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Review

Electrophoresis: The march of pennies, the march of dimes

Pier Giorgio Righetti *

University of Verona, Department of Agricultural and Industrial Biotechnology, Strada Le Grazie 15, Verona 37134, Italy

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Abstract

The present review encompasses ca. 65 years of history of developments in electrokinetic separations, taking as a starting point the year 1937, i.e. the official launching of Tiselius' moving boundary electrophoresis (MBE). The 1950s have been particularly rich in introducing novel methodologies in zone electrophoresis (ZE), thus bringing about the decline of MBE. Among them of extraordinary importance was the development of electrophoresis on agar gels coupled to immuno-diffusion at right angles, which brought a big revolution not only in biochemistry but also in clinical chemistry. Also the by now forgotten paper electrophoresis was a landmark in separation science, in that it implemented, in its "fingerprinting" version, the first genuine two-dimensional (2D) map, coupling orthogonally a charge to a hydrophobic scale separation, while permitting for the first time the detection of spot mutations, i.e. single amino acid replacements in a polypeptide chain, that paved the way to modern genetic analysis. Equally important was the introduction of starch-block electrophoresis, that brought about the notion of sieving and the first discontinuous buffers, refined, in the 1960s, by Ornstein and Davies with their classical papers combining multiphasic buffer systems to polyacrylamide gels, that went down to history as disc-electrophoresis. The 1960s also contributed with two fundamental techniques, isoelectric focusing (IEF) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) that permitted to discriminate proteins solely on the basis of surface charge and molecular mass, respectively. The 1970s gave other fundamental contributions, such as isotachophoresis, the first example of a fully instrumental approach to electrophoresis, both in its analytical and preparative version (Tachophor and Tachofrac), 2D maps combining IEF to SDS–PAGE at right angles and silver staining techniques, that incremented sensitivity by 3 orders of magnitude. The 1980s generated immobilized pH gradients and capillary zone electrophoresis (CZE), two big players that dominated the electrokinetic horizon for all the 1990s and still in vigorous use in present days. The review terminates with a glimpse, in the third millennium, onto microchip technology and hyphenated techniques, notably direct interfacing of various electrophoretic separation methods with mass spectrometry (MS).

Keywords: Electrophoretic methodologies; Isoelectric focusing; SDS-electrophoresis; Isotachophoresis; Immuno-electrophoresis; Disc-electrophoresis; Capillary zone electrophoresis; Two-dimensional maps; Microchip electrophoresis; Hyphenated techniques; Silver stains

Contents

* Tel.: +39 045 8027901; fax: +39 045 8027929.
E-mail address: righetti@sci.univr.it.

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1. Introduction

Eheu fugaces, Postume, Postume,
Labuntur anni, nec pietas moram
Rugis et instanti senectae
Adferet indomitaeque mortis . . .
(Quintus Horatius Flaccus, Carmina 2, 14).

It is a privilege to have been asked to write this review in honour of Professor Csaba Horvath, not only a very good friend of mine, but also one of the most brilliant scientists and most charming human being I have ever met in my long scientific career. I only hope he will not revolt in his tomb for this lousy treatise and bear with me with the same patience and tolerance he always exhibited any time I approached him to illuminate me in the obscure paths of science. Although he went down to history as one of the most sagacious inventors in chromatography (his first prototype of an HPLC instrument was still standing in his office at Yale last time I visited him), he has also made some outstanding contributions in the field of electrophoresis, when he moved into it through the rising star of the 1990s, capillary zone electrophoresis (CZE), bringing to this field his unique knowledge in chromatography. It was the "Marriage of Figaro", although the effervescent music of Mozart was missing. Albeit the leitmotif of this special issue is "separation science: Past, Present and Future" I have decided to concentrate this historical survey especially on the past (though not disdaining the present) for an educational purpose: since journal issues have been placed in the Internet only starting from ca. 1997, it turns out that the vast and incredibly rich body of science of pre-Internet origin is rapidly falling into oblivion. Nobody spends time in libraries any longer (a most unfortunate situation), so all past history is rapidly forgotten and the new generations tend to re-invent the wheel over and over again. If we do not learn from our past we will never be able to build a radiant future. This chronicle will start from an incipit, i.e. the first genuine example of a most powerful analytical technique, the moving boundary electrophoresis (MBE) a la Tiselius and inch its way through modern times. A march that encompasses some 65 years in electrokinetic methodologies.

2. 1937: crossing the columns of Hercules

Was MBE the start of modern separation science? Surely it was. Some people like to quote, as the point of origin, the work of Ferdinand Frédéric Reuss (an officer in the Czar's army in Moscow, although he was born in Tuebingen), who, while moonlighting on the banks of the Moskwa River, instead of fighting Napoleon's army, discovered the phenomenon of electroendoosmosis (EOF, to be rediscovered almost two centuries later in CZE; surely enough, he had filled his U-tube apparatus with sand collected on the river shore, i.e. silica powder!) [1]. His was a rich man experiment: his power supply was a voltaic pile composed of 92 silver rubles and an equal number of zinc plates, something that not even count Volta could afford (more humbly, his pile was a stack of alternate zinc and copper disks, separated by cotton sponges imbued with dilute sulphuric acid). But this would be like claiming that Homer, in his poem the Iliad, laid the foundations of modern war, with its super-extermination weapon power! Tiselius "Moving Boundary Electrophoresis" [2], just like the ultracentrifuge, was a highly sophisticated instrument, equipped with in situ observations devices, able to monitor the movement of proteins molecules in the electric field (perhaps scientists could be the ancestors of present day "pippin toms"). The detection principle was very much the same as that of the ultracentrifuge [3,4]: light shed along the migration path detected the "moving boundaries", i.e. the regions of strong variation of refractive index upon the passage from one macromolecular ion to the next one (in fact to the mixture of all ions having lower mobilities!) (Fig. 1, left side). Since these boundaries would be displayed as sigmoidal transitions, a complex schlieren optics device would transform them into peaks, easily amenable to computational treatment (Fig. 1, right side). The Tiselius instrument made thus possible, for the first time, the accurate measurement of absolute mobilities of proteins [5], since the movement of the macroions was not hindered by the viscous drag of density gradients or by the tortuous channels of support media, as later on occurring in "zone electrophoresis". Moreover, since both, the ascending and descending boundaries in the two limbs of the cell U-tube could be monitored, it was soon realized that departure from enantiography afforded clues to interactions among the species under investigation. Thus, the

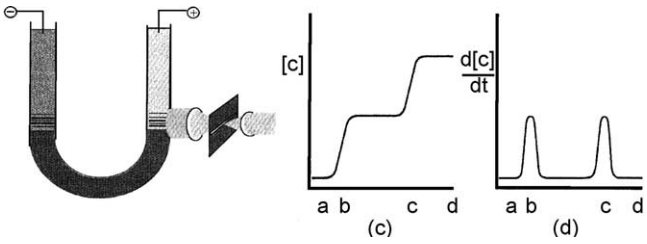

Fig. 1. Left: scheme of the Tiselius U-tube with some protein moving boundaries and detection device (Schlieren optics). Right: profiles of two protein boundaries (*c*, concentration gradients) and of the peaks obtained by taking the derivative of the sigmoidal transitions (d).

very limit of boundary electrophoresis, i.e. the failure to permit complete separation of the components of a mixture, became an advantage for the study of many protein–protein, protein–small ion and protein–small molecule interactions [6].

3. 1939: zone electrophoresis (ZE) versus moving boundary electrophoresis: a fight without quarter

In ZE, the analyte ion are fully separated from each other, the space in between being filled by buffer ions. On the contrary, in Tiselius moving boundary (MBE), only the heads and tails represent clean zones, the space in between being filled by the mixtures of all different ions in the preparation. Since a zone of a pure macromolecule, if surrounded by free buffer, would sediment in the gravitational field to the bottom of the chamber, MBE can never be carried to ZE, no matter how long the experiment is continued. Conversely, ZE, since it physically separates the zones among themselves, needs a support medium, acting as an antigravity device, preventing said zones from sedimenting at the bottom of the cell (the principle of the two methods is illustrated in Fig. 2). A host of such support media have been reported, the first (and worst one) being sheets of filter paper. The first indication on the use of "paper" was given by von Klobusitzky and König [7] who

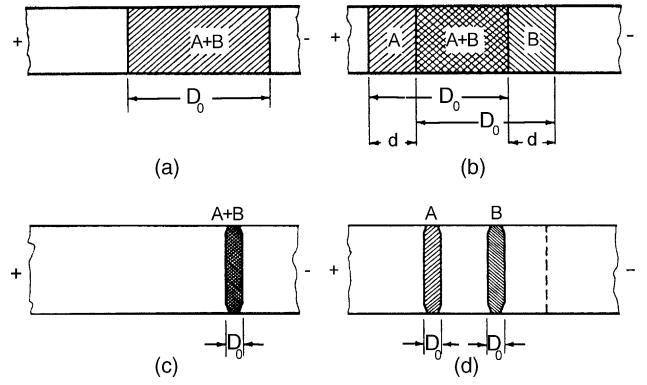

Fig. 2. Diagram of separation during electrophoresis. (a, b): moving boundary electrophoresis; (c, d): zone electrophoresis. a and c show initial protein distribution, b and d that after separation.

employed this support medium for isolating yellow pigments from snake venom. Thus, paper electrophoresis was several years older than paper chromatography, that was first reported in 1944 by Consden et al. [8]. Today, of course, nobody would ever dream of using paper for electrophoresis, if for no other reason because its high content of carboxyls would produce severe streaking due to overimposition of a chromatographic process to electromigration. Yet, in the 1940s and 1950s it enjoyed immense popularity, e.g. for separation of mononucleotides [9], of nucleoside mono-, di- and tri-phosphates [10], of nucleotide coenzyme derivatives [11], of mono-, di-, and tri-nucleotides [12], of amino acids [13] to name just a few [14]. Its popularity was also underlined by the rainbow of nicknames by which it was named around the word: "*papierelektrophorese*" in Germany, Sweden and Switzerland; "*elettroforesi su carta*" in Italy; "*microélectrophorèse sur papier*" in France, "*electroforesis en papel*" in Spain, even "*electroforesa na papire*" in former Czechoslovakia. Fig. 3 (based on a museum jar, now of historical interest only) shows a section of a typical apparatus. A feature of this instrumentation is the presence of carbon tetrachloride (or a similar non-conducting liquid less dense than water) for dissipating heat generated during the run and for preventing evaporation of moisture from the filter paper. Note also how the electrode compartments were physically separated from the electrophoresis tank, so as to avoid contamination from electrolysis ions and oxidation/reduction products at the electrodes. On these large sheets of paper up to 50 samples could be spotted and run simultaneously on a very short time (e.g. 10 min) at high voltage. Some of these ideas (like having the paper strip submerged under organic solvent) were later on exploited for running isoelectric focusing slabs in the alkaline region, so as to prevent CO_2 adsorption [15]. Resurrecting a mummy might not seem an exciting proposition, but I would

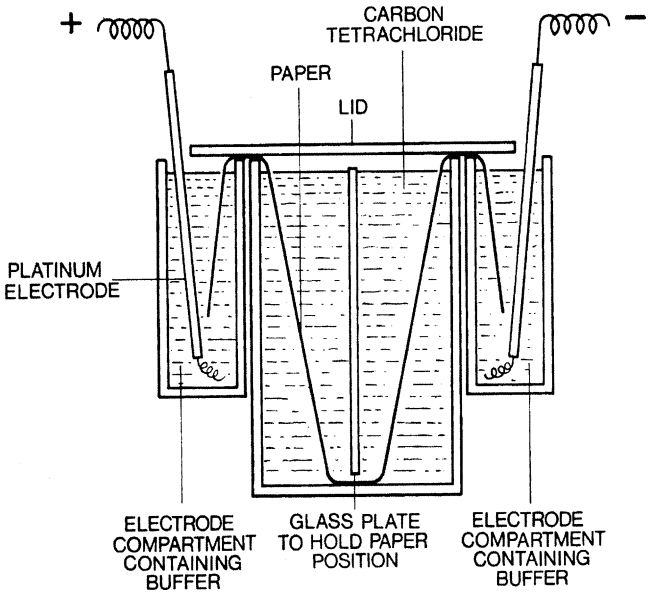

Fig. 3. Scheme of the jar utilized for electrophoresis on large sheets of paper.

like to recall here that paper electrophoresis had more than one valence in modern science. One example will suffice: in 1958–1959, in a now classical series of papers [16–18], Vernon Ingram solved the genetic puzzle of sickle-cell anaemia (Hb S), a pathology that Pauling et al. [19] in 1949, had recognized as the first example of molecular disease. In those days (but even today!) it would have been foolish to think one could detect amino acid substitutions on entire polypeptide chains. Thus, proteins were digested to peptides, which were then separated on the first genuine two-dimensional technique (2D map) ever reported: paper electrophoresis coupled at right-angle to ascending chromatography (see Fig. 4). Aptly so, it was called "fingerprinting", much the same method used today to spot criminals. In the case of haemoglobin (Hb), some 30 peptides of about 10 amino acid each were expected. Now the "criminal" (i.e. the abnormal peptide) could not hide in the crowd any longer: its aberrant behaviour was immediately nailed down, the peptide eluted and, by the magics of the just-developed Pehr Edman degradation [20], the abnormal amino acid characterized. It was a big surprise to find out that such a devastating disease as Hb S was caused by a single amino acid replacement, an Hb$\beta^{6Glu \rightarrow Val}$. With that, modern genetics was born. It is charming to hear the comments of Vernon to his experiments: "I remember that our first fingerprints looked like a modern watercolour left out in the rain" [21]. I do remember too, as a dumbfounded, young post doc at MIT, in the mid 1960s, staring at my friend Corrado Baglioni running around the lab with those giant "cucuruchos" (as a Guatemalan friend would call them), covered with dot and streaks of all violet hues, trying to decode all sorts of Hb mutations [22]. I still wonder if he was doing science or perhaps taking lessons of modern art with maestro Juan Mirò.

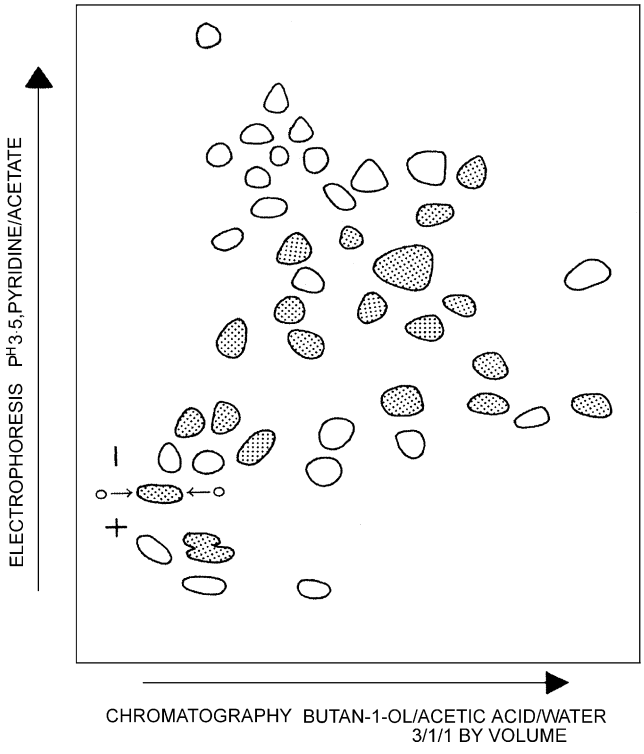

Fig. 4. Scheme of a 2D separation on paper (fingerprinting of peptides). The first dimension is electrophoresis, the second dimension is ascending chromatography at right angles. "O" indicates the sample application point (origin). Note that this is a true 2D techniques, since the first dimension is based on charge, while the second on peptide hydrophobicity.

4. 1950: the rising star of bio-gels

It was in 1950 when Gordon et al. [23] first introduced agar gel electrophoresis. Agar is a polysaccharide derived from marine algae, containing D- & L-galactose subunits, joined predominantly with $1 \rightarrow 3$ bonds. It contains two populations, agaropectin, highly charged with sulphate and carboxyl groups (thus producing strong electroendosmotic flow and sometimes adsorption of proteins) and agarose, an almost neutral variant. A scheme of the experimental set-up is shown in Fig. 5A. In 1959, Wieme [24] introduced a smart variant of this technique, by which the agar gel, supported onto a glass slide, is run in an inverted position. In this way, he eliminates the most noxious contacts with the electrode vessels via paper wicks, which adsorb most of the voltage gradient, due to their low conductivity (further aggravated by the fact that, due to their Ohmic resistance, they tend to dry out and worsen the electrophoretic conditions). In his set-up, the agar gel makes direct contact with agar blocks, imbued with proper buffer, connected via a channel with the

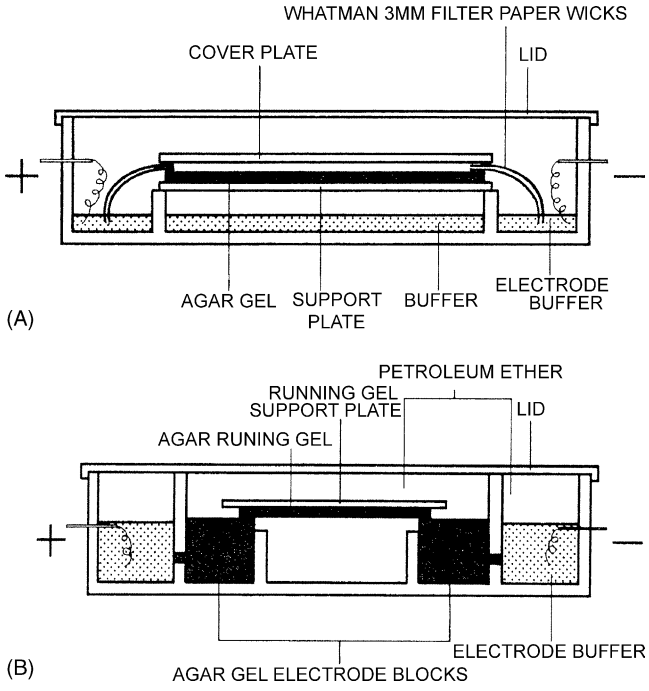

Fig. 5. Upper: scheme of conventional agar-block gel electrophoresis. Lower: drawing of the system of Wieme [24]. Note, in this last case, that the gel plate is run inverted, so as to allow direct contact with agar blocks underneath, thus disposing of paper wicks. Additionally, as the gel is submerged under petroleum ether, heat dissipation is greatly enhanced.

electrodic reservoirs (Fig. 5B). Here too one should appreciate the idea of running the gel submerged under petroleum spirit, which acts both as a heat sink and for preventing water loss from the gel by evaporation. The other smart approach was the use of fairly thin gel layers, typically 1–2 mm thick, minimizing temperature gradients through the gel thickness.

The severe EOF effect which crude agar displays doubtless led to its eclipse as a medium for electrophoresis, although highly purified agarose (whose repeating unit, agarobiose, alternates 1,3-linked β-D-galatopyranose with 1,4-linked 3,6-anhydro-α-L-galactopyranose) especially the one completely desulphated, is used in plenty of immuno-techniques and in isoelectric focusing due to its very large pore size [25]. The unique popularity of agar-gel electrophoresis, though, stems from a paper of Grabar and Williams [26], who reported in 1953 an innovative concept of crossed immuno electrophoresis in agar gels, that was a landmark in all separation techniques. In fact, for the first time, it coupled a separation of a complex matrix (biological fluids, such as sera) with specific detection directly on the same agar plate. The technique was also two-dimensional, in that, orthogonal to a first dimension zone electrophoretic step, an immuno-detection step, driven by simple diffusion, was activated by placing proper antisera in a long trench spanning the length of the electrophoretic migration of all antigens (Fig. 6A and B). It had an immense impact in all biochemical techniques and in clinical chemistry. Although the "boomerang" pattern is only qualitative (the position reveals the identity of the protein), it could also be used for semi-quantitative purposes, in analysis of some

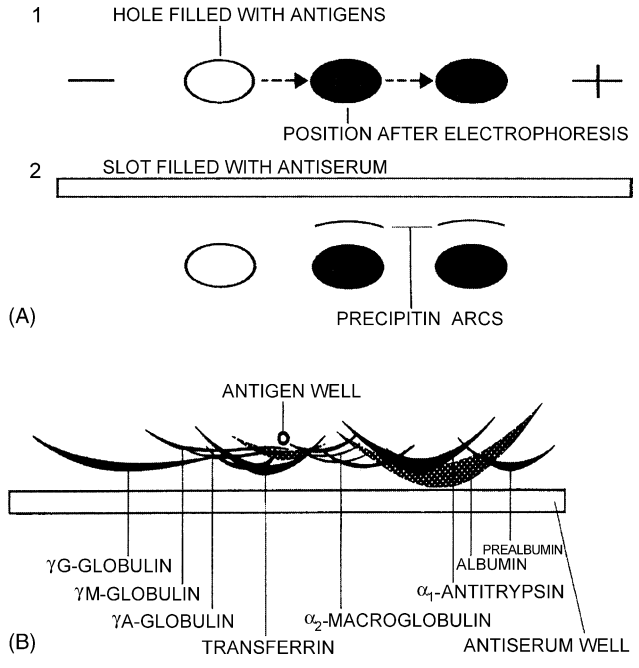

Fig. 6. Upper: drawing of the initial set-up for immunoelectrophoresis according to Grabar and Williams [26]. The trench is filled with antiserum at the end of the electrophoretic step. Lower: drawing of the "boomerang" pattern of the major precipitin arcs obtained in human sera. "Antigen well" indicates the initial sample application slot.

diseases. An avalanche of quantitative methods were then described, such as the most famous Laurell's rocket technique [27] or the crossed-immuno electrophoresis of Clarke and Freeman [28], the latter permitting the visualization of ca. 50 rockets in human sera, whose relative abundance could be assessed by computer-aided area measurements [29]. Ample accounts of all these techniques can be found in a series of special issues of *Scand. J. Immunol*. [30–33] and in [34]: it is surprising how most of these methodologies were developed in the Scandinavian countries, especially in Copenhagen. Although a few of these techniques are still in use today, they surely laid the foundations of present-day immuno-blotting techniques (Western blots) from both 1D sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and 2D gels [35].

5. 1955: the great potato blight!

The discovery by Smithies [36] of the excellent resolving power of starch gels for serum proteins was a major development in electrophoresis. By this technique, Smithies detected haptoglobins in sera. Whereas paper is made of cellulose (linear polymer of 300–2500 glucose units joined by 1,4-β links), potato starch (the best brand being obtained from Canadian potatoes, which forced Smithies to smuggle this powder in frequent boat trips Madison-Toronto, across the great Lakes; not a blight, like the Great Famine that forced 1 million Irish to emigrate to USA towards the end of the XIX century, but a blessing), is made of two polymers, amylose (300 glucose units 1,4-α linked) and amylopectin (same structure, but branched through 1,6-α links). This was a unique event in separation science: since the starch blocks were made in a concentrated (14–15%) polymer matrix, sieving ensued, due to frictional resistance encountered as macromolecules migrated through the relatively small pores of the gel. Thus, the discovery of sieving, in electrophoresis, although not precisely understood, came just about at the same time as Porath was describing sieving of proteins in what went down to history as Sephadex beads for gel filtration [37,38]. The second major discovery brought about by starch–gel electrophoresis was the introduction, by Poulik [39] of discontinuous buffers, with the use of citrate as leading ion and borate as terminating ion. Although starch gels are not any longer in vogue (polyacrylamide gels stamped them out, also because, at the concentration used, they were slightly opaque), it is remarkable that the unique features later on reported with the introduction of polyacrylamides (sieving and disc-buffers) were indeed described quite a few years before the most famous theory of Ornstein and Davis of multi-phasic buffers. Fig. 7A and B gives the two chambers designed by Smithies, the horizontal one in 1955 [36] and the vertical one in 1959 [40]. Although the design of the second chamber is still primitive (the presence of paper wicks does not ensure uniform current densities and regular voltage drops between the electrodes), it overcame a nuisance of horizontal systems: electrodecanta-

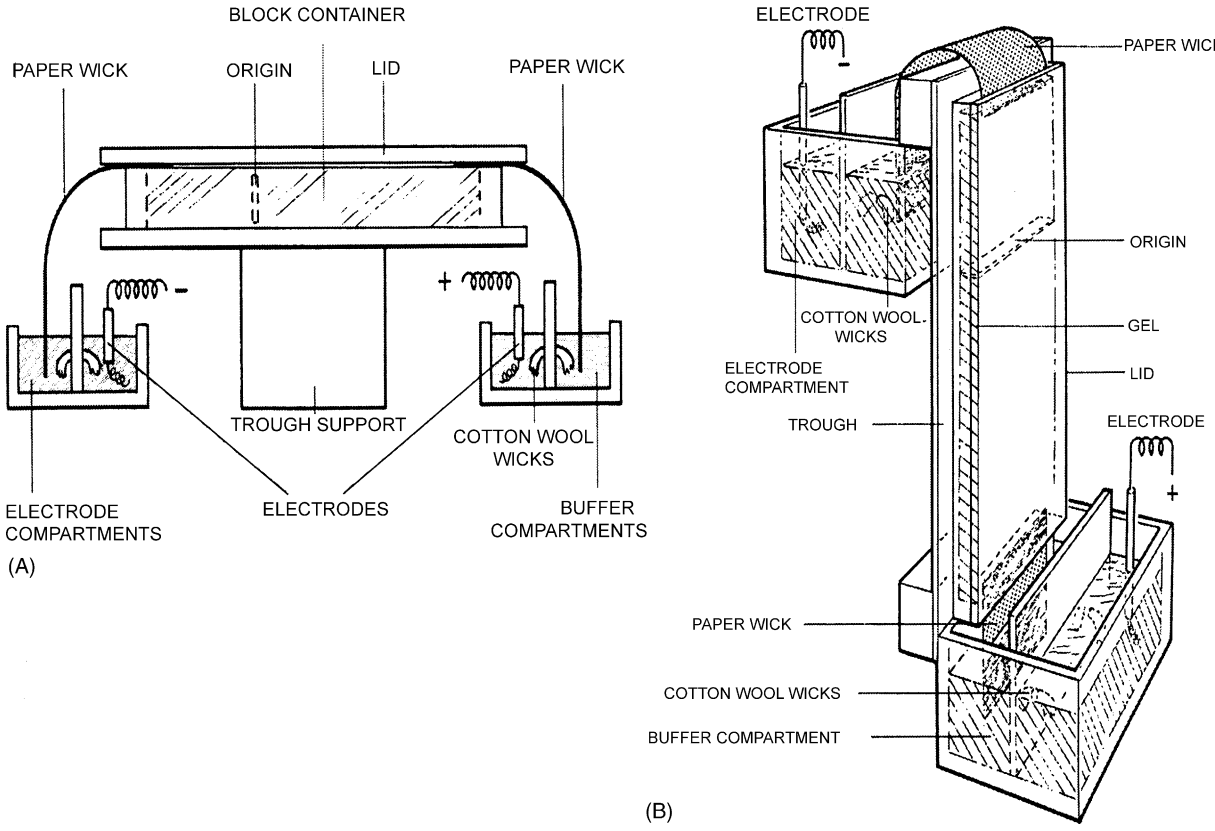

Fig. 7. Left: scheme of the original starch block gel electrophoresis set-up of Smithies [36]. Right: vertical chamber for starch gel electrophoresis according to Smithies [40].

tion. As the sample, applied in a pocket, moves against the gel wall, it is highly concentrated and slides down to the bottom of the pocket. Thus, high mobility proteins, in the horizontal set-up, are found only at the gel bottom, rather than being uniformly distributed throughout the gel thickness. The other major drawback of the system shown in Fig. 7A is the incredible gel thickness (1 cm), exaggerated for today standards (considering that Radola described, already in 1980, for IEF, gel layer thicknesses of barely 50 μm) [41].

6. 1957: a chemically modified, natural polymer strip

When, in 1957, Kohn [42,43] described cellulose acetate (CAc) electrophoresis, this also represented a big revolution in the field of clinical analysis. CAc is basically acetylated cellulose and is a very homogenous medium of uniform pore size containing no more than traces of impurities. Adsorption of proteins by this medium is minimal so that trailing does not occur. This results in much sharper zones than on paper. In addition, the supporting foil is extremely thin (100 μm) and can be easily stained, destained and dried for a permanent record. A standard buffer (for decades): barbitone, pH 8.6, later abolished in force of the law against narcotics. Staining was performed with Ponceau S, Naphthalene black, Azocarmine B, Nigrosine, Leuco-malachite green, Oil Red O for lipoproteins. CAc strips could be made transparent with a mixture of cyclohexanone–ethanol (30:70, v/v). Although modern scientists claim that CZE was the first instrumental approach to electrophoresis, they surely lack a historical background. Cellulose acetate electrophoresis became so widespread the world over in all clinical chemistry laboratories that the instrumentation was made fully automatic: sample loading (applying dozens of samples to acetate strips) staining, destaining, densitometry and peak integration were all performed in a single machine. Although Fig. 8 shows different set-up modes for the acetate strip, the simplest approach was to stretch said strip in a horizontal fashion between the two electrodic vessels. This eliminated one of the most noxious approaches typical of all other systems, namely the use of paper wicks for the electric contact between the gel extremities and the electrode reservoirs; here the contact was direct. Although the use of cellulose acetate in clinical chemistry has dwindled considerably, in several countries (including Italy), this is still the standard approach to fast serum analysis.

7. 1961: electrophoresis in silica gels

Electrophoresis in thin layers of silica gels was first reported in 1961 by Honnegar [44]. For amino acids and amines, it was conducted in 2 M acetic acid and 0.6 M formic

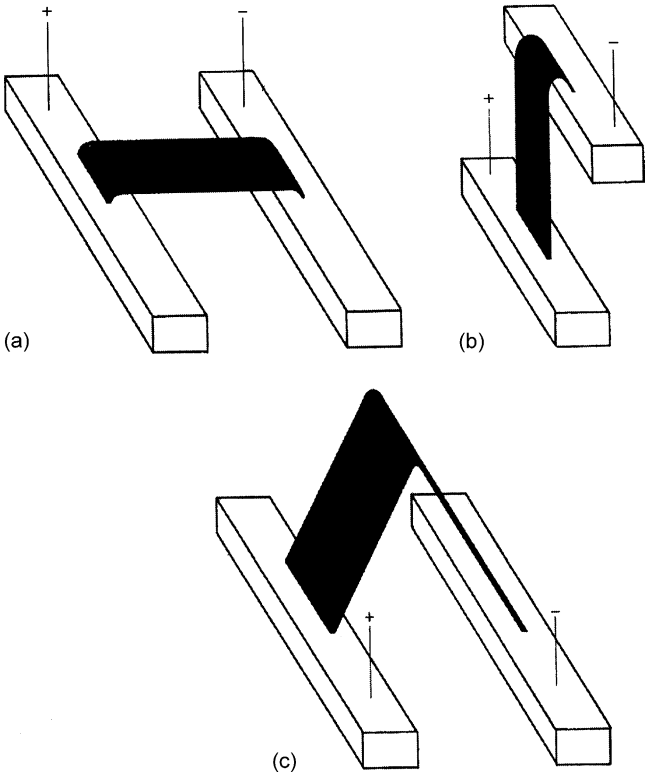

Fig. 8. Drawing of the cellulose acetate electrophoresis set-up in three different configuration of the acetate strip, according to Kohn [42,43].

acid, pH 2.0. For separation of peptide mixtures, a conventional pyridine-acetate buffer, pH 6.5, was generally adopted and voltages up to 1000 V could be applied. As with paper electrophoresis, the silica gel could be dried, after the first dimension electrophoresis, and run chromatographically in a second dimension orthogonal to the first one, thus producing "micro-fingerprints" of peptides [45,46]. Since the slab plates were 20 cm × 20 cm, the resolving power was quite unique for the epoch: e.g., Ritschard [47] resolved 60 components from a tryptic digest of myosin in a single plate. There are some interesting innovation in this methodology: first of all, the use of volatile buffers, enabling easy transfer from the first to the second dimension conditions; secondly, the first use, in electrophoresis, of circulating coolant in the cavity of the supporting metal plate (see Fig. 9), a feature that later on became standard in slab-gel IEF [48]. Other thin layers were adopted in the same set-up: slurries of Pevikon (a copolymer of polyvinyl chloride and polyvinyl acetate) or of cellulose [49]. Interestingly, here too beds made of Pevikon C-870 beads (100 μm diameter) were later-on adopted for analytical and small-scale preparative IEF [50,51]. By the same token, Kohn's acetate foils were re-discovered in 1978 for fast IEF, due to the unhindered protein migration to their p*I* values [52–54] (with a proviso, though: the membranes had to be extensively methylated so as to minimize their EOF flow, amplified by the very low ionic strength of a focusing environment, due to some free carboxyl residues in the acetate support).

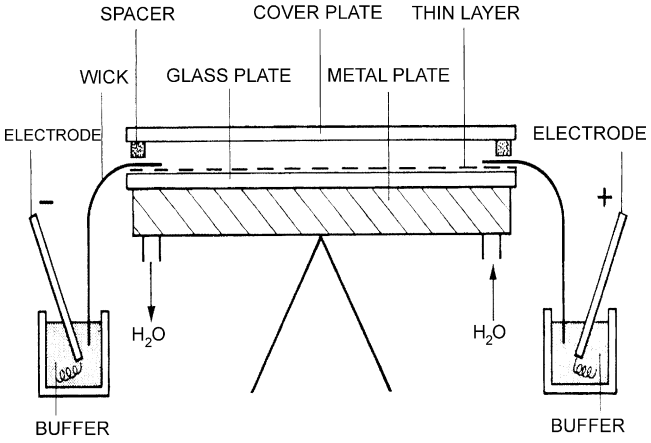

Fig. 9. Scheme of the thin layer silica gel electrophoresis apparatus according to Honnegar [44]. Note the supporting metal chamber with circulating coolant.

8. 1961: isoelectric focusing, the brightest star on the electrophoretic horizon

In 1961 Svensson [55–57] reported the possibility of separating such amphoteric macromolecules as proteins into a non-isocratic field, a most unorthodox electrophoretic protocol calling for a pH gradient in between the electrodic reservoirs. He lacked, though, the much-needed infantry for covering and surveying the grounds, i.e. the pH gradient along the migration path, so as to ensure its temporal stability. One needed not just a few amphoteric ions, able to reach steady-state conditions along the separation space, but a whole army of them, extending, like the Chinese Great Wall, in between anode and cathode. Only in this way would the zones of isoelectric "carrier ampholytes (CA)" form a continuous chain as the electric field would tie them to their isoelectric zones while diffusion would cause them to broaden just enough to penetrate the neighboring ampholyte peaks, thus simultaneously ensuring buffer capacity and conductivity. The synthesis of carrier ampholytes was finally reported by his pupil, Vesterberg [58,59]. This chap had been moonlighting and pouring over textbooks of organic chemistry and surfaced with a remarkable synthesis of the much wanted "carrier ampholytes": a chaotic synthesis, to be sure, as chaotic as a medical student could possibly devise. A most ingenious chaotic process, in fact, by which concoctions of oligoamines (from tetra- to hexa-amino groups) were reacted with limiting amounts of an α-β-unsaturated acid, acrylic acid. Chaos generated order! In a steep voltage gradient, this army of synthetic amphoteres join arms in an orderly fashion, with each assuming a (quasi) Gaussian distribution about its respective isoelectric point (p*I*) value [60]. The IEF technique was launched as a preparative method, requiring 110 and 440 ml columns for operation. An entire experiment, including column set-up, focusing, elution and analysis of hundreds of fractions, required a minimum of one week of hard labor! Although during the 1960s the growth of IEF was painfully slow, by the beginning of the 1970s, especially due to the

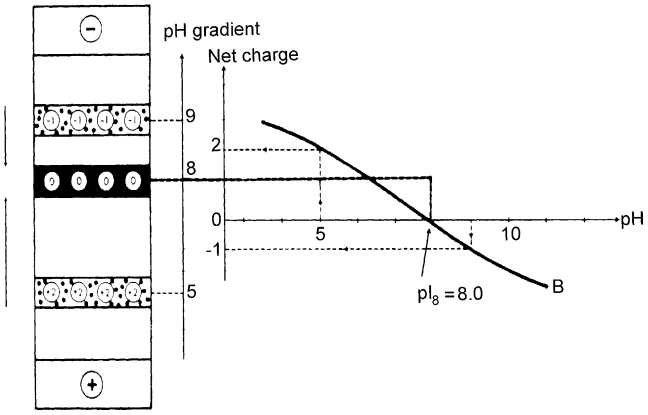

Fig. 10. Pictorial representation of a focusing process in a carrier ampholyte-generated pH gradient. If the same protein ion is applied simultaneously below and above the p*I* value, on the surface of a gel slab, the two ion fronts will migrate towards each other to converge (focus) at the p*I* zone, where both protein fronts will have zero net charge. The right side shows a theoretical titration curve of a protein with a p*I* = 8.0.

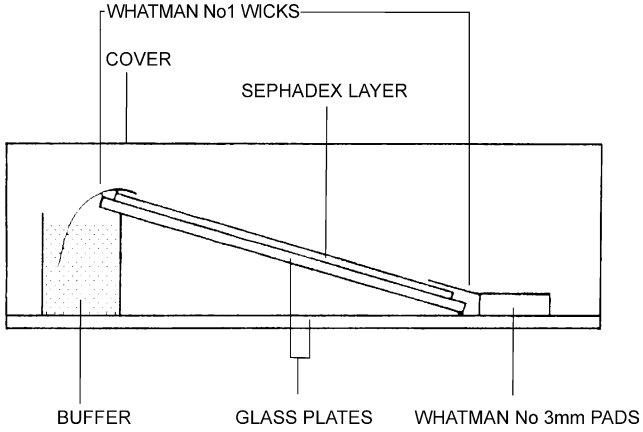

Fig. 11. Scheme of a thin layer Sephadex electrophoresis set-up, according to Fasella et al. [62]. In reality the drawing shows the assembly for the second dimension run, which is a gel filtration step at a right angle after the first dimension electrophoretic step. This too is a true 2D technique, in that the first dimension is driven by charge, while the second by the mass of the protein.

introduction of the analytical counterpart in polyacrylamide gels [61], IEF enjoyed such a marked growth as to soon become a leading separation technique in all fields of biological sciences. Fig. 10 summarizes the IEF principle: it shows how the same protein zones, applied simultaneously below and above the p*I* value, will acquire opposite surface charges and migrate towards each other till merging (i.e., focusing, condensing) in the p*I* zone, a point along the titration curve where its net charge will be equal to zero. Conventional IEF in soluble, carrier ampholyte buffers, permitted a resolution of 0.01 pH units in Δp*I*s between two neighbouring protein zones, a truly remarkable resolving power, not to be matched by any other technique till the invention, some 20 years later, of immobilized pH gradients.

9. 1964: electrophoresis in thin layers of Sephadex beads

In 1964 Fasella et al. [62] described electrophoresis in thin layers of Sephadex G-200 superfine [63]. After swelling, the Sephadex slurry is poured on a glass plate, the excess solvent let to evaporate and electrophoresis run for 300 V for 6 h. The method can become two-dimensional if the plate is run, inclined at 20° angle, in a gel filtration mode, orthogonal to the first dimension (see Fig. 11). Why would one want to resurrect such an obscure technique? Well, Sephadex was rediscovered by Radola [64–66] as a medium for preparative IEF. Detection was by the "paper print" technique, consisting in obtaining a contact print, for a few min, by touching the gel surface with a sheet of filter paper. Said sheet was then stained with, e.g., Cooomassie Brilliant Blue or Violet. The load capacity of this technique was impressive: in a 800 ml of gel suspension, Radola [66] fractionated 10 g pronase E with excellent band resolution. The unique advantage, over the preparative vertical density columns of Svensson, was that isoelectric precipitation would be confined to the p*I* zone and the protein aggregates held in situ by the surrounding grains of Sephadex, whereas in the latter case the flocculated proteins sedimented through the sucrose density gradient towards the column bottom contaminating all other fractions. This preparative technique had become so popular in the 1970s that LKB Produkter had devised a simple fractionating tray, a kind of a guillotine with 19 blades, which would be lowered onto the focused Sephadex bed and divide it into 20 fractions [67]. Each of them, in turn, could be collected with a spatula and the proteins recovered (with very high yield) by a simple "gel filtration" step in a buffer having a pH value removed from the protein p*I*. Additionally, Sephadex beds were used as anticonvective media in a continuous-flow preparative IEF chamber, as described by Fawcett [68], with the capability of separating up to 500 mg protein/day. A variant of this was described by Quarmby [69], who built a flow cell, filled with a stabilizing layer of Sephadex G-75, with a unique collection manifold allowing harvesting of 188 fractions. Where this not enough, IEF in Sephadex layers has recently been revived for prefractionation in proteome analysis [70]. There is another important aspect that I would like to underline: the 2D technique here reported, as well as those described in the case of paper and silica gel electrophoresis, are genuine 2D protocols, in that the separation parameters in each dimension are totally unrelated (charge versus mass or charge versus hydrophobicity), this ensuring maximum spreading and randomisation of spots in the 2D surface. Other 2D methodologies reported in the same epoch, such as separation of serum proteins by paper, followed by starch gel electrophoresis [71,72], or by agar followed by starch electrophoresis [73], or cellulose acetate coupled with starch gel electrophoresis [74], or even starch gel electrophoresis in both dimensions but at two different pH values [75], or the Orthacryl method of Raymond [76] are indeed "pseudo 2D" protocols, in that the separation

parameters in both dimensions are just about the same, this resulting in an aligning of the proteins spots along a diagonal, with not much increased resolution. It is thus seen that the seeds for classical 2D map analysis reported a decade later, as well as for column coupling 2D chromatography in modern proteome analysis were indeed planted in the 1950s and 1960s (see, e.g. the MudPit of Yates III) [77].

10. 1964: polyacrylamide gels and disc electrophoresis: an explosive marriage

In a cornerstone meeting held at the NY Academy of Sciences on 2 and 3 December 1963, some novel and remarkably advanced ways and means to perform zonal electrophoresis were reported. It was like the explosion of a supernova in the firmament of bioanalysis. On the one side, Raymond [76] shot a broadside to the audience by unveiling polyacrylamide gel electrophoresis, with the remarkable gadget of a vertical gel slab electrophoresis apparatus that became the ancestor of all subsequently-designed gel apparati [78] (although his group had already reported this novel, synthetic polymer in 1959) [79]. Moreover, he amply expounded on the concept of "molecular sieves" and "molecular filtration", as already described by Smithies for starch gel electrophoresis [36,40] and by Porath in gel chromatography [37]. On the other side, manning the Almiranta of the Invincible Armada, responded with heavy fire Ornstein [80] and Davis [81], the first giving all the background and theory of disc electrophoresis, the second one providing ample experimental evidence and methodology to support Ornstein's claims (see Fig. 12). What a duetto! The side dishes were not bad at all, though: Williams and Reisfeld reported new pH and conditions for extending disc electrophoresis along the pH scale, down to quite acidic pH values [82]; Poulik offered data on starch gel immunoelectrophoresis [83], although, in the long run, it turned out to be no match with the original version of immunoelectrophoresis in agar gel slabs devised by Grabar and co-workers [26,84], amply in use today in its agarose gel version. With Ornstein's work, the epos of UFOs (unidentified flying objects) in electrophoresis was born: due to the defocusing of the origin of ideas, scientists soon forgot the meaning of disc (abbreviation for discontinuous, on account of pH, trailing and leading ions, conductivities and porosities discontinuities in the experimental set-up) and started equating this marvellous technique to flying saucers, on account of the discoid-shape of the protein zones confined into the tiny sausages forming the polyacrylamide matrix. In polyacrylamide gel electrophoresis proteins are separated on the basis of two parameters, surface charge and molecular mass (M_r). Ferguson [85] demonstrated that one can distinguish between the two by plotting the results of a series of experiments with PAGs of varying porosity. For each protein under analysis, the slope of the curve log μ (electrophoretic mobility) versus gel density (T%) is proportional to M_r, while the y-intercept is a measure of surface charge [86]. The concept of isotachophoresis was exploited in disc electrophoresis for the first time, to arrange for a stack of protein ions with Cl^- as a leading and glycinate as terminating ions. Zone could be concentrated by a factor of up to 10,000 fold! Disc-PAGE enjoyed immense popularity in the 1960s and 1970s, not only because of its unique resolving power, but also because the other competing techniques, IEF and SDS–PAGE, had not been introduced as yet. As the latter two techniques gained importance in the 1970s, disc-PAGE steadily declined. Polyacrylamide, though, remained as a *prima donna* in all electrophoretic techniques, due its high versatility in producing any range of pore sizes and to the host of N-substituted monomers and cross-linkers described, endowed with unique properties [87]. The versatility of PAGE was further incremented when Margolis and Kenrick [88–90] proposed porosity gradient gels (both linear and non-linear) for separating complex mixtures of proteins. Just like steady-state techniques (such as isoelectric focusing and isotachophoresis), in which entropic dissipation of peaks is counteracted by forces applied to the system, also this method allows for sharp zones, since, at any given moment, the front of a diffuse band will be retarded in respect to its rear, as it moves into regions of smaller pore sizes. This method is also called "pore limit" electrophoresis since, for long running times, all the proteins in the mixture reach asymptotically zero migration, precisely where the surrounding pore matches the average diameter of the particle. The method is still much in vogue today, especially in the second dimension of a 2D map, although not any longer exponential, but linear gradients are used. It is also often coupled to discontinuous buffers, for extra sharp bands. It also turned out that such porosity gradient gels were an excellent tool for proper M_r assessments of proteins [91,92].

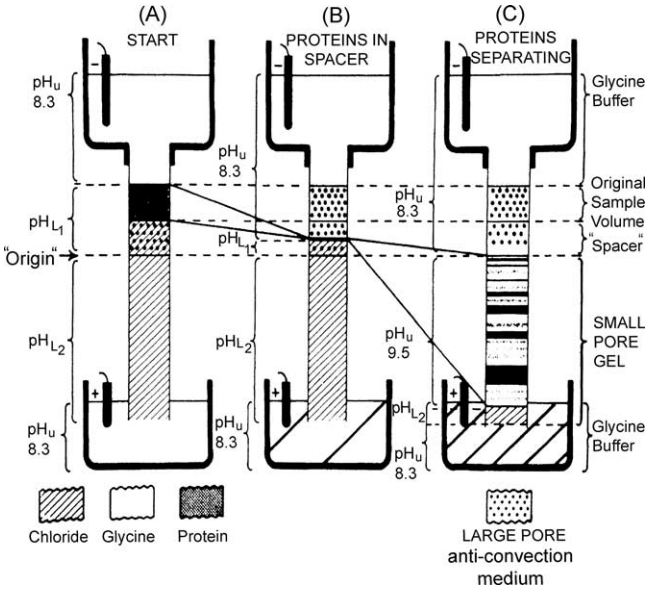

Fig. 12. Set-up of disc electrophoresis according to Ornstein [80] and Davis [81]. (A) Starting conditions with sample trapped in sample gel; (B) formation of the thin stack of proteins according to an isotachophoretic process; (C) zone electrophoretic separation in the small-pore gel.

11. 1967: carnival at Copacabana!

In 1967 a Carioca trio [93], tired of celebrating carnival only in a short time window, decided to promulgate it all the year around. It was a perennial disguising: let proteins swim in a solution of an anionic surfactant, above the critical micellar concentration (sodium dodecyl sulphate, SDS, seemed well suited for this task). They would surely be coated by micelles of this chemical, to the point of swamping the original amphoteric charge of polypeptide chains and forcing them to behave just like nucleic acids, odd macromolecules in which the charge to mass ratio becomes nearly constant above ca. 400 bp in length [94]. Most proteins would just adsorb SDS to a magic ratio of 1.4 mg SDS/mg protein [95] (suggesting that the number of SDS molecules bound is approximately half the number of amino acid residues in a polypeptide chain): if one would then drive them into a porosity gradient gel [88–90], may be even by exploiting discontinuous buffers [96], one would end up with razor-blade-sharp zones, whose mobility, when plotted against the log of molecular mass, would result in a linear relationship [97]. Subsequent work [98,99] proved in fact that this constant ratio SDS/protein holds true for a very large number of proteins, although with some notable exceptions. Two classes of proteins show anomalous behaviour in SDS electrophoresis: glycoproteins (because their hydrophilic oligosaccharide units prevent hydrophobic binding of SDS micelles) and strongly basic proteins, e.g., histones (because of electrostatic binding of SDS micelles through their sulfate groups). The first can be partially alleviated by using alkaline Tris/borate buffers [100], which will increase the net negative charge on the glycoprotein and thus produce migration rates well correlated with molecular size. The migration of histones can be improved by using pore gradient gels and allowing the polypeptide chains to approach the pore limit [101]. SDS–PAGE is today, by far, the most popular and widely adopted electrophoretic technique in laboratories around the world. It is routinely used mainly for the following purposes: (a) estimation of protein size; (b) assessment of protein purity; (c) protein quantitation; (d) monitoring protein integrity; (e) comparison of the protein composition of different samples; (f) analysis of the number and size of polypeptide subunits; (g) when using, e.g., Western blotting; (h) as a second dimension of 2D maps. Additionally, SDS has found a most important application in a class of electrophoretic separations called by Terabe and co-workers [102,103] “micellar electrokinetic chromatography” (MEKC). MEKC is a unique mode of CZE, in that it is capable of separating uncharged compounds, an apparently impossible proposition in an electric field. The fact is that neutral molecules can interact with the pseudostationary phase (the SDS micelles) by partitioning into them according to a hydrophobicity scale. Thus MEKC can be viewed as a hybrid of reversed-phase liquid chromatography (RPLC) and CZE, since the separation process incorporates hydrophobic and polar interactions, a partitioning mechanism and electromigration. MEKC has opened a spectacular window in electrokinetic methodologies, bringing about an extraordinary resolving power coupled to the possibility of analysing in an electric field just about any uncharged molecule, a true electrophoretic paradox!

12. 1970: the Wabash cannonball

It was my friend Haglund [104] who introduced in 1970 the term isotachophoresis (ITP, i.e. electric transport at the same velocity), occurring when a locomotive (the leading ion), after hooking up all the wagons (the various ions of given mobilities) and ending with the slowest moving species (the trailing ion) is lunched on the electrophoretic track. Just like the train of the famous song of Seeger [105], at steady-state, all species have to move at the same velocity as the locomotive, of course. This is a unique electrokinetic methodology, in that entropy, dissipating the zones, is effectively counteracted by the steep voltage steps existing at every given boundary. If an analyte diffuses in front, it is decelerated; if it diffuses behind, it is accelerated. IEF, of course, acts on a similar principle for preventing zone dissipation: when the focused protein diffuses away from the p*I*, it will acquire either a net positive or negative charge, forcing the voltage gradient to push it back to its zone. The second major advantage of ITP is the one that produces the spectacular effects of disc electrophoresis: stacking into extremely thin starting zones (a few μm thick, from a loading zone up to 1 cm thick, a concentration factor, for proteins, of up to 10,000 fold), since the concentration in each zone has to follow the Kohlrausch [106] autoregulating function [107,108]. The major drawback: there is no solution of continuity among the different zones, so that, upon on-line detection, it is impossible to discern the various ionic zones. Aware of that, protein biochemists tried to cure the problem by introducing “mobility gradients”, i.e. a large number of molecules with mobilities intermediate between those of the various protein ions, so as to space them apart. The only way to do that was to use narrow range carrier ampholytes, which would act, precisely, as spacers [109–111]. Given the CAs complexity, though, it was not certain that a genuine “isotachophoretic train” would be formed prior to run termination, which means that the users, perhaps unknowingly, were converting the ITP into a CZE system! Biochemists using ITP were also aware of the drawbacks in protein analysis due to EOF flow and consequent macromolecule adsorption, so that the use of methylcellulose for preventing these noxious phenomena was proposed [112,113]. Analytical ITP instrumentation was first commercialised by LKB Produkter in 1973 with the name LKB 2127 Tachophor [114]. The instrumentation was very advanced and fully automated. It contained two detectors, one thermal (thus universal, in that each ion zone would give a thermal signal) and one for UV absorbance. Separation took place in a PTFE capillary of 0.5 mm i.d., immersed in a kerosene liquid for cooling. The liquid was thermostated by Peltier elements, allowing separation from 3 to 29 °C (see Fig. 13). Four

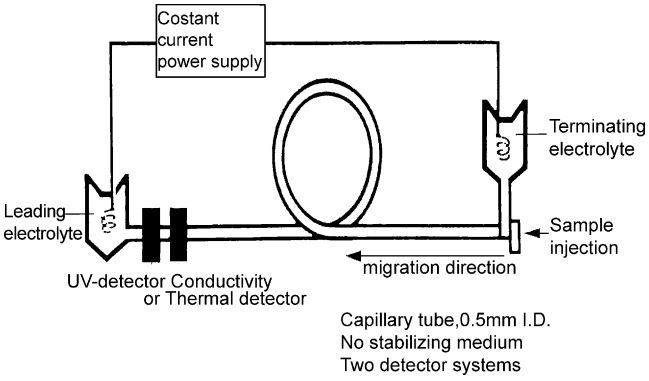

Fig. 13. Scheme of the analytical isotachophoretic assembly of the Tachophor. Note that the thermal detector generates two signals: the original one and its derivative, needed for measuring with precision the length of each zone.

lengths of capillary were available (23, 43, 61 and 80 cm). The power supply could deliver up to 30,000 V with a constant current ranging from 0 to 500 μA. These very high outputs gave high resolution and short separation times (often 5–30 min). A conductivity detector was also available [114]. The analytical ITP system could also be modified for preparative purposes (see Fig. 14). In Fig. 14A, a m_1 semipermeable membrane separates the leading electrolyte reservoir from the capillary compartment in order to prevent hydrodynamic flow. For preparative purposes (Fig. 14B), a T-tube is inserted after the detector (D), through which the sample is swept by a flow of leading electrolyte, provided that the liquid flow rate from the pump is greater than the corresponding migration

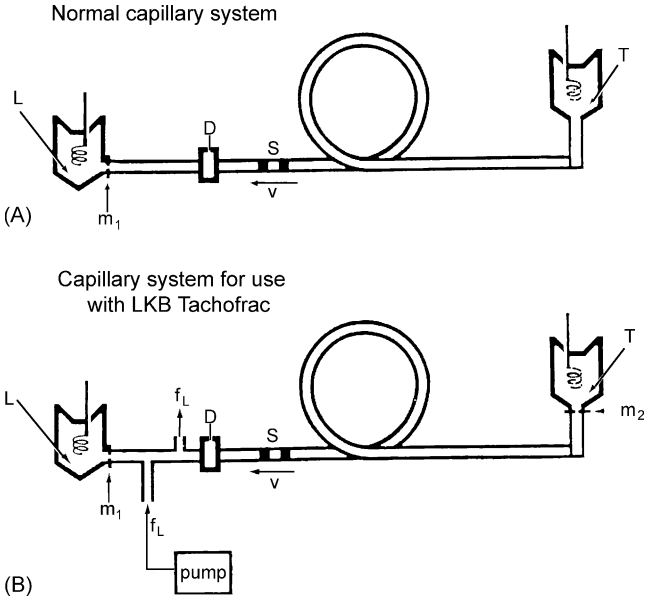

Fig. 14. Scheme of the analytical Tachophor (with a membrane m_1 at the port of the leading electrolyte reservoir) and of the preparative instrument (Tachofrac) for eluting the bands at the end of the run. The fraction collector (a moving strip of acetate foil) is not shown. L and T: leading and terminating ion reservoirs, respectively. D: detector. S: sample zones. f_L: flow of eluting electrolyte.

rates of the ions. Since the sample zones may have volumes as low as 10–20 nl, a traditional fraction collector cannot be used. In the Tachofrac, the sample zones are collected on a cellulose acetate strip passing by the outlet of the T-tube. As the strip moves at a fixed rate, an event marker tags the strip and chart of the recorder [115]. Although ITP experienced a steady growth in the 1970s, and a rather large pool of users, it steadily decayed in the 1980s and disappeared completely in the 1990s. Perhaps the seeds for the decay of ITP were planted by a young German post doc, who had been enjoying a one year leave of absence in Chrambach's laboratory at NIH. This guy got terribly excited at the use of discontinuous buffers in isotachophoresis and decided he would investigate the matters thoroughly. In those days, we were happy with that dozen or so of combinations of leading/trailing ions, carefully selected by LKB Produkter and theoreticians so as to optimise the most common separations typically encountered in daily practice. But not Jovin [116,117]! You know how Germans are, they have to cover the grounds extensively. As a result, this chap come forward not just with a few dozens of such systems, but with no less than 4269 computer-generated buffer recipes, calculated throughout the full pH range and at two temperatures, 0 and 25 °C. And that was the reason for the sudden decline of ITP: imagine us poor users having to disentangle ourselves not any longer with a dozen recipes (and that already brought daily uncertainties!) but with now no less than 4269! A genuine disaster, the collapse of the entire system.

I have described ITP in extenso for a reason: I believe that we in separation science should make an act of contrition and recite a "*mea culpa*": when CZE was "invented" 10 years later, nobody gave credit to the work developed in ITP, that amply showed that the technique was already there! CZE was simply "re-invented" by cannibalising all ideas and instrumentation well known to all of us "electrophoreticists" and amply documented in the early 1970s. Years later, the CZE tribe, in a crisis of resipiscence, gave some credit to the work of Hjertén [118], who also had come very close to a CZE set-up already in 1967, but, curiously, very few gave proper credit to those who invented and developed ITP, no doubt containing all the seeds of present-day CZE.

13. 1975: Galileo and the outer space

Now that we had two electrokinetic methodologies perfectly orthogonal (in the Giddings' sense) [119] (IEF screening on the basis of surface charge, SDS–PAGE solely on the basis of mass), combining them at right angle would have offered a unique map in which the protein spots would be maximally spread in the two-dimensional space having as coordinates charge (p*I*) and mass (in the SDS–PAGE second run). Such a technique would have been like the telescope of Galileo, that first allowed us to explore, if not the deep outer space, at least our solar system. Three laboratories reported this 2D technique simultaneously and independently

in 1975, although most of the credit went just to O'Farrell [120–122]. Perhaps because his system was the most elaborate: in fact, he was able to resolve and detect about 1100 different proteins from lysed *E. coli* cells on a single 2D map and suggested that the maximum resolution capability might have been as high as 5000 different proteins (something of galactic dimensions for the epoch!). Apart from the meticulous attention to detail, major reasons for the advance in resolution obtained by O'Farrell, compared to earlier workers, included the use of samples labelled with ^{14}C or ^{35}S to high specific activity, and the use of thin (0.8 mm) gel slabs for the second dimension, which could then be dried down easily for autoradiography. This detection method was able to reveal protein zones corresponding to one part in 10^7 of the sample (usually 1–20 μg was applied initially, since higher loads caused zone spreading, although up to 100 μg could be loaded). Coomassie blue, in comparison, was about 3 orders of magnitude less sensitive and could reveal only about 400 spots. For the first dimension, O'Farrell adopted gel rods of 13 cm in length and 2.5 mm in diameter. The idea was to run samples fully denatured, in what became known as the "O'Farrell lysis buffer" (9 M urea, 2% Nonidet P-40, 2% β-mercaptoethanol and 2% carrier ampholytes, in any desired pH interval). For the second SDS–PAGE dimension, O'Farrell [120] used the discontinuous buffer system of Laemmli [96] and, for improved resolution, a concave exponential gradient of polyacrylamide gel (usually in the intervals 9–15 or 10–14%*T*, although wide porosity gradients, e.g., 5–22.5%*T*, were also suggested). It is thus seen that, since its very inception, O'Farrell carefully selected all the best conditions available at the time; it is no wonder that his system was adopted as such in the avalanche of reports that soon followed and that there were hardly any modifications to this technique, a very rare event in science (as soon as a method is published, usually a cohort of modifications is immediately reported, in the hope that the "second discoverer" will be the winner!). Although the notion of resolving up to 5000 polypeptide chains might have seemed as an exaggeration, history soon vindicated O'Farrell's claim. As a lesson to scientists of the third millennium, who only think and act in terms of miniaturization, some scientists in the 1980s reasoned in completely opposite terms, moving towards larger and larger 2D gels. This strategy paid handsomely. It all started with Young [123], who named his gels, humbly, "giant" gels, since they had a napkin-size: 39 cm × 37 cm! He developed them out of a sense of frustration, after 2.5 years of research for proteins induced by adrenal glucorticoid administration, of which he could detect none. Yet, when the gel size was increased by roughly six-fold in area, and the amount of protein loaded increased up to 100-fold, he could see a constellation of protein changes induced by the hormone. An even more bountiful harvest was made by Klose and Zeindl [124] when they adopted slightly larger gels (42 cm × 33 cm, 0.85 mm in thickness) for analysing an epithelial-like human larynx carcinoma cell line, uniformly labelled with ^{14}C-amino acids: >10,000 different polypeptide spots could be revealed in a single 2D map, a remarkable achievement, indeed.

14. 1979: Eldorado or "Silverado"?

Everybody knows the legend of Eldorado, but what came out of Carl Merrill's laboratory in 1979 was not so much gold but silver, thence the mention to the famous movie of Kasdan [125], the last of the great "westerns". Perhaps nobody at the beginning thought that this would represent a landmark in separation science, but I feel that his location in this historical panorama is fully justified, in view of the enormous impact it had in the field of analysis as performed in 2D maps, an impact that it is still strongly felt in present days. In 1979 Merrill et al. [126,127] introduced silver staining techniques for protein detection in polyacrylamide gels, especially in SDS–PAGE and 2D maps. This was a quantum jump, since it increased the sensitivity of protein detection by 2000-fold over Coomassie blue staining, from tenths of a microgram to tenths of a nanogram. True, the work of Becquerel [128], while engaged in the discovery of radioactivity, provided the means for use of radioactively-labelled compounds. Detection of strong β-emitters, such as ^{14}C and ^{32}P, can be achieved by using autoradiography, with excellent results, as we have seen [124], although weak β-emitters, such as ^{3}H, require enhancement by fluorography [129,130]. However sensitive radioactive labelling might be, difficulties encountered with radioactive isotope use, such as waste disposal, have discouraged its use, in favour of silvering or other protocols, such as biotin-streptavidin labelling [131]. All silver stain methods depend on the reduction of ionic to metallic silver, to provide metallic silver images [132]. Merrill's group has developed the three main silver staining methods currently in use: diamine or ammoniacal stains [126,127]; non-diamine, silver nitrate stains [133]; silver stains based on photodevelopment [134]. Most silver stains produce monochromatic brown or black colour, although other hues may be produced. Lipoproteins may stain with a bluish hue, while glycoproteins may stain yellow, brown or red [135,136]. This colour effect is due to the diffractive scattering of light by the microscopic silver grains [137]. The colour produced depends on the size of the silver grains, the refractive index of the gels, and the distribution of the silver grains in the gel. In general, larger silver grains produce black images, while smaller particles (>02 μm in diameter) generate yellow to reddish images [138].

15. 1981: the early warnings of capillary zone electrophoresis

As stated in section 13, ITP contained all the germs and seeds of present day CZE, including most of the instrumental development. But in the mid 1970s one important bit was sorely missing: a true capillary in which electrophoresis would take place. Hjertén's machine had to work with straight

glass tubes of 3 mm i.d., hardly capillary dimensions! The Tachophor exploited plastic tubings of 0.5 mm i.d. Perhaps the one who came closest to modern-day CZE was Virtanen, who defended his Doctor of Technology dissertation in 1975 [139]. His electrophoretic cell was a Pyrex tubing, with typical length of 50–100 cm, drawn to an inner diameter of only 250–500 μm, truly close to modern CZE instruments, although, curiously, it was U-shaped just as in the Tiselius apparatus. The merit for the development of CZE goes to an obscure couple working at Chapel Hill in North Carolina: they had the idea of adopting true capillaries, flexible silica tubings, protected by a polyamide coating, of IDs ranging from 20 to 100 mm, thus genuine capillary dimensions, able to prevent analyte sedimentation via capillary forces, and permitting very high voltage gradients due to truly minute conductivities in such a very narrow bore. The separations produced were quite spectacular [140–144]. The CZE field expanded dramatically at the beginning of the 1990s, when the first commercial instruments appeared on the marked and experienced a spectacular growth. The literature in this field is enormous and I will only mention here a few books that summarize some of its aspects [145–151].

Since this issue is dedicated to Horvàth, I am happy to recall here some outstanding work he has produced also in the field of electrophoresis, though most of us know him as a "pure chromatographer". In 1995, he introduced CZE at sub-zero temperatures for separation of *cis*- and *trans*-isomers of small peptides [152]. Peptides containing Pro residues are known to exist in both the *cis* and *trans* conformation due to the rigidity of the peptidyl-proline bond. At temperatures near ambient, the relaxation time of the *cis–trans* isomerization is on the time scale of minutes, thus commensurate with the migration times in CZE under usual operating conditions. When the separation was conducted at temperatures as low as—17 °C, two conformers of the heptapeptide Tyr-Pro-Phe-Asp-Val-Val-Gly-NH_2 were fully resolved. In the case of Tyr-Pro-Phe-Gly-Tyr-Pro-Ser-NH_2, all the four conformers, due to the presence of two peptidyl-Pro bonds, were also resolved. Big deal, one could object, since his group had already performed such deeds by RP-HPLC already in 1984 [153–155]. But here there was an extra bonus to collect, although quite painstakingly. I remember visiting him during this work: no commercial CZE instrument was meant to be operated at sub-zero temperatures, so he had to re-write the programs for allowing this "forbidden" operation. In addition, the Beckman P/ACE 2200 instrument had to be kept in a cold room, while an external circulating bath would allow to reach −12 to −17 °C. Humidity condensation on the electronic circuits would provoke sparks and damage the instrument. It was a constant fight with the Beckman people for paying the repair bills! The extra bonus was that, since the separation was not encumbered by interaction with a stationary phase, as is the case with the retention data in HPLC, measurement of molecular properties and other physico-chemical data was made possible. Thus, from electrophoretic mobilities, the hydrodynamic radii of the *cis–trans* conformers of two dipeptides, Phe-Pro and Leu-Pro, could be estimated. In both cases, the *trans* isomers had 1.3 times greater Stokes radii than the *cis* conformers (see Fig. 15).

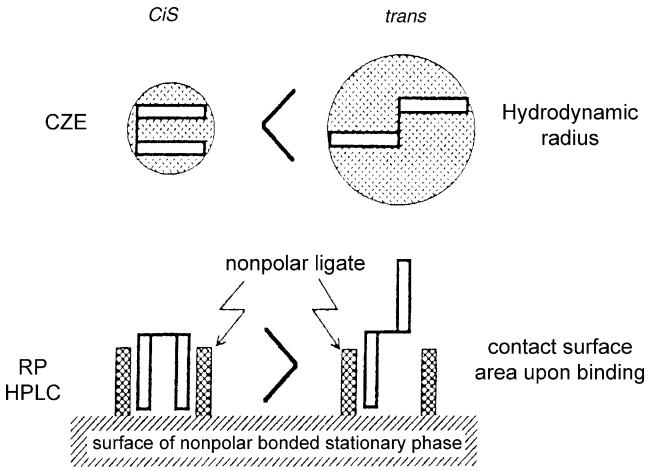

Fig. 15. Illustration of the putative origin of selectivity for the *cis*- and *trans*-dipeptide conformers in CZE and RP-HPLC. The *trans* isomer has a larger Stokes radius than the *cis*-form and therefore has a lower electrophoretic mobility in CZE. In HPLC, the *trans*-form is less retained because its contact surface with the ligates of the non-polar bonded stationary phase is smaller than that of the *cis*-isomer (from Ma et al. [152], by permission).

I want to close this section on CZE with two additional examples. One is the hyphenation of CZE, and especially of capillary IEF (cIEF), with mass spectrometry (MS), implemented by several groups, but especially by Smith's laboratory with the use of Fourier transform ion cyclotron resonance-MS (FTICR-MS) [156,157]. Spectacular results were obtained, with the display, in a 2D map format, of some 900 "spots" (i.e., unique putative protein masses) from total lysates of either *E. coli* or *D. radiodurans*, with a resolution, in the cIEF step, of $\Delta pI = 0.004$ pH units! The other is the recent work of Mathies' group on high throughput DNA sequencing with a microfabricated 96-lane capillary array electrophoretic "bioprocessor" [158–160]. Have a look at Fig. 16: yes, the 96 channels (with an electrophoretic path that is still a robust 16 cm long channel, reduced to half of the space by a smart U-turn in the middle) are dug in a compact disc. As I look at this remarkable gadget, I dream of a future bathed in "electrophoretic music", whereby, as the compact disc slowly rotates to be read by the optical device, we will hear may be the majestic ouverture of an organ fugue by J.S. Bach, or the heavenly music of the "Goldberg Variations", as elicited by the magic fingers of Gould!

16. 1982: the birth of the steady-camera

Although 2D maps experienced a remarkable growth by the end of the 1970s and throughout the 1980s, there was a big impediment: the erratic spot profile obtained by performing the first dimension in conventional IEF with soluble carrier ampholytes, a la Svensson-Vesterberg, if you like. There

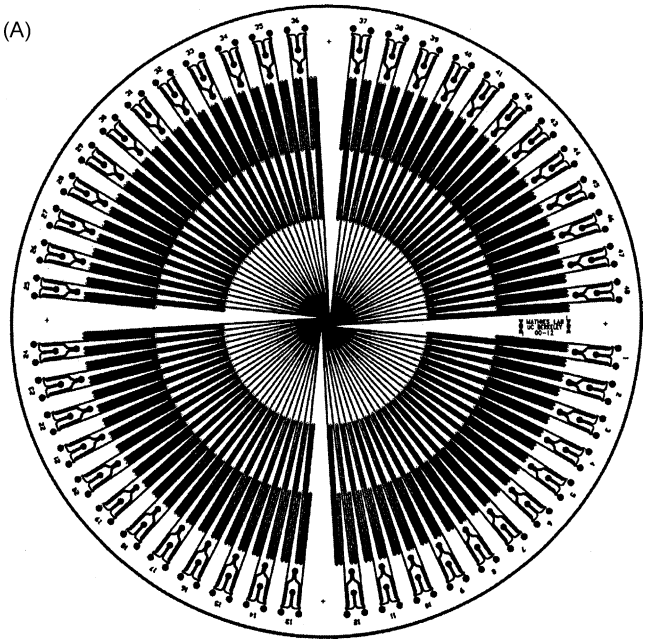

Fig. 16. Overall layout of the 96-lane DNA sequencing microchannel plate. The injector ports are visible on the outer ring as U-shaped channels. Each separation column is 200 μm wide, 30 μm deep, and is folded twice upon itself for an effective length of 15.9 cm by using four symmetrical tapered hyperturns with taper lengths of 100 μm and radii of curvature of 250 μm (not shown; from Paegel et al. [159], by permission).

were no fixed stars in the firmament of 2D maps: the apparent p*I* values kept changing, from batch to batch of CAs and, of course, from brand to brand, as manufactured by different companies (a chaotic synthesis, as you might remember). The situation was so frustrating that the Anderson's recommended carbamylation train standards for mapping the pH gradient course [161] and even preparing large volumes of stock solutions of CAs, obtained by carefully blending the various commercial products. The help was at hand, of course, since in 1982 Bjellqvist et al. [162] lunched another supernova in the sky of bioanalysis: immobilized pH gradients (IPGs), which were soon demonstrated to be able to steady the camera when taking a picture of the small firmament of stars in a 2D map, while affording exquisite resolution when run in narrow and ultra-narrow pH ranges. This new work of art was unveiled in April 22, 1982, at the electrophoresis meeting organized by Stathakos in Athens and acclaimed with standing ovations. At least that is what we thought. In reality, we presented these data to an almost empty room, since most of the delegates had never been to Athens before and surely enjoyed lovely spring weather on the Acropolis, on the Licabetto, on the Plaka, strolling just about around any corner in the capital except at the Hellenic Academy of Science, where the meeting was held [163]. IPGs went largely unnoticed for about a decade, even though they brought about some out-of-(terrestrial) space results in bio-separations. Together with that, IPGs brought "democracy" for the first time in electrokinetic processes. Up to their introduction, 2D maps had been conducted only in linear pH gradients, which penalize acidic proteins, jammed in the overcrowded zone of the pH 4–6 region, where >60% of all proteins focus [164]. Already in 1985, we were able to describe broad-range, non-linear IPG, strongly flattened in the crowded region, with a sharp upward turn at alkaline pH values [165]; these ranges are by far the most popular in today 2D map analyses.

17. 1989: Piazzolla's bandoneon

I had no intention to finish this review with the multicompartment electrolyzers (MCE) with isoelectric membranes [166–169], since this fractionation technique did not seem to have met the favour of users, although a commercial instrument had been produced by Hoefer. Perhaps the fabrication of membranes was not so well understood by the users, perhaps they felt the technique was too complex and cumbersome; it is a fact that the MCE looked like a disaster and surely Hoefer would have gone broke if it had to survive just on that item. Although for the whole decade we published extensively on all sorts of applications and purification of rDNA proteins, including the novel idea of using the MCE as an immobilized enzyme reactor [170,171], just about none seemed interested in picking up the idea. Then, in the year 2000, the MCE concept took up momentum, as we realized that it could be used for proteome pre-fractionation and miniaturized the instrument [172–174]. It had been lamented up to present times that 2D maps could only see the tip of the iceberg, the vast mass of the proteome being "submerged" and subtracted to the scrutinizing eye of the classical 2D approach. Yet, when pre-fractionation with the MCE was applied to entire cell lysates, the results were astonishing. For instance, by properly exploiting this pre-fractionation device, Pedersen et al. [175] were able to capture and detect much more of the "unseen" yeast membrane proteome. Here is a summary of their results: 780 protein isoforms identified; 323 unique proteins (genes) detected, of which 105 were integral membrane proteins (33%), 54 were membrane associated proteins (17%), 159 were total membrane/associated proteins (50%) and 90 were proteins with CBI < 0.2 (27%). The importance of some of these finding is here highlighted: integral membrane proteins are rarely seen in 2D maps; proteins with CBI (codon bias index) <0.2 represent low abundance proteins and are scarcely detected in 2D maps unless enriched by some pre-fractionation protocol [176]. There have also been important spin-off of the MCE, such as the off-gel IEF in multichamber device of Ros et al. [177] or the parallel IEF chips of Zilberstein et al. [178], to name just a few. In fact, at the beginning of the third millennium, sample pre-fractionation by all sorts of electrophoretic devices and chambers has been revived and becoming a most promising tool in proteome analysis. However, since there is no more space in this review, the readers are referred to a review amply covering this topic [179] and to the most recent (at this writing) book on proteome analysis [180]. Part of the miniaturized instrument is shown schematically in Fig. 17. In this exploded view, two

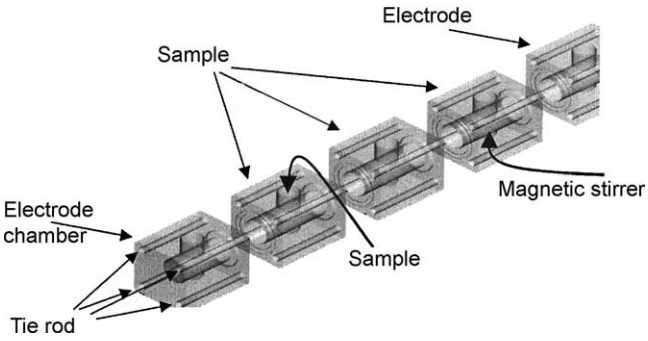

Fig. 17. Exploded view of the miniaturized multicompartment electrolyzer operating with isoelectric membranes. An assembly with only five chambers is shown (by courtesy of Proteome Systems Ltd.).

terminal electrodic chambers are used to block, in between, three sample chambers, although the electrolyzer can be built with up to eight chambers, accommodating, in between, the isoelectric, buffering membranes used to define a given pH interval in which a set of proteins, having p*I*s encompassed in between a set of two adjacent membranes, will be trapped. Thus, as you assemble the instrument with a variable number of chambers (thus expanding or contracting it), you might hear the exciting music of Astor Piazzolla, the maestro of Argentinean tangos, playing with his bandoneon (an accordion) some milonga or contromilonga or, perhaps, una "*Balada para un organito loco*".

18. 2005: conclusions

"Postumo, Postumo, alas, years flow away rapidly and compassion will not help in slowing down wrinkles, incumbent senility and inexorable death". So much for the verses of Horatius. Now for the curious title. The march of pennies originated in Baltimore shortly after 1849, when the enraged inhabitants found out that their illustrious poet and novel writer, Edgar Allan Poe, had been buried anonymously in plain earth, outside the consecrated precinct of the church. The mayor claimed that the town had no money for a better burial, but in reality it was the Church that had denied proper burial since they held him excommunicated. A school teacher had the brilliant idea of asking pupils to donate a penny: the movement rapidly propagated through the States, to the point at which the triumphant Baltimorean could bring carts and carts loaded to the brim with pennies to the town hall and ask the mayor to build a decent tomb for Poe or be buried under the tons of pennies brought forward. A marble tomb of E.A. Poe can now be visited in Baltimore and you might chance upon school classes visiting Edgar and placing strings of pennies on the sarcophagus. The march of dimes was started in the early 1960s as associations asked the USA citizens to donate at least a dime for biomedical research, which from then on took up at a brisk pace. Envious of this success, and wishing to have a share of the action, major movie companies offered to people to go to see two movies for a dime. It was an instant success! Too bad that these things do not happen today anymore.

This humble review was meant precisely for that: to show that indeed, if today we enjoy this unique success in separation science, we owe it to the countless scientists who have donated not just a penny or a dime, but most of their life for a slow but continuous progress. This work of mine is dedicated to these hard workers that made all of this possible: they might have been already captured by the inexorable fate lamented by Horatius, but they now live again in our memories. It was a just tribute, and I am sorry that I could only mention a few of them, as space and time permitted.

Acknowledgements

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