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Review

Recent developments in electrophoretic methods

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CONTENTS

1. INTRODUCTION

At the first International Symposium on High Performance Capillary Zone Electrophoresis (CZE) in Boston, Vesterberg[1] gave a comprehensive account of the history of electrophoretic methods, including the pioneering work of the nineteenth-century physicists. His survey correctly emphasized the importance of the Uppsala School of Separation Science, where most of developments in both chromatography and electrophoresis have been initiated. He also extensively covered the field of isoelectric focusing (IEF), to which he has made some fundamental contributions. In addition, there has been a recent historical survey[2,3] of electrophoresis based on the development of gel matrices, the latter playing a fundamental role in many electrokinetic techniques. In this review, it was therefore decided to concentrate on the last 10 years of electrophoresis, and to give particular emphasis to nucleic acid separations, often overlooked at electrophoresis meetings, still frequented by numerous protein chemists. The review includes recent developments in two-dimensional (2D) maps and their coupling to blotting techniques, which allow direct sequencing and fingerprinting of polypeptides from complex samples without a need for prior chromatographic steps. Some of the improvements in 2D techniques have been obtained

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through the use of immobilized pH gradients (IPGs), which currently offer the highest resolving power in electrophoresis. An alternative approach to 2D mapping (which generates a pure charge/mass fractionation) is chromatofocusing, which separates polypeptides on a 2D plane having hydrophobicity and mass as coordinates. This is achieved by running directly the eluate from a reversed-phase high-performance liquid chromatographic (HPLC) column into a sodium dodecyl sulphate (SDS) gel. The two techniques are clearly complementary and could help in creating a three-dimensional map of polypeptides, having charge, hydrophobicity and mass as coordinates. The review ends with a brief discussion of CZE.

2. NUCLEIC ACID ELECTROPHORESIS

The revolution in modern biology over the past decade has been driven largely by the development of an array of new methods for the isolation, analysis and manipulation of DNA molecules. One of the key areas has been the ability to determine the precise chemical structure of large DNA molecules, *i.e.*, the sequence of the four bases on the DNA strand. The last step in this process is an electrophoretic analysis by which a series of oligonucleotides, differing in length by a single nucleotide, are separated, detected and read in order of sequence. The other major breakthrough has been the ability to separate very large DNA fragments, in the mega-base pair range, by a technique generally known as pulsed-field gel electrophoresis (PFGE). Both developments have opened up new dimensions in the field of electrophoretic DNA analysis, which previously was mostly dominated by electrophoretic separations in "submarine" gels, *i.e.*, dilute agarose gels run under a thin veil of buffer, for the analysis of restriction fragments of DNA. Such analyses, coupled to probing with radioactive or biotinylated probes, is routine in, *e.g.*, screening of defective human genes and parentage testing.

3. DNA SEQUENCE ANALYSIS

In the early 1970s, two groups developed rapid methods for sequencing DNA: the enzymatic method, proposed by Sanger *et al.*[4] at the Medical Research Council in England, and the chemical method, described by Maxam and Gilbert[5] at Harvard, work for which they shared the Nobel Prize. In enzymatic sequencing, a cloned copy of the DNA region to be sequenced is used as the template for an enzymatic reaction which copies the DNA sequence into a new strand. Four separate incubations are prepared, each containing a dideoxy nucleotide, which will act as chain terminator on incorporation at one end of the growing oligonucleotide. In the chemical sequencing method, four base-specific chemical reactions generate four sets of DNA fragments. In both instances, the key that permits DNA sequencing is the ability, by electrophoresis on very thin (*e.g.*, 200–400 μm thick) denaturing polyacrylamide gels, to separate DNA fragments differing by size increments of one nucleotide each, with extremely high resolution. In conventional DNA sequencing, the DNA fragments are labelled with radioisotopes, separated on the sequencing gel and visualized by the image they generate on a radiographic film in contact with the dried gel. This generates a "snapshot" image of the gel at the time at which power was disconnected.

The enzymatic sequencing method seems to be the predominant one, as it has

been adopted for the two largest sequencing projects presently reported, the analysis of the 172 000-base Epstein-Barr viral genome[6] and the 48 000-base λ genome[7]. Nevertheless, neither method could be of any practical use for very large-scale sequencing projects, such as the analysis of the human genome, which is encoded in $3 \cdot 10^9$ bases of DNA. At this level of complexity, automation is strongly desirable. This has been made possible by abandoning radioactive tags in favour of fluorescent probes, which allow direct data acquisition in real time during the electrophoretic separation. This also has the added advantage of increased sensitivity, which is greatly needed, as there are only of the order of 10^{-15}–10^{-16} mol of DNA in each band in the gel[8]. There are essentially three methods available: (a) use of four fluorophores linked to the primer (*i.e.*, to the short DNA fragment which serves as starting point for the enzymatic synthesis, by annealing to the template DNA molecule)[8,9]; (b) use of four fluorophores linked to the chain-terminating dideoxy nucleotides[10]; and (c) use of a single fluorophore[11]. The first two methods allow electrophoresis in a single lane, whereas the last still requires four separate electrophoretic lanes, as is customary in radioactive labelling (*i.e.*, one for each of the four terminating nucleotide analogues).

Fig. 1A and B shows the set-up developed by Smith and co-workers[8,9] and introduced commercially by Applied Biosystems (Fig. 1C). Basically, four different fluorescent dyes (fluorescein, NBD, tetramethylrhodamine and Texas red) are linked to the primer and used for four different incubation reactions for A, C, G and T. The products of each reaction are then combined and co-electrophoresed on a single lane of a polyacrylamide gel. A fluorescence detector near the bottom of the gel reveals the fluorescent bands of DNA as they pass by during electrophoresis, and determines their colour. In the system of Prober *et al.*[10] (adopted by DuPont in the Genesis 2000 DNA sequencer) the four fluorescent tags, instead of being attached to the primer, are linked to the chain-terminating base analogue itself. This accomplishes two operations in one step: first, it terminates the synthesis on dye–analogue incorporation, just as in conventional enzymatic sequencing, and second, it attaches a fluorophore to the end of the DNA molecule at the same time.

The optical instrumentation in the DuPont instrument is shown in Fig. 2. As the fluorophores are much more closely spaced, the ratio of the fluorescence emission at two wavelengths is measured, thus identifying the fluorophore on the basis of its spectral fingerprint. In the single fluorophore system developed by Ansorge *et al.*[11] (and introduced commercialized by Pharmacia–LKB Biotechnology), tetramethylrhodamine is bound to the primer and four electrophoretic lanes are probed by a laser beam entering through the gel from the side.

The limit of the above techniques still rests on the fact that, in a single run, it is impossible to obtain sequence data longer than *ca.* 400 nucleotides. Above this limit, there is not enough spacing among the different bands, which migrate almost as a continuum. By using a novel matrix, HydroLink[12], having properties intermediate between those of agarose and polyacrylamide, as shown in a Ferguson plot, we have been able to extend this upper limit to at least 600 bases[13]. It apppears that also CZE could hold a major promise for DNA sequencing[14].

4. SEPARATION OF MEGA-DNA FRAGMENTS

Conventional agarose gel electrophoresis separates native DNA molecules,

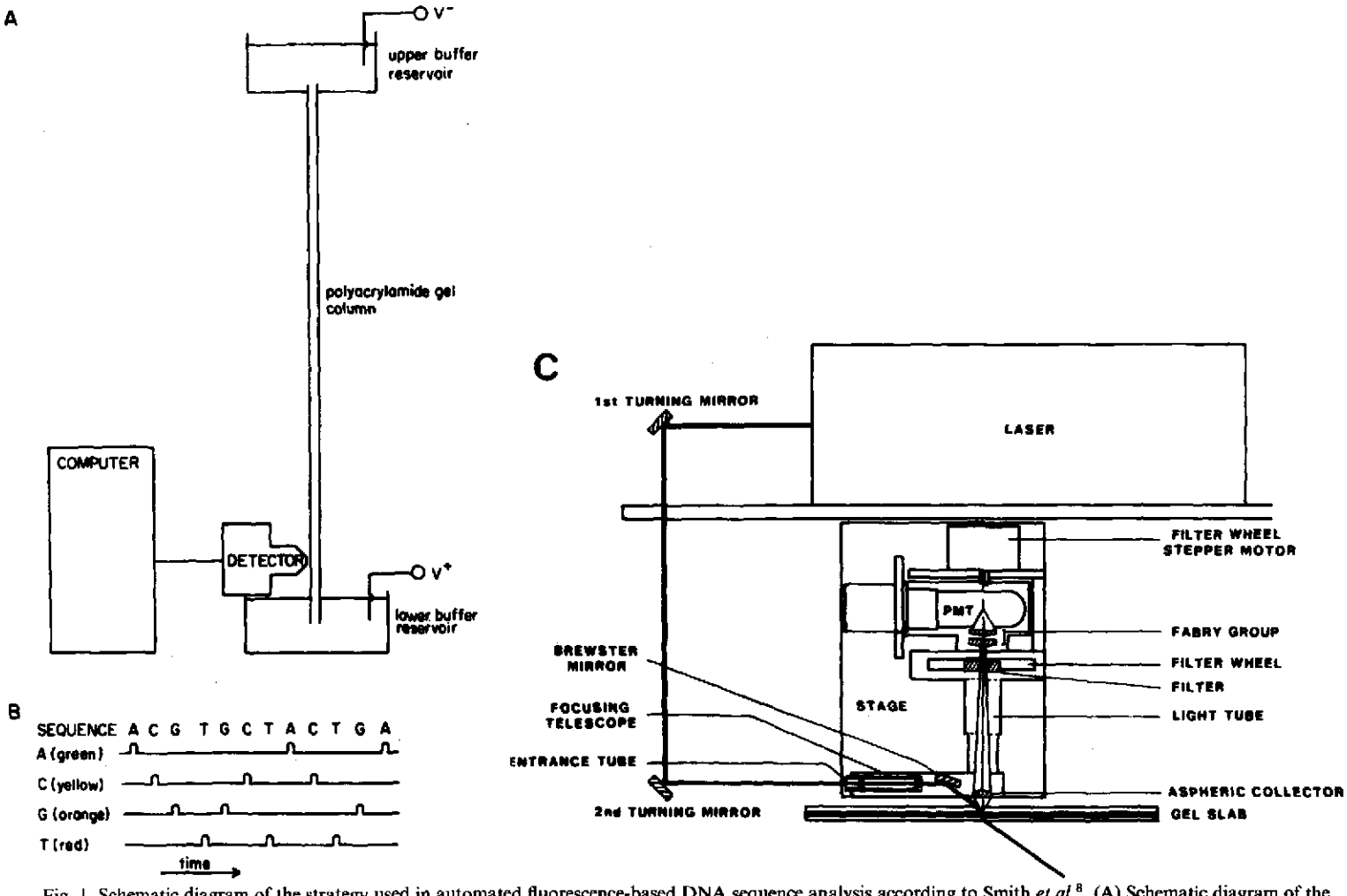

Fig. 1. Schematic diagram of the strategy used in automated fluorescence-based DNA sequence analysis according to Smith *et al.*[8]. (A) Schematic diagram of the prototype apparatus; (B) idealized representation of the data and the manner in which the measured fluorescence corresponds to the DNA sequence; (C) block diagram of the Model 370A fluorescence-based automated DNA sequencer of Applied Biosystems. Reproduced by permission from Smith and co-workers[8,9].

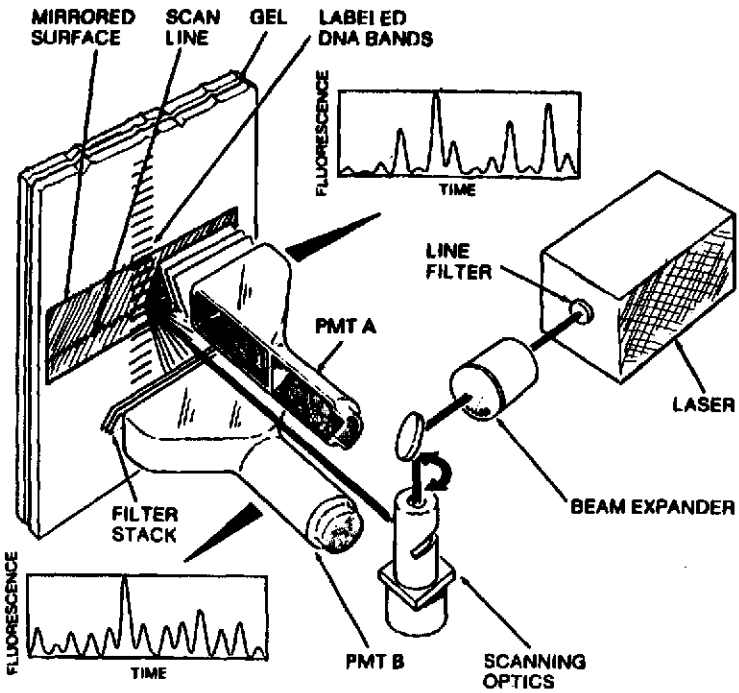

Fig. 2. Fluorescent detection system of the Genesis 2000. Schematic diagram of the optical system used for scanning excitation and for measurements of fluorescence from multiple sequencing lanes in an electrophoresis gel. The light from the argon ion laser is filtered to isolate the 488-nm emission line. The beam is deflected by a mirror into the scanning optics which are mounted on the shaft of digitally controlled stepping motor. A lens focuses the beam into a spot in the plane of the gel. A second mirror directs the beam to a position on the scan line defined by the rotational position of the motor shaft. Sequencing of multiple samples is achieved by directing the beam sequentially to each of the sequencing lanes on the gel. On entering the gel, the beam excites fluorescence in the terminator-labelled DNA. Fluorescence is detected by two elongated, stationary photomultiplier tubes (PMT A and B) which span the width of the gel. In front of each PMT, a filter stack is placed with one of the complementary transmission functions. Baseline-corrected ratios of signals in the PMTs are used to identify the labelled DNA fragments currently in the excitation region. Excitation efficiency and fluorescence collection are increased by the mirrored outer surface of the glass plate in the electrophoresis gel assembly. Reproduced by permission from Prober *et al.*[10].

within the range 1000–40 000 bases, on the basis of molecular mass. Above this threshold, DNA molecules exhibit size-independent mobilities and co-migrate in an agarose gel matrix[15]. As the average pore size of a 1% agarose gel is *ca.* 90–120 nm[16] and as, *e.g.*, the length of a small-size DNA, like the λ phage, is *ca.* 16 μm[17], with a radius, as an extended random coil, of *ca.* 500 nm, it would seem that for entering the gel these molecules must orient longitudinally with respect to the gel pores. Hence, the forward motion of the large DNA molecules is thought to be serpentine in nature, as they migrate through the gel pores, much as the movement of a snake in a field of grass. With the beginning of serpentine motion (called reptation)[18], the capacity for the gel matrix to sieve large DNA molecules is lost because the charge on the DNA and the friction exerted on the moving molecules are both proportional to size.

In 1983, Schwartz *et al.*[19] addressed the problem of separating large DNA molecules based on data from viscoelastic techniques for DNA molecular size determinations. They reasoned that, by forcing large molecules to change direction periodically and taking into account the strongly size-dependent relaxation time of large DNA, it might be possible to obtain size-dependent DNA separations. By arranging for a complex electrophoresis tank electrode geometry in conjunction with an electric switching unit, they were able to apply periodically across the gel two different electric fields at right-angles to each other in the horizontal plane[20], and large DNAs were again resolved on the basis of molecular mass. PFGE was thus introduced, and was shown to be able to separate DNAs in the 20 000–2 000 000-base size range. Subsequently, variations of the PFGE method were introduced, including orthogonal-field-alternation gel electrophoresis (O-FAGE)[21], field-inversion gel electrophoresis (FIGE)[22], electrophoresis using a contour-clamped homogeneous electric field (CHEF)[23], transverse alternating-field electrophoresis (TAFE)[24] and rotating-gel electrophoresis (RGE)[25–27]. Intact chromosomal DNA molecules have been resolved on these gel systems from a number of lower eukaryotes, including *Saccharomyces cerevisiae*[20,21,24] and several parasitic protozoa[26,27]. Such electrophoretic karyotypes have complemented classical genetic mapping in *S. cerevisiae* and have facilitated the study of karyotype[28,29], ploidy[30], gene location[31], chromosome polymorphism[32] and chromosomal rearrangements[33,34] in parasitic protozoa which are not amenable to genetic or cytogenetic analysis. PFGE methods have also been used for the separation of large human chromosomal DNA fragments generated with restriction enzymes that cleave the DNA infrequently[35], and in the analysis of amplified DNA in cell lines containing double minute chromosomes and homogeneously staining regions[36].

There are some basic problems with the first systems used (PFGE and O-FAGE), however. Owing to the non-uniformity of the electric field across the gel, the speed and migration path of DNA would vary from lane to lane and with distance from the wells, along curved trajectories, rendering comparison very poor. In CHEF[23], where the electrodes are clamped around the gel in an hexagonal array (see Fig. 3), the resulting field geometry allows migration of the DNA bands in a straight path towards the opposite gel extreme. The same applies to FIGE[22] (Fig. 4) and to TAFE[24] (Fig. 5). In fact, with the advent of these systems, it became clear that the term O-FAGE was a misnomer: for optimum separation and performance, the two alternating electric fields did not have to be orthogonal, but at a rather obtuse angle (*ca.* 120°)[37]. FIGE is in reality an extreme case, where the two electric fields are coupled at 180°, net forward migration being achieved either by employing longer switching intervals between the periodic inversion of the uniform electric field, or higher field strengths in the forward than in the reverse direction[38]. In a 2D O-FAGE/FIGE run, an unexpected phenomenon of parabolic migration of DNA fragments is experienced, by which small and large DNAs move forward, whereas some intermediate sizes (depending on the pulse frequencies) are arrested in the application well. For this reason Olson[38] proposed a three-state model (see Fig. 4) in which some DNA fragments could resonate with given field frequencies, thus spending all their time dwelling in the application slots. The latest version of PFGE is rotating-gel electrophoresis, by which a conventional, continuous electric field is applied and pulsing is obtained by rotating the gel platform (Fig. 6).

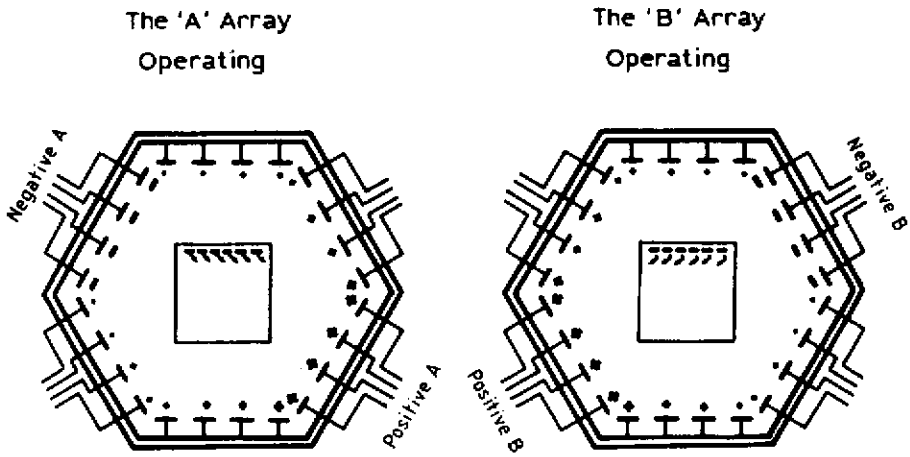

Fig. 3. Diagrammatic representation of the contour-clamped homogeneous electric field (CHEF) electrode format showing the clamped potentials at each electrode (represented by the size of the positive sign) when the A and B electrodes are in operation. The central square represents the agarose gel and the arrows indicate the direction of movement of the DNA out of the wells on activation of the A (right-pointing arrows) or B (left-pointing arrows) electrode couples. This electrode array has been adopted by Pharmacia–LKB to transform the Pulsephor (an O-FAGE-type apparatus) into a CHEF-type instrument. The clamped electrode potentials produce a homogeneous electric field throughout the gel and a field alternation angle of 120°. Reprinted by permission from Dawkins[37].

Whereas it appears that PFGE instrumentation has advanced considerably, at the theoretical level the mechanism of DNA migration is still not fully understood. Recent work by Stellwagen and Stellwagen[39] has shed more light on PFGE. It appears that, when the DNA molecule is much smaller than the median gel pore diameter, the DNA coil does not have to stretch or to orient. However, when the median pore diameter of the gel is smaller than the contour length of DNA, the DNA molecule becomes both stretched and oriented. However, whereas molecules smaller than *ca.* 4000 bases become completely stretched, larger molecules are only partially stretched. The orientation and stretching of DNA molecules in the gel matrix indicate that end-on migration, or reptation, is a likely mechanism for DNA electrophoresis in agarose. A unique finding[39] is that the effect of the pulsed electric field is exerted not

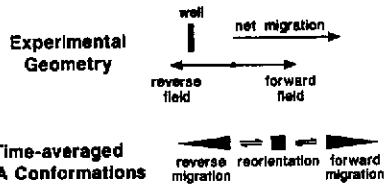

Fig. 4. Scheme of field-inversion gel electrophoresis (FIGE). The top of the diagram indicates the simple electrophoretic geometry. Note that the forward and reverse fields can be of equal strength with longer forward than reverse switching intervals, or the switching intervals can be equal with a higher forward than reverse field, or some combination of inequalities in the field strengths and the switching intervals can be used to achieve net forward migration. The bottom of the diagram presents a simple three-state model for the conformational changes that accompany the field-inversion events. Reproduced by permission from Olson[38].

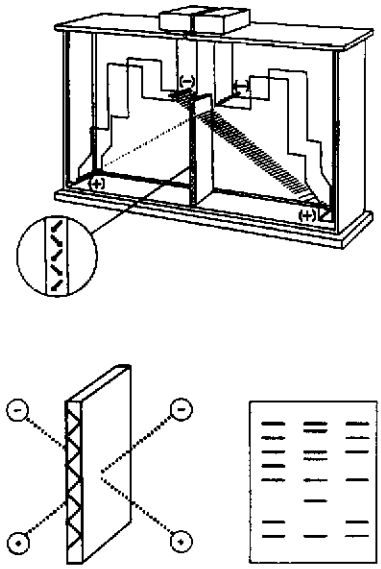

Fig. 5. Schematic diagram of transverse alternating-field electrophoresis (TAFE). The electrodes (+ and −) are wires stretched across the width of the box parallel to the gel faces (note that the gel is standing vertically and that both sides are exposed to the current). The angle formed at the sample loading wells between the fields generated by the electrodes is 115°. TAFE works like other pulsed-field techniques by causing periodic reorientation of DNA molecules under the influence of two alternating electric fields. The technique employs a three-dimensional geometry, causing DNA to zig-zag through the thickness of the gel (see the inset). The result of this movement is that the face of the gel displays perfectly straight migration paths (see lower part of the drawing). Reproduced by kind permission from Beckman Instruments, Palo Alto, CA, U.S.A.

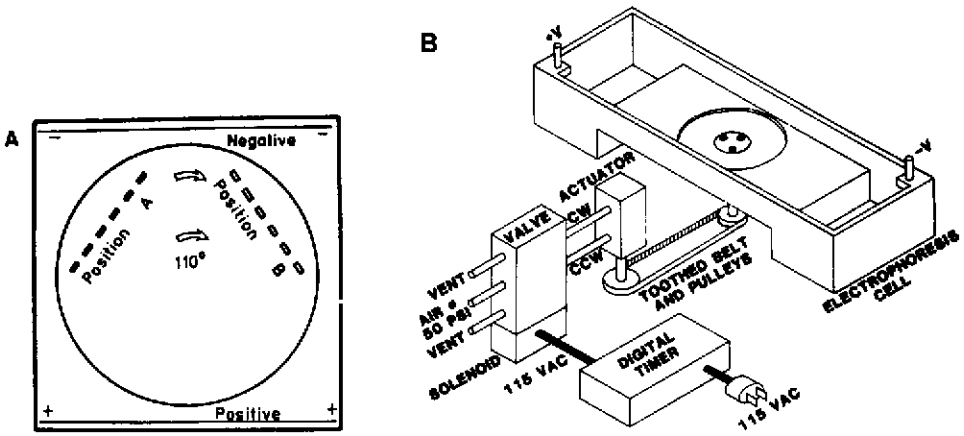

Fig. 6. (A) Diagrammatic representation of the rotating-gel electrophoresis (RGE) apparatus. The gel is subjected to a homogeneous electric field, but the circular gel rotation between positions A and B produces a field alternation on the samples. The optimum angle of the gel rotation is between 110 and 120°. The electric field is interrupted only for the few seconds it takes the gel to rotate to its new position. (B) Schematic diagram of the pneumatic apparatus of Sutherland *et al.*[34]. The rotating platform holding the gel inside the electrophoresis cell is coupled via a toothed belt and pulleys to a pneumatically driven rotary actuator. Reproduced by permission from Sutherland *et al.*[34].

only on the DNA molecules, but also on the gel matrix. It appears that with very short pulses of high amplitude, individual agarose chains or bundles of chains, or dangling ends of the matrix, could be oriented in the field. At longer pulse times and at lower voltages, yet another phenomenon is apparent: larger sections of the agarose gel (microdomains) become oriented in the field. The entire matrix becomes in a way more "fluid", and this could explain why very large DNA molecules can migrate through the gel. This is a fascinating new finding, and this effect will have to be taken into consideration in all theories of DNA migration under pulsed fields.

5. ELECTROPHORESIS AS A PROBE OF MACROMOLECULAR STRUCTURE

We are all used to the idea of electrophoresis as a powerful tool for macromolecular separation and characterization. However, recent work on DNA electrophoresis has proved that this technique is also a fine probe of structural conformation of this macromolecule. Some striking examples can be given. It is known that DNA molecules of the same size may assume different topologies, including supercoiled, bent, branched or cruciform structures. Recent work with CHEF has shown that such molecules exhibit anomalous shifts in mobility with respect to linear DNA in response to changes in electric field strength[40]. A case in point is shown in Fig. 7: in lane 2, a mixture of linear and supercoiled DNAs, applied in the same sample slot, are

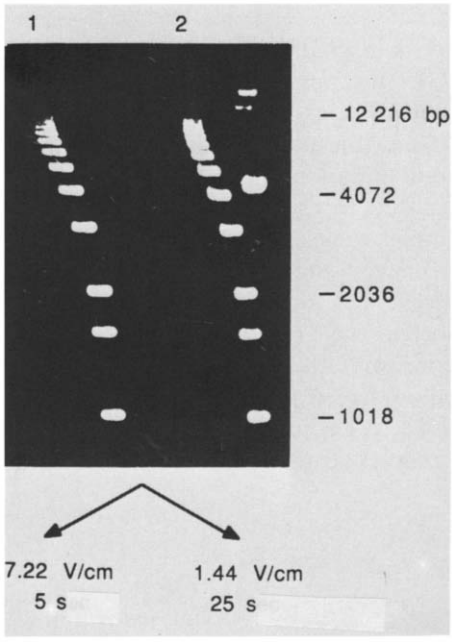

Fig. 7. Separation of DNA by topology. Lane 1 contains linear DNA markers (Bethesda Research Labs. 5615 SA/SB). Lane 2 contains a mixture of the linear DNA markers and pRSV cat plasmid DNA purified by equilibrium centrifugation in caesium chloride. The 5027-base pair (bp) supercoiled plasmid forms the major band that is displaced from the arc of linear molecules. The fainter bands (upper right corner) correspond to the nicked plasmid and supercoiled and nicked dimers. Electrophoresis was performed through a 1% agarose gel in Tris–borate–EDTA buffer (pH 8.3) at 9°C for 22 h. The field strength alternated between 7.22 V/m for 5 s and 1.44 V/cm for 25 s. Reprinted by permission from Chu[40].

seen to be separated along the migration path. The supercoiled 5027-base pair plasmid (and its dimer) are displaced from the arc of linear DNA molecules and resolved from fragments of the same size by a lateral displacement. This is a unique effect of the CHEF technique; previously, for resolving such topologies, a 2D method had to be employed, as follows.

Electrophoresis was performed under standard conditions in the first dimension. Then the gel was soaked in a DNA intercalating agent such as chloroquine to alter supercoiling. Finally, the gel was rotated by 90° with respect to the electrodes, and subjected to electrophoresis in the second dimension[41,42], where the topoisomers would ultimately be resolved.

Even in the absence of a pulsed electric field, conventional electrophoretic analysis can yield a wealth of information on DNA molecular structure. For instance, in the case of branched DNA, Ferguson plot analysis has given excellent information about junction structure, formation, stoichiometry, supramolecular assembly and accessibility to chemical attack[43]. According to Seeman *et al.*[43], gel electrophoresis has been a valid substitute for NMR spectroscopy and crystallography in DNA analysis, even though the two latter techniques can be expected to yield eventually the conformational details of junction structure at higher resolution. According to Fried[44], measurement of protein–DNA interaction parameters can be performed by electrophoretic mobility shift assays. Under appropriate conditions, both equilibrium constants and rate constants for binding reactions can be obtained through analysis of electrophoretic patterns.

In addition, by performing electrophoresis in gradients of denaturants (*e.g.*, temperature-gradient gel electrophoresis, TGGE), it is possible to analyse conformational transitions and sequence variations of nucleic acids, as well as protein–nucleic acid interactions[45]. For example, when analysing these thermal transition curves under electrophoresis, it is possible to detect point mutations induced in cDNA clones by site-directed mutagenesis[45]. Moreover, the effects of a single amino acid exchange on the thermal stability of a protein–DNA complex can easily be assessed by TGGE. Thus, in the case of the Tet repressor from *E. coli*, containing a Trp → Phe mutation at positions 43 and/or 75, by TGGE analysis it was found that no alteration in binding was induced in the Trp_{43} mutant, whereas the Trp_{75} mutation strongly affected the complex DNA–tetracycline (Tet) repressor. Other types of denaturing gradients (urea and formamide) have been used to screen for DNA sequence polymorphism in the human factor VIII gene[46]. These are just some examples of the power of electrophoresis in elucidating some complex structural variations in macromolecules.

6. TWO-DIMENSIONAL MAPS

2D Protein maps have also shown a marked growth. By coupling sequentially a pure charge (IEF) to a pure size [SDS–polyacrylamide gel electrophoresis (SDS-PAGE); the latter orthogonal to the first] fractionation, one can distribute the polypeptide chains on a surface having as coordinates charge and mass (IEF-SDS or ISO-DALT, according to the Andersons' nomenclature)[47]. When the first dimension is performed in immobilized pH gradients, the technique is called IPG-DALT[48]. Fig. 8 gives an example of a 2D map of [^{35}S]methionine-labelled proteins from human

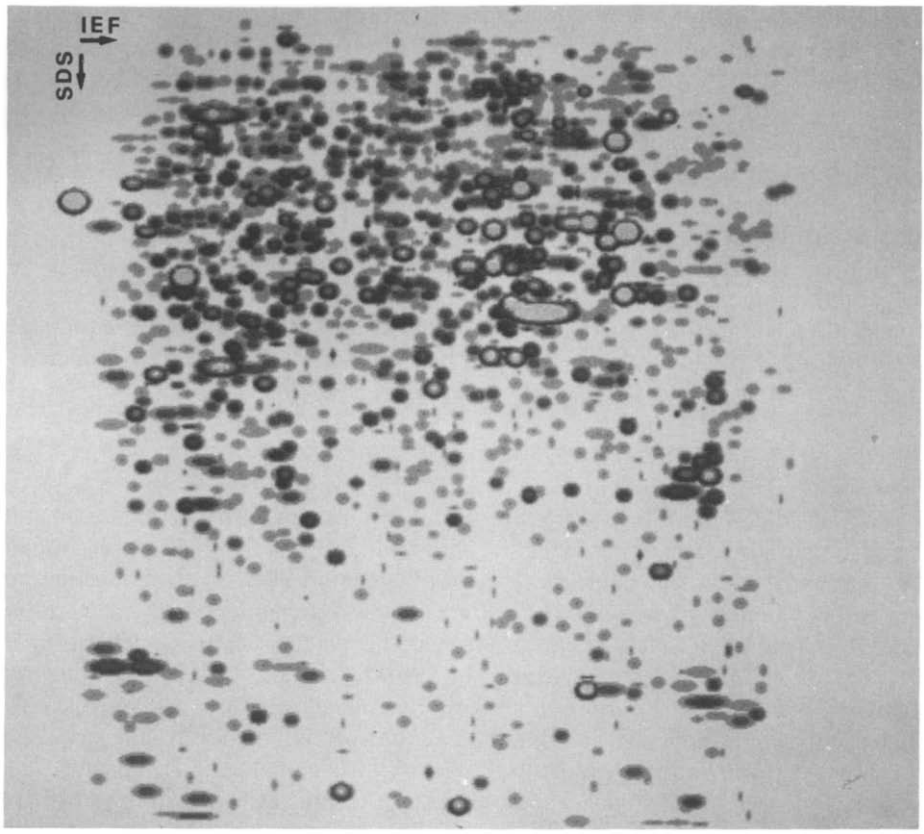

Fig. 8. Synthetic image of the two-dimensional (ISO-DALT) fluorogram of [^{35}S]methionine-labelled proteins from human ephitelial amnion cells. There are 1244 spots in this map. Reproduced by permission from Celis *et al.*[49].

epithelial amnion cells[49]: there is almost an artistic beauty to it, even though the image shown here is synthetic, *i.e.*, it has been redrawn by a computer after digitizing the experimental 2D map and cleaning the background, etc. The technique was reported for the first time by Barrett and Gould[50] and then described more extensively in 1975 by at least three workers: O'Farrell[51], Klose[52] and Scheele[53].

What is the value of 2D maps? In giant gels (*e.g.*, 30 × 40 cm size)[54] and on prolonged exposure of radiolabelled material (up to 2 months), the technique is capable of resolving as many as 12 000 spots in a total mammalian cell lysate. Hence it is likely that, in a properly run 2D map, a spot will represent an individual polypeptide chain, uncontaminated by other material co-migrating in the same gel area. On this assumption, and provided that enough material is present in an individual spot (about 1 μg), it is possible to elute it on a glass-fibre filter and to do microsequencing on it[55].

There are in fact two different strategies. According to Choli *et al.*[56], without removing the proteins from the membrane, direct microsequencing, enzymatic or chemical fragmentation or hydrolysis for amino acid analysis can be carried out. Simpson *et al.*[57], however, preferred to extract the Coomassie-stained polypeptides

from the blotting membrane in which they are trapped [usually poly(divinylidene difluoride) membranes] by using a mixture of SDS and Triton X-100. The proteins are then separated from the surfactant mixture by a chromatographic procedure on reversed-phase sorbents at high organic solvent concentration, *i.e.*, under conditions that prevent detergent binding. The proteins are then recovered from the sorbent by adding trifluoroacetic acid and by a decreasing gradient of organic solvent. After proteolytic fragmentation the peptides are analysed on a microbore column and, if needed, eluted for microsequence analysis.

In both instances a general strategy seems to be emerging: owing to the extremely high resolving power of 2D techniques, the possibility of blotting, the ease of the methodology and the availability of unsophisticated and inexpensive equiment, this methodology might replace HPLC techniques for the isolation and microsequencing of proteins.

7. IMMOBILIZED pH GRADIENTS

In 1982, IPGs were introduced, resulting in an increase in resolution of one order of magnitude when compared with conventional IEF[58]. By 1980, it was apparent to many IEF users that there were some inherent problems with the technique, which had not been corrected in more than 20 years of use and were not likely to be solved. In particular, a major phenomenon was the near-isoelectric precipitation of samples of low solubility at the isoelectric point (p*I*) or of components present in large amounts in heterogeneous samples. The inability to reach stable steady-state conditions (resulting in a slow pH gradient loss at the cathodic gel end) and to obtain narrow and ultra-narrow pH gradients, aggravated the situation. Perhaps most annoying was the unreproducibility and non-linearity of pH gradients produced by the the so-called "carrier ampholyte" buffers[59]. IPGs proved to be able to abolish all these undesirable phenomena.

IPGs are based on the principle that the pH gradient, which exists prior to the IEF run itself, is copolymerized, and thus insolubilized, within the fibres of a polyacrylamide matrix. This is achieved by using, as buffers, a set of six commercial chemicals (called Immobiline, by analogy with Ampholine) having p*K* values distributed in the pH range 3.6.–9.3. Previously, not much was known about the Immobiline chemicals, except that they are acrylamido derivatives, with the general formula $CH_2=CHCONHR$, where R denotes a set of two weak carboxyls, with p*K* 3.6 and 4.6, for the acidic compounds, and a set of four tertiary amino groups, with p*K* 6.2, 7.0, 8.5 and 9.3, for the basic buffers. We have recently been able to decode these structures and to give their synthetic routes and purification protocol[60,61]. We have reported a total of ten such buffers: eight are weak acids and bases, with p*K*s covering the pH range 3.1–10.3, and the other two are a strongly acidic (p*K* 1.0) and a strongly basic (p*K* > 12) titrant which were introduced in 1984 by Gianazza *et al.*[62] for producing linear pH gradients covering the entire pH 3–10 range (computer simulations had shown that, in the absence of these two titrants, extended pH intervals would exhibit strong deviations from linearity at the two extremes, as the most acidic and most basic of the commercial Immobilines would act simultaneously as buffers and titrants)[63].

With these additional Immobilines, it has been possible to extend the fraction-

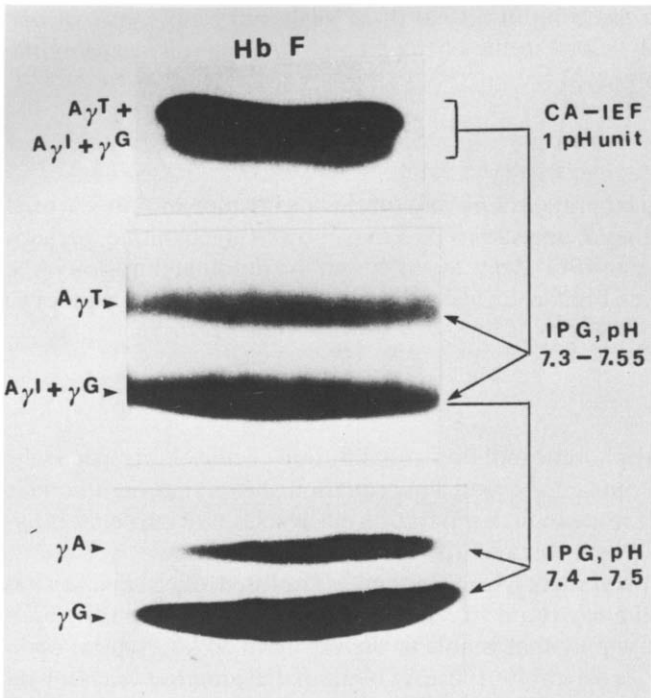

Fig. 9. Focusing of umbilical cord lysates from an individual heterozygous for foetal haemoglobin (Hb F) Sardinia (for simplicity, only the HbF bands are shown, and not the two other major components of cord blood, *i.e.*, Hb A and Hb F_{ac}). Top: focusing performed in a 1 pH unit span in the presence of carrier ampholytes (CA-IEF). Note that broadening of the Hb F zone occurs, but not splitting into well defined zones. Centre: same sample, but focused over an IPG range spanning 0.25 pH unit. Bottom: focusing of the lower band in central panel, but in an IPG gel spanning 0.1 pH unit. The resolved Aγ/Gγ bands are in a 20:80 ratio, as theoretically predicted from gene expression. Their identity was ascertained by eluting the two zones and fingerprinting the γ chains. Modified from Cossu and Righetti[76]; reproduced by permission of Elsevier.

ation capability to strongly acidic proteins[64], as the pH gradient could be extended to as low as pH 2.5, and to strongly alkaline species[65]. Given the evenly spaced pK values along the pH scale, it is clear that the set of ten chemicals proposed (eight buffers and two titrants) is adequate to ensure linear pH gradients along the pH 2.5–11 axis (the ideal ΔpK for linearity would be 1 pH unit between two adjacent buffers). For a more detailed treatise on how to run an IPG gel and how to use IPG recipes, the reader is referred to an extensive manual[66] and to a recent review[67].

Owing to the much increased resolution of IPGs, a number of so-called "electrophoretically silent" mutations (bearing amino acid replacements with no ionizable groups in the side-chains) have now been fully resolved. An illustration is shown in Fig. 9: two foetal human haemoglobin (Hb F) phenotypes, Aγ and Gγ, with an Ala → Gly substitution in residue 136 of the γ chains, are fully resolved in IPGs, although their difference in surface charge (ΔpI) is barely 0.003 pH unit. This is an exceptional separation: in reality, what we are showing is that IPGs are able to detect not just a

difference of a single amino acid (out of a total of *ca.* 600), but a difference of one carbon atom out of total of >2000 in the haemoglobin molecule (disregarding the three protons of the methyl group).

8. CAPILLARY ZONE ELECTROPHORESIS

It is proposed not to elaborate extensively on this technique, and only a brief discussion will be presented. CZE appears to be a most powerful technique, perhaps equalling the resolving power of IPG. If one assumes that longitudinal diffusion is the only significant source of zone broadening, then the number of theoretical plates (N) in CZE is given by[68]

$$N = \mu V/2D$$

where μ and D are the electrophoretic mobility and diffusion coefficient, respectively, of the analyte and V is the applied voltage. This equation shows that high voltage gradients are the most direct route to high separation efficiencies. For proteins, it has been calculated that N could be as high as 10^6 theoretical plates.

Fig. 10 is a schematic diagram of a CZE system[69]. The fused-silica capillary has a diameter of 50–80 μm and a length up to 1 m. It is suspended between two reservoirs, connected to a power supply that is able to deliver up to 30 kV (typical operating currents being of the order of 10–100 μA). One of the simplest ways of introducing the sample into the capillary is by electromigration, *i.e.*, by dipping the capillary extremity into the sample reservoir, under voltage, for a few seconds.

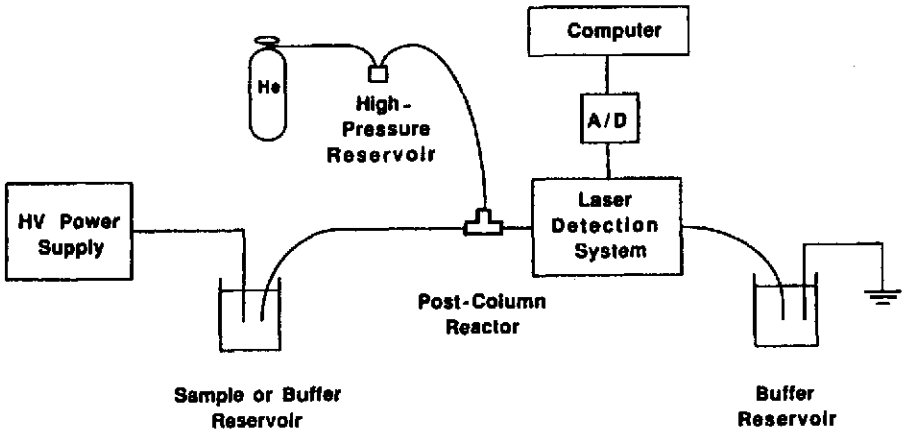

Fig. 10. Scheme of a CZE apparatus. The high-voltage (HV) power supply can deliver up to 30 kV. The fused-quartz capillary generally has and I.D. of 50–80 μm. The detector consists of a beam from a high-pressure mercury–xenon arc lamp source oriented perpendicular to the electric migration path, at the end of the capillary. The sample signal (generally emitted fluorescence) is measured with a photomultiplier and a photometer connected to the analogue–digital converter of a multi-function interface board mounted on a microcomputer. In the scheme reproduced here, postcolumn sample derivatization is obtained by reacting the sample zones with *o*-phthaldialdehyde. A/D = Analog-to-digital converter. Reproduced by permission from Rose and Jorgenson[69].

Detection is usually accomplished by on-column fluorescence and/or UV absorption. Conductivity and thermal detectors, as employed in isotachophoresis, exhibit too low a sensitivity in CZE. The reason for this stems from the fact that the flow cell where sample monitoring occurs has a volume of only *ca.* 0.5 nl, allowing sensitivities down to the femtomole level. In fact, with the postcolumn derivatization method in Fig. 10, a detection limit for amino acids of the order of femtograms is claimed, which means working in the attomole range[69].

By forming a chiral complex with a component of the background electrolyte (Cu aspartame), it is possible to resolve racemates of amino acids[70]. Even neutral organic molecules can be made to migrate in CZE by complexing them with charged ligands, such as SDS. This introduces a new parameter, a hydrophobicity scale, in electrokinetic migrations. For more on CZE, readers are referred to the Proceedings of the First International Symposium on High-Performance Capillary Electrophoresis[71] and to other papers presented at this meeting. Review papers have also appeared recently[14,72,73].

However, it should not be thought that there are no problems with CZE. According to Pickering[74], CZE is a good complement to HPLC, but not without its own problems. To quote: "in absolute terms, mass sensitivity is good, but relatively high sample concentrations are needed, which means that reports of femtomoles analysed must be taken with some reservations. Selectivity is not outstanding, and reproducibility and quantitation raise questions that need to be addressed".

9. CHROMATOPHORESIS

This survey concludes by mentioning the latest development, chromatophoresis[75]. In a way, this is a variant of 2D techniques, but it is unique under many respects. Classical 2D maps couple a charge (IEF) to a mass (SDS-PAGE) fractionation, whereas in this new analytical technology, reversed-phase HPLC is coupled in a real-time automated system to SDS-PAGE.

Fig. 11 gives an example of how this is achieved: proteins eluting from the HPLC column pass through a UV detector (UV) to a heated micromixing chamber (protein reaction system, PRS). Polypeptides in the eluate are denatured and complexed with SDS on mixing with the standard protein reaction cocktail (PRC) containing SDS, 2-mercaptoethanol and buffer. The SDS–protein complexes reach the SDS gel slab through an outlet lying flush on the surface of the stacking gel of a discontinuous polyacrylamide gradient gel. The capillary delivering the column eluate is moved across the gel surface by a computer-controlled tracking system, in such a way that the entire gel width accommodates, by the end of this sweeping process, the entire column eluate. Eluate delivery to the SDS-gel slab is performed under a moderate voltage gradient, so that each liquid element dispenses immediately its protein content to the stacking gel, thus minimizing side diffusion (there are no sample application slots). The height of the stacking gel is in general higher than in conventional gels, so that the stacking process can be continued for up to 1 h, if needed; hence, by the time the entire column eluate reaches the interface of the running gel, all the protein zones are aligned horizontally.

This seems to be a useful approach and it could provide a clue as to where the field of electrophoresis is moving: perhaps toward a more intense integration with

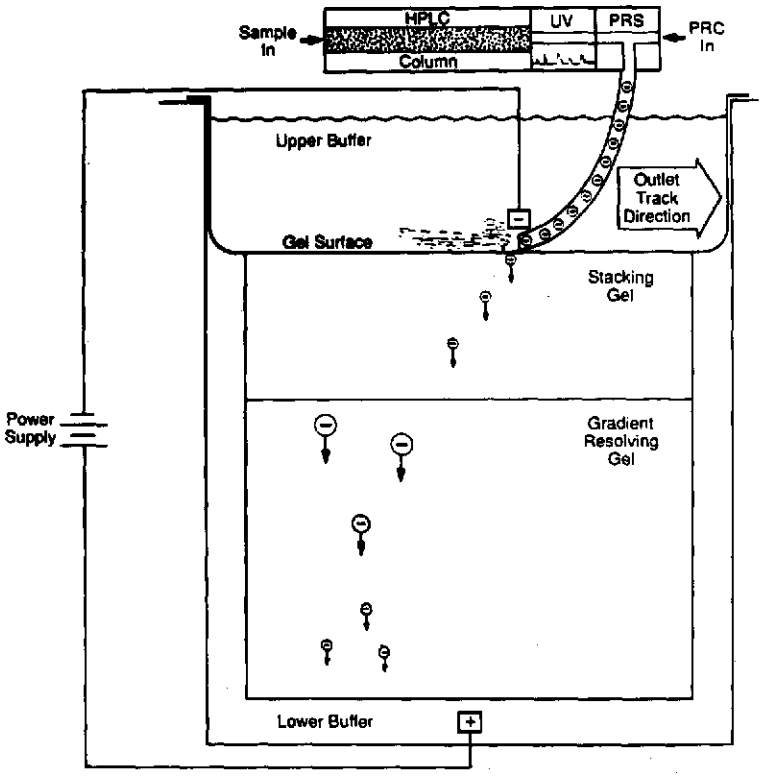

Fig. 11. Schematic illustration of the electrophoretic transfer of proteins in the chromatophoresis process. For details, see text. Reproduced by permission from Burton *et al.*[75].

chromatography. It now appears that the circle is closing: we are going back to moving boundary electrophoresis, as Tiselius, when starting electrophoresis at the Faculty of Science in Uppsala, also greatly encouraged work in chromatography. The two sciences started together then, divorced on the way and might end up united again.

10. CONCLUSION

A review in any field is always a damnation and a blessing: a damnation for the reviewer, who will come under attack by those scientists not mentioned, and a blessing for the reader, who will greatly benefit from a well written and well organized review. It is hoped that this review will prove useful, but I am willing to be damned, as I am sure I have unjustly left out many good articles which had to be sacrificed for lack of space or personal ignorance. A review also runs often the risk of being a personal recollection of the author's own experience and developments in a given field. In order to avoid that, I have on purpose expanded the field of nucleic acid electrophoresis, as I can claim no contributions in this field, in the hope also of focusing on this fascinating field.

Finally, some recent, special volumes of *Journal of Chromatography, Biomedical Applications*[77–79], dedicated to selected topics in biomedical chromatography and electrophoresis can be mentioned, where readers will find ample additional information.

11. ABBREVIATIONS

A	adenine
A/D	analog-to-digital converter
bp	base pairs
C	cytosine
CA	carrier ampholytes (Ampholine)
CHEF	contour-clamped homogeneous electric field
CZE	capillary zone electrophoresis
2D	two-dimensional
DNA	deoxyribonucleic acid
FIGE	field-inversion gel electrophoresis
G	guanine
Hb	haemoglobin
Hb A	human adult haemoglobin
Hb F_{ac}	human acetylated foetal hemoglobin
HPLC	high-performance liquid chromatogrpahy
HV	high voltage
IEF	isoelectric focusing
IEF-SDS	same as ISO-DALT
IPG	immobilized pH gradient
IPG-DALT	same as ISO-DALT except that immobilized pH gradients are used for the first dimension
ISO-DALT	isoelectric focusing followed by sodium dodecyl sulphate electrophoresis at right-angles
N	number of theoretical plates
NBD	4-chloro-7-nitrobenzo-2-oxa-1-diazole
NMR	nuclear magnetic resonance
O-FAGE	orthogonal field-alternation gel electrophoresis
PAGE	polyacrylamide gel electrophoresis
PFGE	pulsed-field gel electrophoresis
PMT	photomultiplier tube
RGE	rotating-gel electrophoresis
SDS	sodium dodecyl sulphate
T	thymine
TAFE	transverse alternating field electrophoresis
TGGE	temperature-gradient gel electrophoresis
Trp	tryptophan
UV	ultraviolet

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ABSTRACT

13. Given the recent extended review by Vesterberg [*J. Chromatogr.*, 480 (1989) 3–19] of electrokinetic methods, this survey has been restricted to the last decade, which has seen tremendous progress in several fields. DNA electrophoresis has experienced strong developments, both in the sequencing strategies (which have been largely automated with the use of fluorescent probes) and in pulsed field analysis of mega-DNA fragments, which has seen such developments as inverse-field, contour-clamped and rotating gel platforms, all allowing for straight band migration in each lane. Chromosome size mapping has now become a reality. Two-dimensional (2D) maps have also shown a dramatic improvement in performance, largely through the development of immobilized pH gradients, giving highly reproducible protein spots in the 2D plane and allowing the exploration of very narrow pH regions. Blotting techniques, combined with 2D mapping, allow sequence analysis and fingerprinting of a single polypeptide spot in a complex sample without resorting to lengthy chromatographic purification steps. Chromatophoresis generates a novel type of 2D mapping, based on hydrophobicity *vs.* size, rather than on charge *vs.* size, by direct coupling of a reversed-phase high-performance liquid chromatographic (HPLC) eluate to sodium dodecyl sulphate electrophoresis. The new rising star, capillary zone electrophoresis, offers speed, a large number of theoretical plates, selectivity and small sample requirements in a highly automated equipment.

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