-dimensional gel does not occur. Proposals to dilute Immobiline concentrations and to add increased amounts of CA instead, are not only contradictory to equilibrium IEF, the main purpose for using IPG, but also unnecessary. The use of IPG in combination with prefabricated gels is certainly the most promising approach for highly reproducible 2-D patterns in routine.

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5 References

[1] Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Görg, A., Westermeier, R. and Postel, W., *J. Biochem. Biophys. Methods* 1982, *6*, 317–339.

[2] Görg, A., Postel, W., Weser, J., Patutschnick, W. and Cleve, H., *Am. J. Human Genet.* 1985, *37*, 922–930.

[3] Cleve, H., Patutschnick, W., Postel, W., Weser, J. and Görg, A., *Electrophoresis* 1982, *3*, 342–345.

[4] Görg, A., Weser, J., Westermeier, R., Postel, W., Weidinger, S., Patutschnick, W. and Cleve, H., *Hum. Genet.* 1983, *64*, 222–226.

[5] Righetti, P. G. and Gelfi, C., *Rev. Franc. Transf. Immun. Hematol.* 1984, *27*, 671–690.

[6] Bianchi Bosisio, A., Rochette, J., Wajcman, H., Gianazza, E. and Righetti, P. G., *J. Chromatogr.* 1985, *330*, 299–306.

[7] Görg, A., Postel, W., Günther, S. and Weser, J., *Prot. Biol. Fluids* 1986, *34*, 701–705.

[8] Hanash, S. M., Strahler, J. R., Somerlot, L., Postel, W. and Görg, A., *Electrophoresis* 1987, *8*, 229–234.

[9] Görg, A., Postel, W., Günther, S., Weser, J., Strahler, J. R., Hanash, S. M., Somerlot, L. and Kuick, R., *Electrophoresis* 1988, *9*, 37–46.

[10] O'Farrell, P. H., *J. Biol. Chem.* 1975, *250*, 4007–4021.

[11] Klose, J., *Humangenetik* 1975, *26*, 231–243.

[12] Anderson, N. G. and Anderson, N. L., *Anal. Biochem.* 1978, *85*, 331–340.

[13] Dunn, M. J. and Burghes, A. H. M., *Electrophoresis* 1983, *4*, 97–116.

[14] Celis, J. E. and Bravo, R., *Two-Dimensional Gel Electrophoresis of Proteins*, Academic Press, Orlando, FL 1984.

[15] Strahler, J. R., Hanash, S. M., Somerlot, L., Weser, J., Postel, W. and Görg, A., *Electrophoresis* 1987, *8*, 165–173.

[16] Damerval, C., de Vienne, D., Zivy, M. and Thiellement, H., *Electrophoresis* 1986, *7*, 52–54.

[17] Görg, A., Postel, W., Günther, S. and Weser, J., *Electrophoresis* 1985, *6*, 599–604.

[18] Görg, A., Postel, W., Westermeier, R., Gianazza, E. and Righetti, P. G., *J. Biochem. Biophys. Methods* 1980, *3*, 273–284.

[19] Gianazza, E., Celentano, F., Dossi, G., Bjellqvist, B. and Righetti, P. G., *Electrophoresis* 1984, *5*, 88–97.

[20] Görg, A., Postel, W., Günther, S. and Friedrich, C., *Electrophoresis* 1988, *9*, 57–59.

[21] Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J. R., Hanash, S. M. and Somerlot, L., *Electrophoresis* 1987, *8*, 122–124.

[22] Görg, A., Postel, W., Westermeier, R., Gianazza, E. and Righetti, P. G., in: Allen, R. D. and Arnaud, P. (Eds.), *Electrophoresis '81*, de Gruyter Berlin 1981, pp. 257–270.

[23] Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, H., *Science* 1981, *211*, 1437–1438.

[24] Laemmli, U. K., *Nature (London)* 1970, *277*, 680–685.

[25] Westermeier, R., Postel, W., Weser, J. and Görg, A., *J. Biochem. Biophys. Methods* 1983, *8*, 323–330.

[26] Iborra, G. and Buhler, J. M., *Anal. Biochem.* 1976, *74*, 503–511.

[27] Goldsmith, M. R., Rattner, C. E., Koehler, M. M. D., Balikov, S. R. and Bock, S. C., *Anal. Biochem.* 1979, *99*, 33–40.

[28] Görg, A., Postel, W. and Westermeier, R., *Anal. Biochem.* 1978, *89*, 60–70.

[29] Görg, A., Postel, W., Westermeier, R., *Z. Lebensm. Unters.Forsch.* 1979, *168*, 25–28.

[30] Radola, B. J., *Electrophoresis* 1980, *1*, 43–56.

[31] Görg, A., Postel, W., Günther, S. and Weser, J., in: Dunn, M. J. (Ed.), *Electrophoresis '86*, VCH, Weinheim 1986, pp. 435–449.

[32] Gianazza, E., Rabilloud, T., Quaglia, L., Caccia, P., Astrua-Testori, S., Osio, L., Grazioli, G. and Righetti, P. G., *Anal. Biochem.* 1987, *16*, 247–257.

[33] Righetti, P. G. and Gianazza, E., *Bull. Mol. Biol. Med.* 1985, *10*, 387–402.

[34] Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J. R., Hanash, S. M. and Somerlot, L., *Electrophoresis* 1987, *8*, 45–51.

[35] Hochstrasser, D., Augsburger, V., Funk, M., Appel, R., Pellegrini, C. and Muller, A. L., *Electrophoresis* 1986, *7*, 505–511.

[36] Fawcett, J. S. and Chrambach, A., *Electrophoresis* 1986, *7*, 260–266.

[37] Gianazza, E., Tedesco, G., Catto, N., Bontempi, L. and Righetti, P. G., *Electrophoresis* 1988, *9*, 172–182.

[38] Klose, J. and Feller, M., *Electrophoresis* 1981, *2*, 12–24.

[39] Fawcett, J. S. and Chrambach, A., *Prot. Biol. Fluids* 1985, *33*, 439–442.

[40] Altland, K. and Rossmann, U., *Electrophoresis* 1985, *6*, 314–325.

[41] Rimpilainen, M. and Righetti, P. G., *Electrophoresis* 1985, *6*, 419–422.

[42] Görg, A., Postel, W. and Günther, S., in: Schäfer-Nielsen, C. (Ed.), *Electrophoresis '88*, VCH, Weinheim 1988, pp. 57–72.

[43] Hanash, S. M., Baier, L. J., Mc Curry, L. and Schwartz, S. A., *Proc. Natl. Acad. Sci. USA* 1986, *83*, 807–811.

[44] Righetti, P. G., Gelfi, C. and Gianazza, E., *Chimica Oggi* 1986, *3*, 55–60.

[45] Altland, K., von Eckardstein, A., Banzhoff, A., Wagner, M., Rossmann, U., Hackler, R. and Becher, P., *Electrophoresis* 1987, *8*, 52–62.

[46] Righetti, P. G., Gelfi, C., Bossi, M. L. and Boschetti, E., *Electrophoresis* 1987, *8*, 62–70.

[47] Gåveby, B.-M., Andrasko, J., Ineva-Flygare, L., Johannesson, U., Görg, A., Postel, W., Domscheit, A., Mauri, P. L., Pietta, P., Gianazza, E. and Righetti, P. G., *J. Biochem. Biophys. Methods* 1988, *16*, 141–164.

[48] O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H., *Cell* 1977, *12*, 1133–1142.

[49] Brewer, J., Grund, E., Hagerlid, P., Olsson, I. and Lizana, J., in: Dunn, M. J. (Ed.), *Electrophoresis '86*, VCH, Weinheim 1986, pp. 226–229.