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NEW DEVELOPMENTS IN ISOELECTRIC FOCUSING

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CONTENTS

1. INTRODUCTION

Since our last review on isoelectric focusing (IEF) in this journal[1], the technique has been extensively modified. For the reader anxious to follow this development over the years, several books have appeared during this decade as proceedings of meetings, organized on this topic as well as other electrophoretic techniques[2-8]. For the real *aficionados*, a new meeting (Electrophoresis '81) has been announced by R. C. Allen and P. Arnaud, to be held in Charleston, SC, U.S.A., April 7–10, 1981. The year 1980 has also witnessed the birth of the Electrophoresis Society with the companion, official journal "Electrophoresis", published by Verlag-Chemie. This society will work closely with the Japanese Electrophoresis Society which (much to the ignorance of the rest of the world) has been around no less than 30 years. Other books, covering general theoretical and methodological aspects of the

technique[9] as well as its biomedical and biological applications[10] have been published. An interesting treatise, devoted partly to the electrophoretic, partly to the
isoelectric analysis of mammalian cells, subcellular organelles, bacteria and viruses
has also recently appeared[11]. As a general reading, a new book, *Electrokinetic Separation Methods*[12] has appeared, which covers in 21 chapters practically all aspects of
electrophoresis. Several chapters[13–17] in it deal with different aspects of IEF. A host
of reviews covering practically all facets of IEF have been published over the years.
Just to name a few recent ones with their fields of coverage: preparative IEF[18],
biochemical and clinical applications[19,20], general applications[21,22], methodological
aspects in comparison with electrophoresis[23] and recent developments[24].

It is not the aim of the present article to be a super-review of already published
reviews. While this might fatten our *curricula vitae*, it would unduly subtract vital
space in scientific journals to other scientists. Therefore we will start from where we
left off last year[24] and cover only the most recent developments, thus quoting here
mainly papers which bear on this subject. As basic references for newcomers in the
field, we suggest the classical laboratory manual[25] covering IEF from its onset till the
year 1975 and the articles subsequently published reviewing most recent aspects[17,19,24].

2. NEW APPROACHES TO THE SYNTHESIS OF CARRIER AMPHOLYTES

We have already reviewed the synthesis and properties of Vesterberg-type
carrier ampholytes (CAs)[26–28], of modified, itaconic acid CAs[29] and of Grubhofer-
type CAs[30]. In 1979, the properties of a new generation of buffering ampholytes
(Pharmalytes) have been described in the literature[31–33]. As we have already described
them in detail[24], we will only briefly survey them here. The basic synthetic process
involves the copolymerization of amines, amino acids and dipeptides with epichlorohydrin. By a suitable choice of amines and amino acids, five narrow pH intervals
are directly generated. Since, within each narrow pH interval, seven different amines
are cross-linked with epichlorohydrin, and the average degree of polymerization is
six, logic suggests that no more than a handful of different ampholytes could be
generated. The striking new idea here is the introduction of the concept of steric
isomerism. By using D,L-epichlorohydrin, D,L-amines and D,L-amino acids, the
possible number of amphoteric species could be increased enormously, since all the
diastereoisomers of the ampholytes, in contrast with the mirror-image compounds,
will have different (even if very slightly) isoelectric points. The number is further
increased by the fact that also penta- and heptamers are generated, together
with branched and cyclic forms. A possible, hypothetical structure of a Pharmalyte
constituent ampholyte containing six amines has been given[31]. Structurally, the
Pharmalyte ions contain mostly tertiary amino groups, most of them linked to at
least one β-hydroxyl group. The main buffering groups, with respective buffering pH
ranges, are: α-amino carboxylic (pH 2–3); glycyl-glycine residues (pH 3–4);
β-hydroxylamines (pH 4–9) and dialkylaminopropyl (pH 9–11).

An interesting variant of the basic synthetic approach of Vesterberg[26] has
been reported by Charlionet *et al.*[34]. It is known that, in classical acrylic acid-type
ampholytes, most of the buffering power in the pH range 2–11 will have to be found
in the different pK values of the amino groups of the polyamino backbone. Actually,
a judicious blend of α-carboxyls (as in Pharmalyte), β-carboxyls (acrylic acid) and

of β- and γ-carboxyls of itaconic acid[29] in carrier ampholytes should provide a good buffering capacity in the pH range 2–6 but still the overall quality will be dictated by the properties of the polyamines. By increasing the heterogeneity of the starting polyamines (usually, in the past, an appropriate mixture of triethylenetetramine (TETA), tetraethylenepentamine (TEPA), and pentaethylenehexamine (PEHA), has been used[28]) ampholytes of improved qualities are synthesized (for the basic requirements of carrier ampholytes see Rilbe[35–37] and Vesterberg[38]). Unfortunately, due to their carcinogenic properties, higher polyamines (especially PEHA) are not any longer available in the market. In an effort to improve the quality of available polyamines, Charlionet et al.[34], before coupling them to acrylic acid, react them with the following compounds: acrylamide, N,N'-methylene bisacrylamide (Bis), 2,3-epoxy-propanol-1 and 1,2-7,8-diepoxyoctane. By these reactions, primary and secondary amino groups in polyamines are transformed into secondary and tertiary amines, respectively. Moreover, while the reaction with monofunctional compounds (acrylamide and epoxypropanol) is used solely for amino group modification, the reaction with the bifunctional agents (Bis and diepoxyoctane) leads to cross-linking of two different polyamines together. While the reaction of acrylamide, Bis and acrylic acid follows the well-known addition of nucleophilic groups of polyamines to the β-carbon of α-β-unsaturated compounds, the reaction of an epoxide group with a polyamine is expected to follow the reaction sequence:

$$R\text{--}NH_2 + CH_2\overset{O}{\overgroup{-}}CH_2 \longrightarrow R\text{--}NH\text{--}CH_2\text{--}\overset{OH}{\underset{|}{CH}}\text{--}$$

$$\underset{R}{\overset{R}{>}}NH + CH_2\overset{O}{\overgroup{-}}CH_2 \longrightarrow \underset{R}{\overset{R}{>}}N\text{--}CH_2\text{--}\overset{OH}{\underset{|}{CH}}\text{--}$$

thus leading to secondary hydroxyl groups and secondary and tertiary amines. By using empirically-found stoichiometric amounts, a highly heterogeneous mixture of modified polyamines is obtained which, upon reaction with acrylic acid, leads to carrier ampholytes with good conductivities and buffering capacities in the pH range 3–10. These ampholytes, for use in α_1-antitrypsin typing (α_1-AT), could be further fractionated into very narrow ranges, encompassing less than 0.5 pH units (e.g. pH 4.4–4.7 range). In these very shallow pH gradients, α_1-AT phenotypes differing in pI values by as little as 0.001 pH units could be resolved. Since the resolution limit was previously given as 0.02 pH units[39], this represents an amazing 20-fold increase in resolving power. It must be emphasized that a previous claim by Allen et al.[40] of a resolution of 0.0025 pH units has not been substantiated by the present authors.

The year 1980 has given birth to yet another variant of Vesterberg synthesis, the Just-type CAs[41], which we had nicknamed "the mysterious strain"[24], since their synthesis had been announced at every international meeting since 1976, but all details withheld, so that the scientific community had lost any hope of ever being informed on them. This suspense book has reached its last chapter, and the plot has been unravelled: once again the synthetic approach involves the addition of α-β-un-

saturated compounds to oligoethylene oligoamines, notably PEHA (although the source of this chemical is being kept secret). However, several variants make the Just synthesis a very interesting approach. First of all, instead of acrylic acid, its methyl ester is used. Secondly, the reactants are brought together not batchwise, but in a flow-through system, by mixing them in appropriate ratios with the aid of the Ultrograd gradient former. Typically, 1 M PEHA and 4 M acrylic acid methyl ester in methanol are poured in each reservoir of the gradient former. The reaction is started with a percentage concentration ratio of 80:20 (PEHA:acrylic derivative; basic carrier ampholytes) and is completed with a ratio of 35:65 (acidic carrier ampholytes). Fig. 1A shows the pH distribution of the carrier ampholytes obtained with PEHA and other oligoamines and Fig. 1B the type of template used in the Ultragrad for continuously varying the PEHA:methyl acrylate ratio. The mixed reactants travel in a capillary column at 40°C for 1.5 h and are collected in a fraction collector, thus being automatically divided into narrow pH ranges (in Fig. 1A seven narrow ranges are represented by the dots on the curve). In order to maintain the number of ampholyte molecules synthesized per unit time fairly constant, and depending on the initial PEHA concentration, the amount of –NH equivalents, going from basic to acidic carrier ampholytes, is kept constant by proportionally increasing the time available for the synthesis of one pH unit (this is seen in Fig. 1B as a progressive widening of the pH axis). After evaporating the methanol from the reaction products, the methyl ester is hydrolyzed for 2 h at 120°C. The advantages of using methyl acrylate are two fold: its reaction rate is enormously higher as compared with acrylic acid (half-life of 3.5 min vs. > 30 h at room temperature); in addition, since the ester is uncharged and volatile, its excess is easily removed by vacuum distillation.

3. PHYSICO-CHEMICAL PROPERTIES OF CARRIER AMPHOLYTES

We will not review here the general properties of Ampholine, which are described by Davies[42] and Haglund[43], nor more specific functions, such as chelating power, which also have been described[44,45]. Comparisons among Ampholine, Servalyte and Pharmalyte have also been reported[46,47]. New properties of Ampholine have recently been measured, and shall be reviewed here in more detail. One of the major problems in IEF experiments has been the total lack of knowledge of the molarity and ionic strength (I) of focused carrier ampholytes. The only parameter measurable with certainty after an IEF experiment is the pH, whose course is easily mapped both, in sucrose density gradients and in gels[48]. On the contrary, when performing electrophoresis, these three physico-chemical parameters which define the buffer medium, are rigorously known by the experimenter. This has made impossible, up to now, any comparison between electrophoresis and IEF data and has ingenerated uncertainty in data, especially when performing IEF of cells, since their measured pI is a strong function of the environmental ionic strength[11]. Recently, however, McGuire et al.[49] have been able to measure accurately the molarity of focused Ampholine. By focusing 1% Ampholine in a free liquid curtain, in the Hannig free flow apparatus, and measuring the osmolarity of the 48 eluted fractions, they have derived a molarity of 9–10 mM in the pH range 3.5–10. This is in fairly good agreement with the value 15 mM given by Gelsema et al.[50], by assuming an average molecular weight ($\overline{MW}$) for Ampholine of 700 daltons. The ionic strength, however, which in IEF is vanishingly
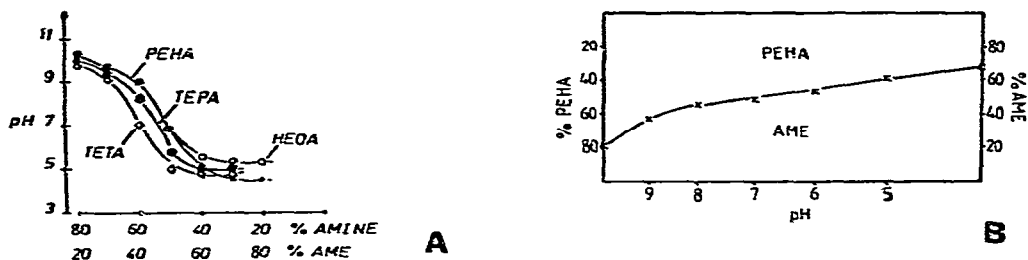
Fig. 1. (A) pH of the hydrolyzed reaction products obtained by coupling various percentage ratios of 4 M acrylic acid methyl ester (AME) with various 1 M amine solutions in methanol (HEOA = heptaethylene octamine). (B) Template used for the synthesis of PEHA ampholytes covering the pI range 3.5–10. Each one-unit pH interval is made to contain the same amount of PEHA by progressively widening the pH axis toward acidic pH values. The two solutions were mixed with the LKB Ultrograd gradient former. Both figures from Just[41].

small, has been a much more elusive parameter to measure. It has been known for a long time that the classical definition of ionic strength of Lewis and Randall[51]:

$$I = \tfrac{1}{2}\Sigma c_i z_i^2$$

(regrettably, due to a printer error which escaped our attention, this equation, reported by one of us[52], was misprinted as the square root of $\Sigma c_i z_i^2$) could not be applicable to such conditions as found in IEF. On the other hand, direct measurement of I during an IEF experiment has so far baffled any attempt. Righetti[52] has circumvented that by using as a probe red blood cells (RBCs), exploiting their well known pI dependence from the buffer ionic strength[53]. By plotting data obtained from electrophoresis and IEF of RBCs, it was possible to extrapolate a value of $I = 0.5$ mg ion/l for focused 1% Ampholine (see Fig. 2). Also theoretical considerations and practical measurements of conductivity point to a value of $I = 0.5$ to 1 mg ion/l. The following equations have been proposed:

$$I = 1/20\ \bar{C}_{amph} + C_H \text{ in the pH range 2.5–7 and}$$
$$I = 1/20\ \bar{C}_{amph} + C_{OH} \text{ in the pH range 7–11,}$$

where $\bar{C}_{amph}$ is the molarity of focused carrier ampholytes and C_H and C_{OH} the molarities of protons and oxydryl ions, respectively, at a given pH.

Another highly controversial aspect of carrier ampholyte chemistry is the definition of their actual molecular weight. Several research groups have proposed all possible ranges of MWs: 5000–7000 daltons (Gierthy et al.[54]); 800–1200 daltons for Servalyte, 1000–6000 daltons for Ampholine and 1000–15000 daltons for Pharmalyte (Radola[55,56]); even an upper limit of 20000 daltons has been suggested[57]. These high MWs, apparently, are not just exhibited by a tiny fraction of the population of carrier ampholytes, but by a substantial proportion of them[55,56], especially in the alkaline pH ranges[54]. On the other hand, direct measurements made by gel filtration and osmometry have given, for Ampholine[43], a $\overline{MW} = 700$, with only 0.7% of the species above 1000 daltons and 0.03% in the proximity of 4000 daltons[58]. As scientists
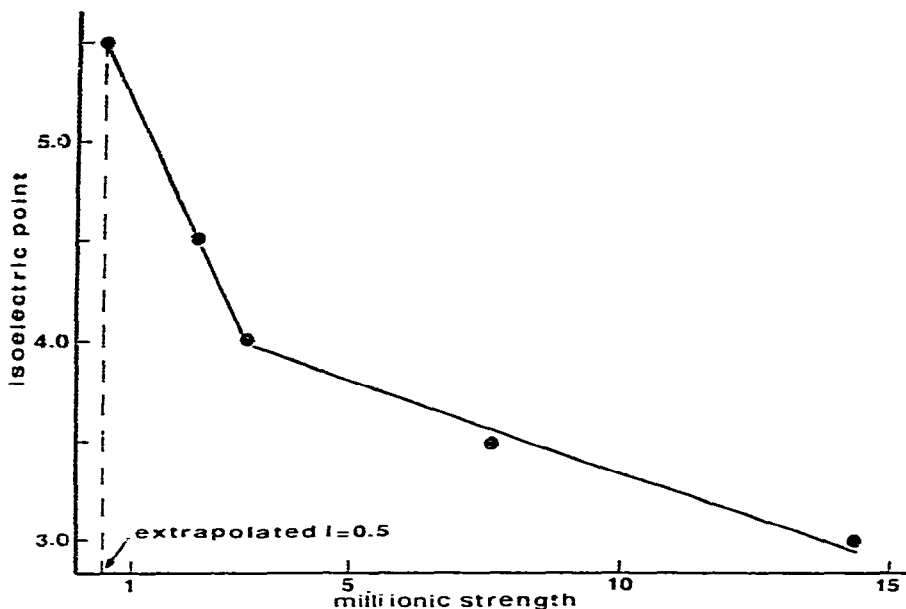
Fig. 2. Dependence of the isoelectric point of red blood cells from the medium ionic strength. The first point to the left has been obtained from IEF experiments by Just *et al.*[133] and McGuire *et al.*[49], while points 2–5 are from electrophoretic data tabulated by Seaman[5]. The ionic strength in IEF is the missing coordinate value of the first point of the curve (from Righetti[52]).

are never inclined to believe manufacturers, it is comforting that these data have been fully substantiated by independent work by Gelsema's group[46], who have found that, in almost all pH intervals, $MW_{Pharmalyte} > MW_{Ampholine} > MW_{Servalyte}$. Moreover, in all three cases, the highest MWs were found in the acidic, not in the basic fraction of carrier ampholytes. This is immediately evident also from theoretical considerations[59]. The actual MWs calculated were[46]: $\overline{MW} = 710$ for Ampholine and $\overline{MW} = 870$ for Pharmalyte. We feel it is important to critically evaluate these data, since a proper assessment of carrier ampholyte MWs is fundamental to all protein chemists using IEF. The supporters of the high MW hypothesis fail to account for the following experimental observations: (a) precipitation of proteins with up to 100% saturation with ammonium sulphate removes more than 99.99% carrier ámpholytes[60]. High-MW species would have been most certainly precipitated; (b) IEF of peptides[61-63] is made possible by the fact that, above a minimum critical MW (corresponding to 12–14 amino acids, *i.e. ca.* 1500 daltons), they are fixed and stained by simultaneous exposure to a dye–trichloroacetic acid (TCA) mixture. Under these conditions, carrier ampholytes are fully soluble.

The MW determinations of Gierthy *et al.*[54] rely on electrophoretic migration in sodium dodecyl sulphate (SDS) gels. In this system (except for Servalytes, which perhaps do not stain), the most alkaline pH ranges (pH 9–11) exhibit the highest MWs (5000–7000) while, if anything, the opposite should be true[43,59]. We feel that these authors have failed to realize that SDS-electrophoresis of basic proteins is

unreliable for their MW assessment[64]. Moreover, Poduslo and Rodbard[65] have demonstrated that the error in MW determination increases as the actual MW of the basic protein decreases. Thus, in the case of two basic proteins from myelin of rat central nervous system, having MWs of 18,400 and 14,300, the apparent MWs found in SDS gels were 21,800 and 19,000, respectively. Moreover, if the carrier ampholytes bound little or no SDS, as could be suspected if they were indeed small molecules, the negative charge density of basic carrier ampholytes would be much lower than that of acidic ones, resulting in a much higher apparent MW in SDS-electrophoresis. The $\overline{MW}$ data of Goerth and Radola[55], obtained by thin-layer gel chromatography on Bio-Gel P-10 in 25 mM phosphate, pH 7.2 and 2 M urea, are less susceptible to criticism. We can only offer as an explanation that Servalyte, Ampholine and Pharmalyte interact to a different extent among themselves, giving aggregates of different composition and stoichiometries, which then exhibit a much higher apparent MW. In fact Gianazza et al.[59] have recently demonstrated that Ampholine aggregates exist even during the IEF process, and that 8 M urea is required to split them apart. Moreover, they have found basic carrier ampholytes to be more hydrophobic than acidic ones, as they bind substantially higher amounts of neutral detergents, such as Nonidet P-40 (NP-40). At the light of these data, we feel that the supporters of the low-MW data[43,46,58] are most probably on the right track.

4. NEW DEVELOPMENTS IN PREPARATIVE TECHNIQUES

For a general survey of preparative techniques, see Righetti[18] and for more recent developments see our last review[24]. Particularly attractive appears the principle of "steady-state rheo-electrolysis", recently described by Rilbe[66], and a similar approach by Martin and Hampson[67], since they could have virtually unlimited fractionation capabilities with practically no consumption of expensive carrier ampholytes. However these systems appear to be hindered at present by a lot of practical problems, and they will probably require long developing times. We will comment here upon some recently described systems. Jonsson et al.[68] have adopted a parallelepipedic density gradient column for rapid preparative IEF (see Fig. 3), which can be run either horizontally or vertically. This column allows a complete IEF run to be terminated within 4 h, a remarkable saving in time in comparison with the standard technique in Rilbe's vertical density gradient columns, which require at least 15–20 h even under high-voltage conditions. These short focusing times are due to a mixed-type operation, whereby the column is operated horizontally for a total of 2.5 h, thus ensuring quick steady-state conditions, then slowly rotated to the upright position, where focusing is continued up to a total of 4 h. This column, however, is limited to small sample loads (10–20 mg protein).

A new approach, called recycling isoelectric focusing, has been described by Egen et al.[69]. It is well known that continuous-flow techniques[70], which appear essential for large scale preparative work, are disturbed by parabolic and electroosmotic flows, as well as by convective flows due to thermal gradients. Egen et al.[69] have improved this system by separating the actual flow-through focusing cell, which is miniaturized, from the sample and heat-exchange reservoir, which can be built up to
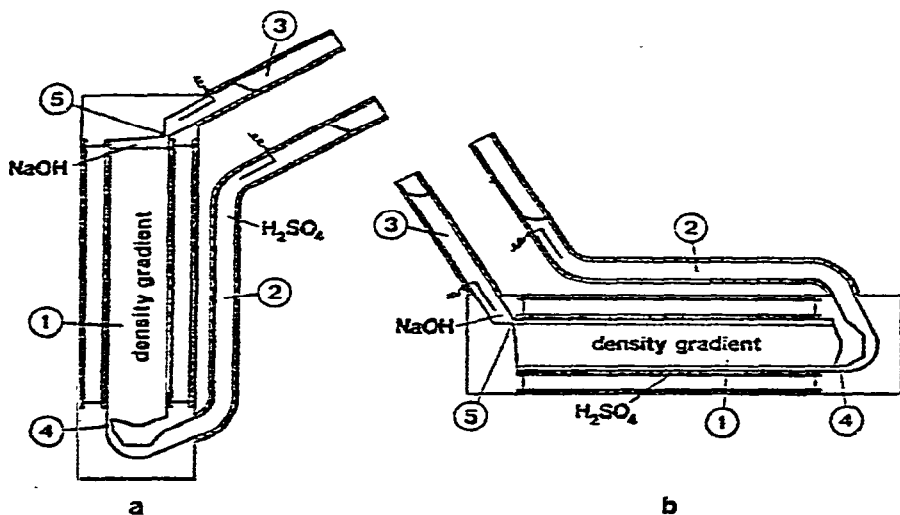
Fig. 3. Schematic drawing of a parallelepipedic density-gradient column, constructed for isoelectric focusing either in the vertical (a) or in the horizontal (b) position. 1 = Separation compartment; 2 and 3 = inclined tubes containing the electrodes; 4 and 5 = slits facilitating filling and emptying of the separation compartment (from Jonsson et al.[68]).

any size. Minimization of parabolic flow, electroendoosmosis and convective liquid flow is achieved by flowing the sample to be separated through a thin focusing cell (the actual distance anode to cathode is only 3 cm) built of an array of closely spaced filter elements oriented parallel to the electrodes and parallel to the direction of flow. Increased sample load is achieved by recirculating the process fluid through external heat-exchange reservoirs, where the Joule heat is dissipated. During each passage through the focusing cell only small sample migrations toward their eventual pI is obtained, but through recycling a final steady-state is achieved. The IEF cell has ten input and output ports for sample flow-through, monitored by an array of ten miniaturized pH-electrodes and ten UV sensors. The entire system is controlled and operated by a computer. Schemes of the entire apparatus set-up and of the flow IEF cell can be seen in Fig. 4A and B. By activating pumps at the two extreme channels, the computer can alter the slope of the pH gradient so as to keep a focused sample in a given channel, thus counteracting any affect of the cathodic drift, which results in a net migration of the sample zones towards the cathode.

Continuing on its classical line of multicompartment electrolyzers[71], Rilbe's group[72] has now built an improved apparatus which allows sample fractionation in the gram range. The cell contains 46 separation compartments, its total volume is about 7.6 l and its length is 1 m. The compartments are closed, and internal cooling and stirring are affected by slow rotation of the whole apparatus in a tank filled with cold water. The apparatus can be run with an electric load of up to 5 kV, and an isoelectric focusing takes 2–3 days. Drawings of the entire assembly and of one end cell and electrode compartment can be seen in Figs. 5 and 6. Fourteen grams of whey
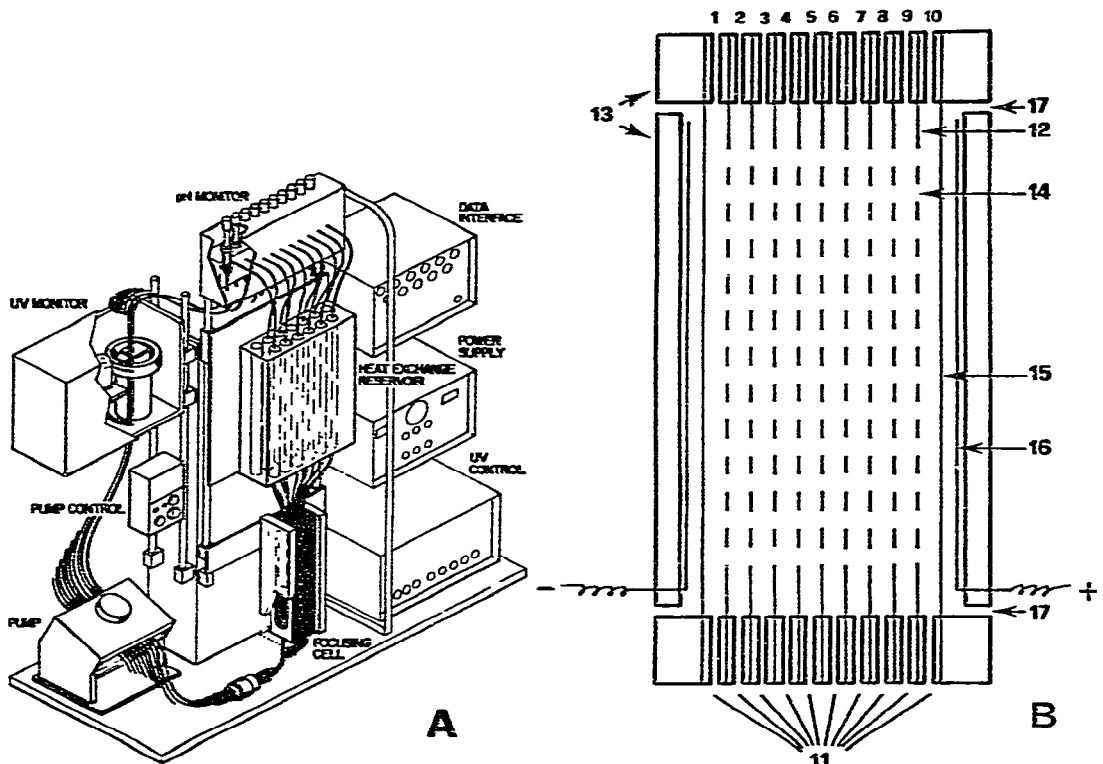
Fig. 4. (A) Schematic drawing of the recycling isoelectric focusing apparatus (RIEF). The 10 fractions from the reservoir are pumped through 10 flow-pH electrodes and then through 10 flow-UV cells to be cycled, via a multichannel pump, through a very compact focusing cell (the size of each compartment of the electrolyzer being 20 × 2 × 0.2 cm) and then back to the heat-exchange reservoir (from M. Bier, N. B. Egen, T. T. Allgyer, G. E. Twitty and R. A. Mosher, in E. Gross and J. Meienhofer (Editors), *Peptides: Structure and Biological Function*, Pierce Chemical Co., Rockford, IL, 1979, pp. 79–89). (B) Cross-sectional schematic representation of the RIEF cell. 1–10 = flow channels; 11 = connection to multichannel pump; 12 = plexiglass spacers; 13 = Perspex block (outer frame of the flow cell); 14 = filter elements (polyvinyl chloride) separating each compartment of the flow cell; 15 = dialysis membrane delimiting the electrode compartments; 16 = electrode platinum wires; 17 = input and output ports for recirculating electrolyte (from Egen *et al.*[69]).

protein could be completely separated into its main components, serum albumin (pI 4.60), α-lactalbumin (pI 5.01) and β-lactoglobulin (pI 5.13–5.23). Due to its mammoth size and the very high cost of the chemicals needed to operate it, we feel that, at present, this apparatus is more suitable for industrial type operations, rather than for research laboratories.

5. ANALYTICAL TECHNIQUES: METHODOLOGY

5.1. Agarose isoelectric focusing

Agarose would be an ideal anticonvective medium for IEF since, at the concentrations normally used (1%), it allows practically unhindered migration of
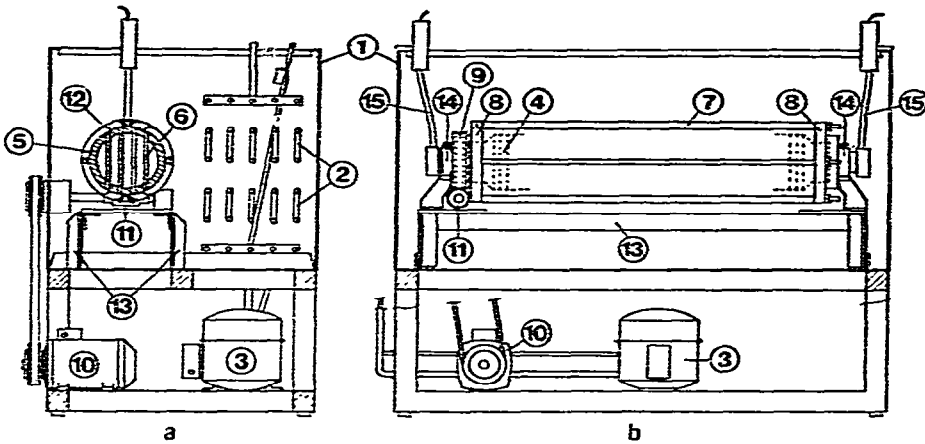
Fig. 5. Cross section (a) and longitudinal section (b) of multicompartment electrolysis apparatus. The main parts are: 1 = cooling tank; 2 = cooling coils; 3 = refrigerating machine; 4 = electrolyzer; 5 = ring-shaped, separation compartment, containing four cooling tubes (6); 7 = four pull rods, for assembling the electrolyzer, attached to a circular end piece (8); 10 = electrical motor for rotation of the electrolyzer via worm gearings (9) and 11); 13 = unit carrying the electrolyzer; 14 = slide bearings; 15 = gas-escape tubes (from Jonsson and Rilbe[72]).

macromolecules in the multimillion MW range. However, notwithstanding early claims in the literature, attempts of IEF in agarose were disastrous due to the presence, even in highly purified commercial products, of sulphate and carboxyl groups, which generate a severe electroosmotic flow and disrupt the pH gradient. Agarose, as purified from seaweeds of the class Rhodophyta (red algae) contains L-galactose-6-sulfate and pyruvic acid, present as the ketal 4,6-O-(1-carboxyethylidene)-D-galactose. By purifying this polysaccharide from *Gracilaria* species at the late stage of maturation (which are known to produce the least sulphated agarose), by alkaline desulfation to 3,6-anhydro-L-galactose and by removal of carboxyl groups (via methyl esterification or other processes) it has been possible to obtain recently a brand of agarose suitable for IEF. This is commercially available from LKB, Stockholm, Sweden (agarose-EF) and from Marine Colloids Division, FMC Corp., Rockland, ME, U.S.A. (Isogel agarose). Agarose IEF has been independently described by Rosén et al.[73–75] and by Saravis et al.[76–78]. Excellent resolution of very-high-MW proteins, such as a_2-macroglobulin (780,000 daltons), 19S immunoglobulins M (Ig M) (900,000 daltons), keyhole limpet hemocyanin ($3 \cdot 10^6$ daltons) and zinc glycinate human tumor marker ($2 \cdot 10^6$ daltons), could be achieved by IEF in agarose matrices. Besides its much larger pore size, agarose also presents other significant advantages over other supports, such as polyacrylamide: (a) it is non-toxic, while acrylamide and Bis are neurotoxins; (b) it is gelled without the aid of catalysts, which in polyacrylamide produce long-lived radicals; (c) it is fully compatible with subsequent immunofixation and crossed electrophoretic techniques; (d) it allows quick and efficient staining and destaining and it is easily dried for permanent records (these last properties are now shared by ultrathin polyacrylamide films, as will be described later). A simple gel casting procedure has been made possible be the use of clear, flexible, polyester-based plastic film (Gelbond, from Marine Colloids) rendered hydrophylic on one side for adherence of gels. A
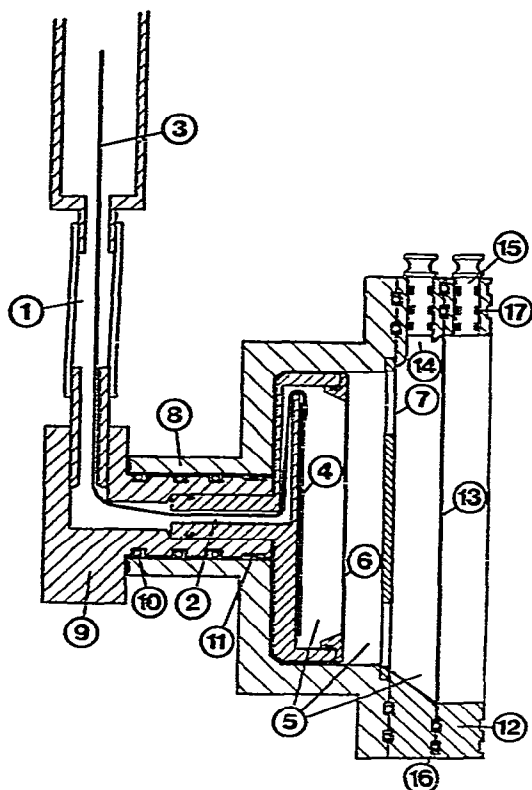
Fig. 6. Longitudinal section of one end of the electrolyzer shown in Fig. 5. 1 = Gas-escape tube, connected to the electrode compartment (5) via a channel (2); 3 = platinum wire for connection to the electrode (4); 6 = membrane preventing the electrode gases from entering the electrolyzer; 7 = circular wall for uniformly spreading the electrode current into the separation compartments; 8 = rotary part, supported by the stationary part (9); 10 = O-rings tightening the bearings; 11 = rubber gasket sealing the electrode compartment; 12 = one of the 46 rings of the separation compartments, divided by membranes (13); 14 = hole for filling and emptying one compartment, fitted with a stopper (15) and O-rings (17) for proper sealing; 16 = set of concentric O-rings for sealing each compartment to the next one (from Jonsson and Rilbe[72]).

piece of Gelbond film (usually 12.5 × 12.5 cm) is placed, hydrophobic side down, on a levelling table and, if needed, rolled flat with the aid of a rubber roller (Fig. 7A and B). In order to avoid too rapid a heat dissipation due to contact with the metal table, which could result in uneven gelling, thermal insulation is suggested by placing a sheet of polystyrol foam beneath the Gelbond film (LKB instruction leaflet). A desired volume of 0.8% agarose is heated at 100°C until dissolved, cooled at 55°C, added with Ampholine to a concentration of 2.5% and poured directly on the Gelbond horizontal sheet (Fig. 8A). The volume of agarose solution is chosen so as to have a thickness of about 1.2 mm of gelled matrix. If thinner gels are required (e.g. 0.8 mm), it is better to let the agarose gel in a vertical cassette, as regularly used for polyacrylamide gel polymerization (Fig. 8B), in order to ensure uniform gel thickness, particularly along the borders. The casting assembly will have to be prewarmed and shall
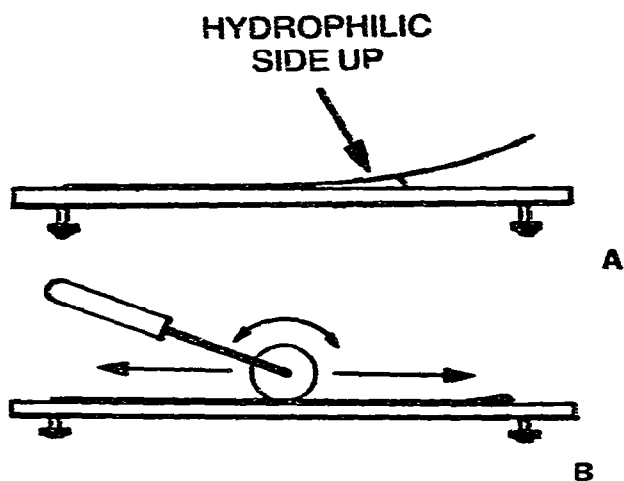
Fig. 7. Placing (A) and rolling flat (B) a Gelbond film on a levelling table for casting agarose gels (from Saravis and Cook, courtesy of Marine Colloids Division; instruction leaflet, 1979).
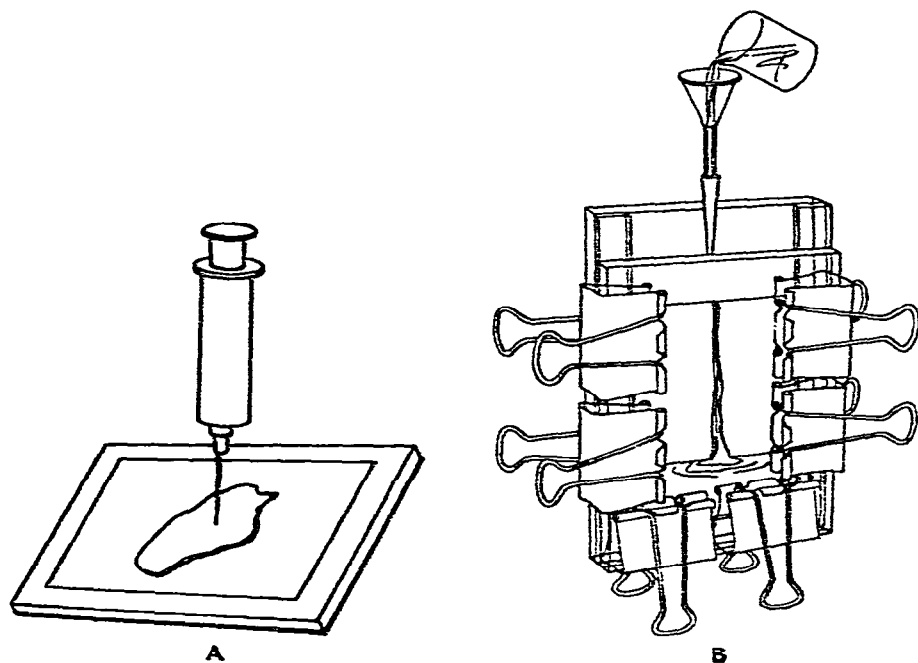

Fig. 8. (A) For casting agarose gels 1 mm thick or thicker, the gel solution is poured directly on an horizontal sheet of Gelbond film (do not use a syringe, though, but an erlenmeyer!). (B) Cassette for casting agarose gels thinner than 1 mm. The casting apparatus should be prewarmed at *ca.* 50°C and should contain the Gelbond film on one face (both figures from Saravis and Cook, courtesy of Marine Colloids Division; instruction leaflet, 1979).

contain, on one face, the Gelbond film. The gelled plates are usually aged overnight at 4°C to increase the mechanical strength of the gels. However, it has been suggested[78] to keep them at room temperature, to eliminate the syneresis (gel collapse and water exudation) observed upon storage at 4°C. The samples are applied as 3-μl droplets onto the gel surface, or soaked into Paratex filter pieces (LKB 2117-103), or with the aid of a 52-μm thick Mylar sheet containing loading slits or, in the case of biopsies, by direct tissue application onto the matrix[77]. The focusing process is usually over within 60 min at a constant wattage of 10 W (1100 V at steady-state)[80]. Since agarose is still not completely charge-free, and there is still a considerable water transport to the cathode, it has been suggested to drain continuously the gel with a thin, cellulose paper foil at the cathode[81], or to run the IEF experiment at 15°C instead of 4°C[80]. At the end of the IEF run, the agarose plate is quickly fixed in 33% methanol, 5% TCA and 3.5% sulfosalicylic acid in water. After the fixation step, the agarose gel is dried onto the Gelbond film by the sequence illustrated in Fig. 9. It is covered with filter paper and blotting towels and left for 30 min under a 500 g weight, then fully dried with a hair dryer and freed from the gel-adhering filter paper by a quick wetting step (steps 3 and 4 in Fig. 9). At this point, the dried agarose film can be quickly stained and destained by classical Coomassie Blue methods[80] or, as suggested by Saravis et al.[78], by using Crowle's stain[82] (2.5 g Crocein Scarlet, 150 mg Coomassie Brilliant Blue R-250, 50 ml glacial acetic acid, 30 g TCA in 1 liter final volume).
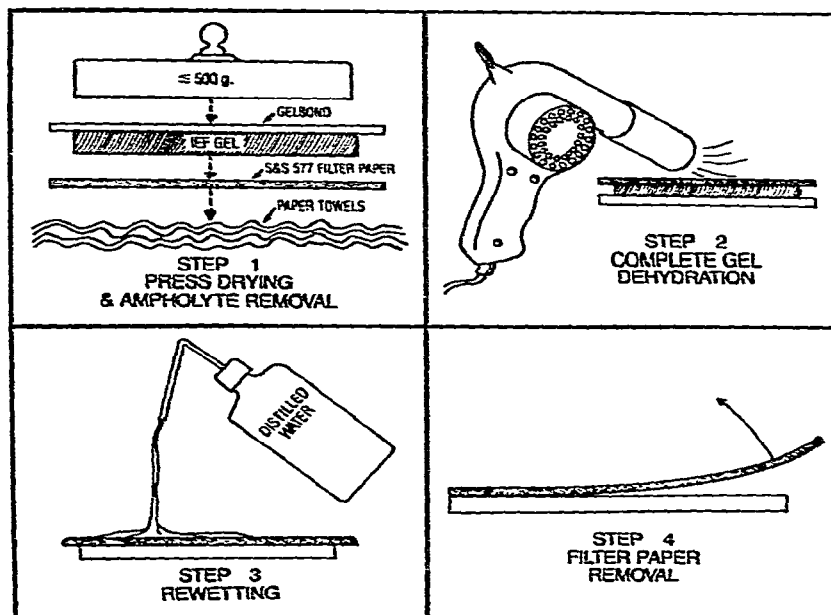
Fig. 9. Procedure for drying an agarose IEF gel prior to staining. After fixing in TCA, the gel is pressed for 30 min against filter paper and paper towels with a weight of 0.5 to 1 kg/dm². The dehydration is completed with a hair-dryer (step 2) and then the filter paper removed from the agarose film by a quick re-wetting step (3 and 4) (from Saravis and Cook, courtesy of Marine Colloids Division; instruction leaflet, 1979).

5.2. Cellulose acetate isoelectric focusing

Cellulose acetate, first introduced for zone electrophoresis by Kohn[83], would also be an ideal support for IEF, since it is practically a non-sieving matrix. However this membrane, as prepared by manufacturers, contains enough carboxyl groups to fully disrupt the IEF process. The strong electroendoosmotic flow is further enhanced by the extremely low ionic strength of focused carrier ampholytes. Harada[84,85] has been the first to describe the use of an acetate membrane, treated with a surface active agent (available from Fuji Photo, Tokyo, Japan, as Separax-EF) for use in IEF, but the method has found little application, possibly due to a still substantial electroosmosis. More recently, Ambler[86,87] has described an extensive methylation process of cellulose acetate strips leading to a support suitable for IEF. Here, again, since the electroosmotic flow is not completely abolished, the run has to be performed in 8% Ampholine (against the customary levels of 2% in polyacrylamide) and 5% glycerol. In narrow ranges, weaker catholytes and anolytes, such as 0.2 M lysine and 0.2 M acetic acid, respectively, should be used. The methylation process with boron trifluoride in methanol, thus far described[86,87], could be applied only to cellulose acetate gel membranes (e.g. Cellogel) but was unsuitable with dry, non-gel membranes, such as Sepraphore III (Gelman, Ann Arbor, MI, U.S.A.). Ambler and Walker[88] have now described a modified methylation process which can be applied also to this last group of membranes, by more gradually processing them through a series of aqueous solutions of increasing methanol concentrations. In our experience, however, the process of IEF in cellulose acetate matrices is still quite erratic and often difficult to control. More work needs to be done in this field both, to improve the quality of the support and to standardize the method. It is also highly desirable, for future use of this technique, that properly treated and carefully controlled cellulose acetate foils are made available to the scientific community by a commercial firm.

5.3. Ultrathin-layer isoelectric focusing

This technique represents one of the most interesting recent developments in polyacrylamide gel slab IEF, and will be described here in detail. The method has been developed by Görg et al.[89–93]. Since ultrathin polyacrylamide gels would be impossible to handle, and would break apart during the process of staining and destaining, they have to be supported by a suitable, tear-resistant backing. Both, polyester foils[91] and cellophane sheets[90] have been tested, the latter being preferred due to a much better adherence of the polyacrylamide matrix to it. Gel layers of 120, 240 or 360 μm thickness can be cast by using as gaskets in the gel cassette either one, two or three U-shaped frames cut out from Parafilm sheets. At the start, one of the two glass plates for gel casting is covered with a wet foil of cellophane, cut to a size extending approximately 2–3 cm over the edges of the glass plate (Fig. 10A). Good adhesion to the glass and removal of entrapped air bubbles is ensured with a rubber roller. Then the redundant portions of the sheet are folded against the bottom of the glass plate, the cellophane dried and the casting chamber assembled in the usual way (Fig. 10B). Since filling of the chamber is not an easy operation, especially with 120- and 240-μm thin gels, the two upper clamps are removed and two paper clips inserted between the glass plates, thus allowing the insertion of the needle of the syringe containing the polymerization mixture (Fig. 11). When the clips are removed and the
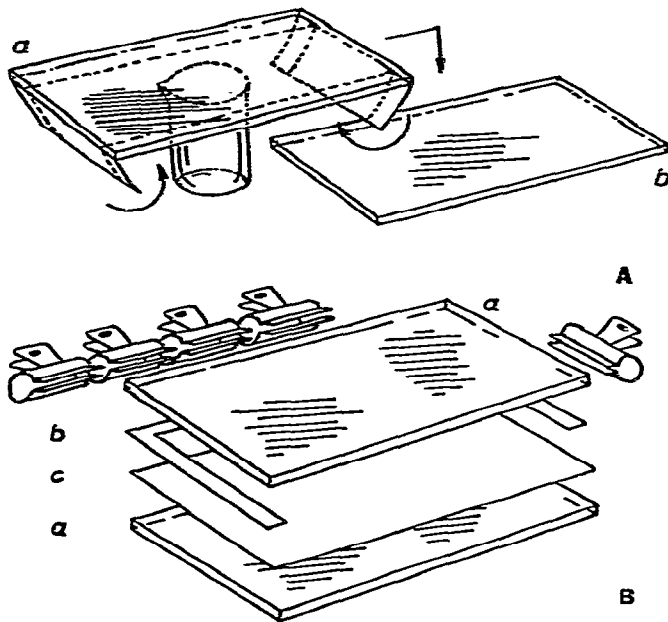
Fig. 10. (A) Procedure for casting ultrathin polyacrylamide gels onto cellophane foils. A wet cellophane sheet, 2-3 cm wider than the 1 mm thin glass used for the gel cassette, is stretched and rolled flat on this glass (a), the excess film folded beneath and then (b) transferred onto the 3 mm thick glass for drying. (B) assembly of the gel cassette; (a) 3 mm thick glass; (b) U-shaped Parafilm gasket (1 layer = 120 µm); (c) 1-mm thin glass coated with cellophane film (from Görg et al.[89-92]).
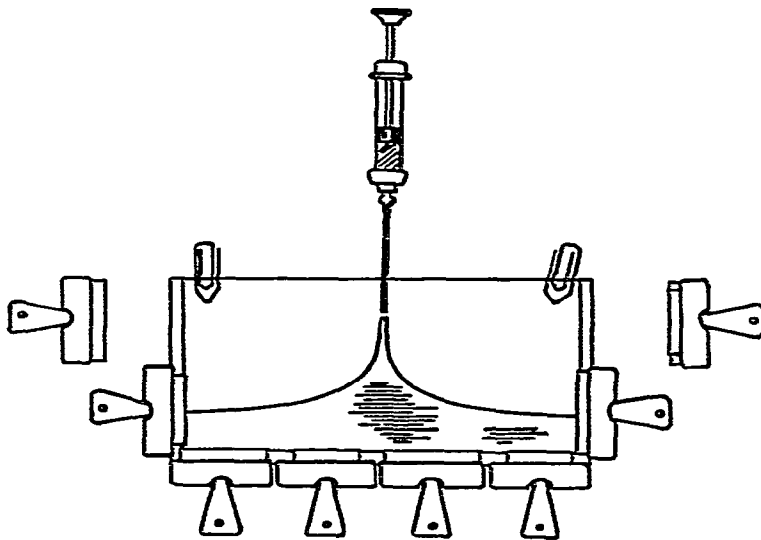

Fig. 11. Moulding chamber for ultrathin gels. The two upper clamps are removed and two paper clips inserted between the glass walls, for easier pouring of the polymerization solution. After the gel mixture has been completely poured in the cassette, the paper clips are removed and the two clamps placed back in the frame, so that the liquid rises to fill up the chamber (from Görg et al.[89-92]).

clamps fastened in place, the liquid level in the chamber rises to the desired height. After polymerization, the excess cellophane foil on the short gel sides is cut away (Fig. 12A) and the cellophane-supported polyacrylamide film transferred onto the cooling block of the IEF cell. At the end of the IEF run, after staining and destaining and a final equilibration step in 5% glycerol, the gel is transferred to a glass plate, the excess cellophane on the long side is folded under and taped completely around to prevent curling and allowed to dry in the air (Fig. 12B). Several advantages are inherent to this technique: (a) resolution is markedly improved in ultrathin gels as compared with conventional gels; (b) heat transfer is much more efficient, thus allowing higher field strengths and sharper zones than in 1–2 mm thick slabs; (c) by adhering to cellophane during all operation steps, the gels can be handled very conveniently and are protected from fracture; (d) staining, destaining and drying are completed in a fraction of the time needed for thicker gels; (e) the demand for carrier ampholytes and other reagents is drastically lowered; (f) zymograms can be developed within a few minutes, thus retaining the high band sharpness of the IEF dimension.
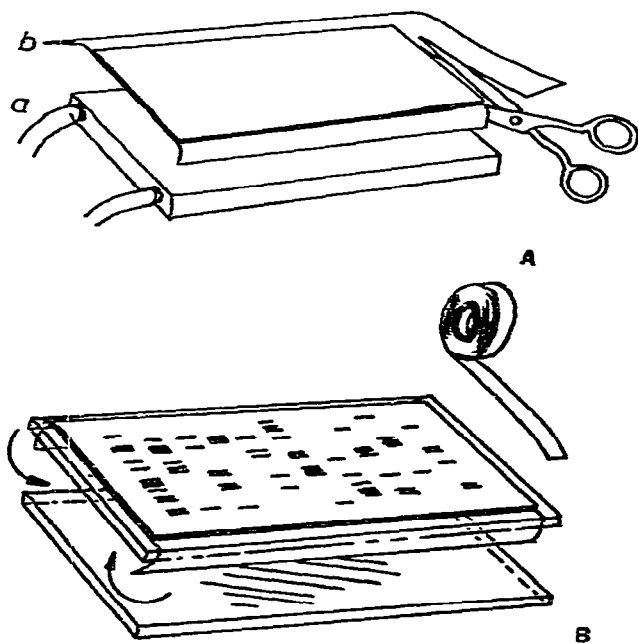
Fig. 12. (A) After the gel is polymerized, before being placed on the cooling block of the IEF chamber, the excess cellophane on the two short sides is cut away, while the one on the two long sides is tucked under the supporting glass surface. (B) After the gel has been stained and destained and equilibrated in 5% glycerol, it is placed on a glass support, with the flaps of the cellophane foil folded under it, transferred onto a second glass slab, taped all around with Scotch tape and left to dry in the air (from Görg et al.[89–92]).

For even thinner gels, in the 50–100-μm range, a modification of the above method, called the "flap technique", has been described by Radola[94,95]. Since it would be impossible, by the conventional cassette technique, to cast such ultrathin gels, the problem has been solved by using a horizontal glass plate with appropriate spacer strips, onto which the total volume of polymerization mixture is poured. The

chamber is sealed by slowly lowering on it the cover plate, so that the gel mixture is spread evenly between the two plates (Fig. 13). The thin gel veil can be made to adhere to polyester films, or can be covalently bound to the glass surface by using a silane coupling agent (methacryloxypropyltrimethoxy silane). Covalent bonding of poly-acrylamide to glass, by the use of Silane A-174, has also been independently described by Bianchi Bosisio et al.[96], when working with highly brittle, highly Bis-cross-linked polyacrylamide gels. Ultrathin gels are particularly attractive when incorporating detergents into gels, since the process of leaching out the detergent micelles prior to staining is terribly lengthy in conventionally thick gels. Valkonen et al.[97] have applied the ultrathin technique to the resolution of β- and γ-globin chains for thalassemia screening in 300-μm thin gels containing 8 M urea and 0.5% Nonidet P-40.
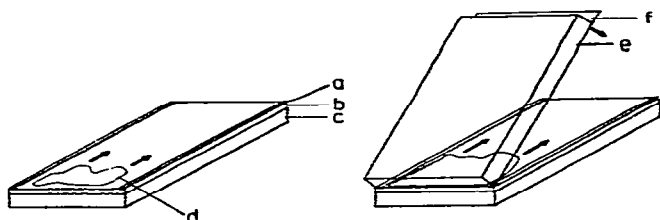
Fig. 13. Gel preparation with the flap technique. (a) 50- or 100-μm spacer strips; (b) silanized glass plate or polyester film; (c) glass base plate; (d) polymerization mixture; (e) glass cover plate; (f) cover film, 100-μm hydrophylic polyester film protruding at the upper end for easier removal of the polymerized gel (from Radola[56]).

5.4. Isoelectric focusing at sub-zero temperatures

This is a useful extension to electrophoretic techniques of the cryobiochemistry methods introduced by Douzou[98]. Early attempts at studying mixed hemoglobin tetramers by IEF below zero were reported by Park[99], who only succeeded in lowering the temperature at about −5 to 10°C. Considerable progress has been recently described by Perrella et al.[100–102]. The major problems to be solved were a suitable thermostatting method, a proper polyacrylamide gel which would not exhibit glass transition below −15°C and an appropriate aqueous–organic, anti-freezing solvent. These problems were solved by using a modified Righetti and Drysdale[1,25] tube apparatus, fitted with a cooling serpentine also for anolyte and catholyte compartments (Fig. 14) and utilizing narrow-bore glass tubes (2 mm I.D.) for rapid Joule heating dissipation. The gel was a copolymer of acrylamide–methacrylate or acryl-amide–ethylacrylate which are able to stand temperatures as low as −30 to −40°C without matrix modifications. As for the aqueous–organic solvents, best results were obtained by dimethylsulphoxide (DMSO)–water mixtures, as previously reported by Righetti et al.[103]. In theory, IEF experiments could be carried out down to −30 or −40°C; in practice, however, the high viscosity of the aqueous–organic medium and the much lower mobility of proteins at subzero temperature, set a limit to a more comfortable −20°C. An additional problem results from precipitation of acidic carrier ampholytes (below pH 5), probably due to aggregation, with severe disturbances of the pH gradient in the zone of the precipitate. Best results are thus obtained with pH ranges above pH 5, setting the temperature limit to −20°C and choosing a DMSO concentration in water of 37%, which ensures reduced viscosity

and is compatible with temperatures down to $-30°C$. By this technique, hybrid tetramers HbA–HbS, starting from the parent molecules HbA and HbS, could be obtained[101], as well as a whole series of intermediates of oxidation of HbCO. Recently, the same authors[103] have also been able to map the course of the pH gradient at $-20°C$ by using the dye-indicator method of Douzou[98]. Generally speaking, in the nominal pH ranges 6–8 or 7–9, the combined effect of 37% DMSO and $-20°C$, as compared with focusing in water at $+4°C$, is to increase all pH values of focused Ampholine by about 1 pH unit. In the case of carbonmonoxy hemoglobin A, its pI value shifts toward the alkaline to the same extent. Cryo-isoelectric focusing appears to be a very promising technique for studying subunit exchange in solution, as well as ligand binding and enzyme substrate complexes.
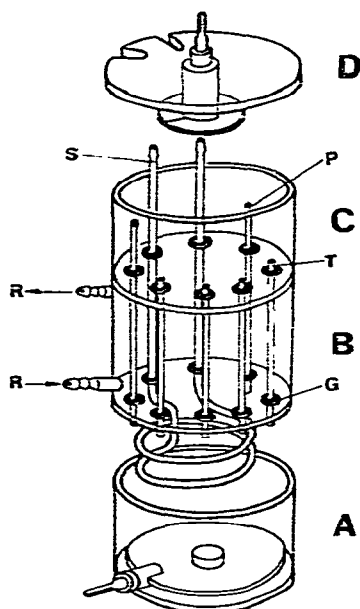
Fig. 14. Modified Righetti and Drysdale apparatus for IEF and electrophoresis at subzero temperatures. A = Anodic compartment; B = refrigerated tube holder; C = cathodic compartment; D = cathode carrying cover; R = coolant inlet and outlet; S = glass coil for refrigeration of liquid in A; P = glass tubes for filling A with a precooled liquid; T = gel containing glass tubes; G = rubber gaskets (from Perrella et al.[100]).

5.5. Flattening of pH gradients —separators and strips

It is well known, from Rilbe's[36] equation on the resolving power in IEF, that good resolution is obtained with compounds having a low diffusion coefficient and a high pH-mobility slope at their pI, as well as by increasing the field strength (V/cm) and by decreasing the slope of the pH gradient. This last proposition is, in practice, achieved by utilizing the so-called "narrow pH ranges", which create pH gradients of 1.5 to 2 pH units over the separation distance. When higher resolution is needed, even shallower pH ranges are available, such as pre-cast gel slabs in the pH 4–5 range for the phenotyping of α_1-AT. Available pH ranges, or home-made batches, can be further

subfractionated by the IEF process[104,105], down to narrow cuts encompassing about 0.4 to 0.5 pH units. As demonstrated by Charlionet et al.[34], this can result in the resolution of protein species differing in pI by as little as one thousandth of a pH unit.

Another way of modifying the pH gradient slope in IEF is the "separator" technique, described by Caspers et al.[106,107]. By adding amphoteric substances (separators or "pH-gradient modifiers"[108]) to an Ampholine pH gradient, its slope is altered and usually flattened in a pH region corresponding to, or in proximity of the pI of the added separator. The following chemicals have been used as separators: tetraglycine (pI 5.2), proline (pI 6.3), threonine (pI 6.5), β-alanine (pI 6.9), 5-amino-valeric acid (pI 7.5), 4-aminobutyric acid, 7-aminocaprylic acid, histidine (pI 7.6) and 6-aminocaproic acid (pI 8.0). All of them, except His, are poor carrier ampholytes (pI–pK values of 3 or 4) and thus focus as broad plateaus in the Ampholine gradient. Therefore, in order to be able to modify the pH gradient, they have to be used at rather high concentrations, usually about 0.3 to 0.6 M. On the other hand, good ampholytes, such as His (or the dipeptide His–Gly, successfully used for the separation of HbA from HbA$_{1c}$, a minor glycosylated Hb[108]) are already effective at concentrations of 10–50 mM and probably act by interposing themselves between the two species to be separated. pH-gradient modifier IEF adds another dimension to IEF by further improving separations which are at the resolution limit of the technique. The successful separation of HbA from the glycosylated component Hb A$_{1c}$, utilized in diabetes screening, by the use of β-alanine as separator, has also been reported by Jepsson et al.[109].

Yet another variant for pH gradient flattening is the "strip" technique recently reported by Altland and Kaempfer[110]. It is based on the assumption that modifying the profile of the gel along the pH axis should be accompanied by changes of pI distances along that axis. Such a modification of the gel thickness along the pH axis can be achieved simply by overlaying a gel strip of a defined thickness and width on the gel at any desired location. In the electric field the overlaid gel strip delivers ampholytes of a distinct pI interval into the strip-free part of the base gel and removes from the latter some ampholytes present in the system; this process results in a flattening of the pH gradient in the IEF gel slab. An example of this technique is given in Fig. 15, which indicates focusing in a pH range of 6–8 with strips added at different locations. If the strip is placed at the anode, this results in a flat pH 7.2–8 gradient; if overlaid at the cathode, conversely, a flat pH 6.0–7.0 gradient will be generated while, if placed symmetrically at both anode and cathode, the central portion of the gradient (pH 6.5–7.5) will be expanded. That this is also the case in daily practice is illustrated in Fig. 16, which shows progressive increments of separation among HbA$_2$, HbA$_1$ and HbF in a gel slab overlaid with two wedge strips placed at both electrodes. For best results, the volume of the overlay gel strip should be at least equivalent to the total volume of the base gel slab, while the area occupied on its surface should not exceed 20–25% of the electrode distance. For these reasons, ultrathin base gel slabs are preferred (200–300 μm) so that the thickness of the overlay gel can be kept in the range 1.5–2 mm. It would be of interest to see if this technique, in conjunction with "separator" IEF, can further flatten pH gradients thus incrementing the resolution obtainable.
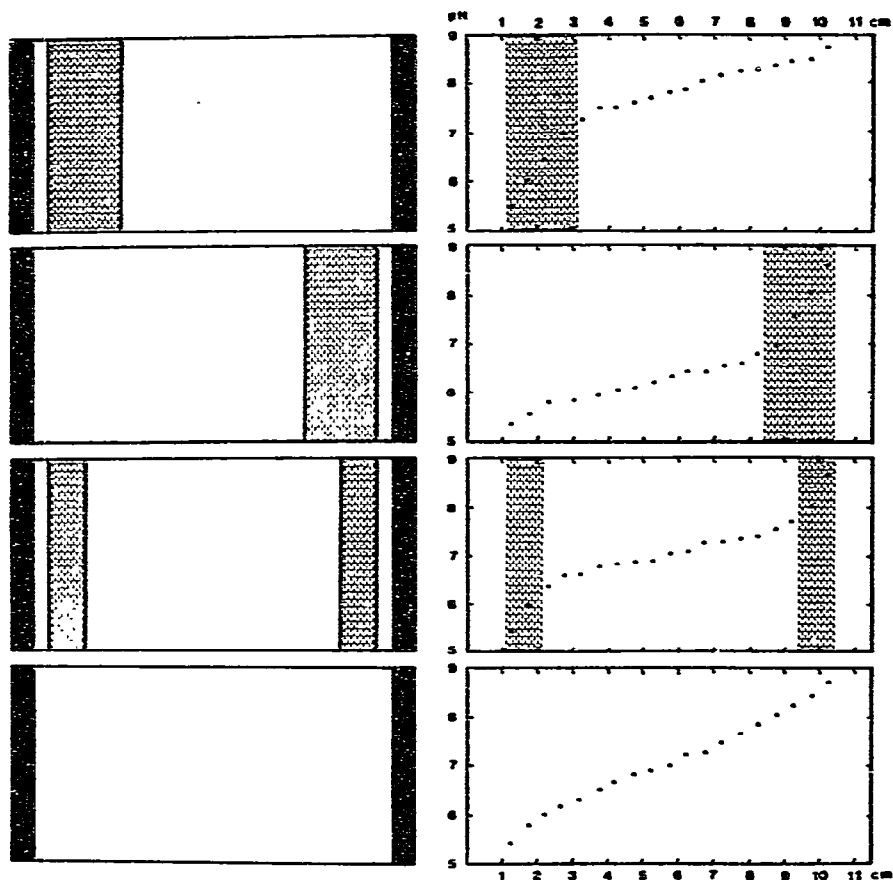
Fig. 15. pH gradients produced by different locations of 2-mm thick gel strips on a 350-μm thin base gel containing 2% (w/v) Servalyte carrier ampholytes in the pH range of 6–8. The locations of the gel strips are indicated by the shadowed areas. Running conditions: 16°C, 4 h at 7.5 mA (constant current), 1000 V (limit) (from Altland and Kaempfer[110]).

6. ANALYTICAL TECHNIQUES: APPLICATIONS

6.1. Detection of neutral mutations

The vast majority of spot mutations so far detected are point variants affecting a charged amino acid. For instance, in the case of human hemoglobin (in Oct. 1973), 174 point mutations had been described[111], distributed as follows: 61 in α chains, 99 in β chains, 8 in γ chains and 6 in δ chains. Of those, only 35 (i.e. ca. 20%) are neutral to neutral mutants (especially Pro $\rightleftarrows$ Leu, which account for about 30% of all neutral mutations) which probably have not been detected by electrophoretic techniques but through altered functional properties and chemical analysis (tryptic digestion, fingerprinting and amino acid analysis of anomalous peptides). If we generalize these findings, given the total frequency of charged amino acids in proteins, this means that only about 40% of all possible mutations will result in charged mutants detectable by electrophoretic techniques. A good 60% will be "silent" mutants, involving neutral for neutral amino acid substitutions, undetectable by any electrophoretic technique. There are hints that careful use of IEF can indeed resolve even
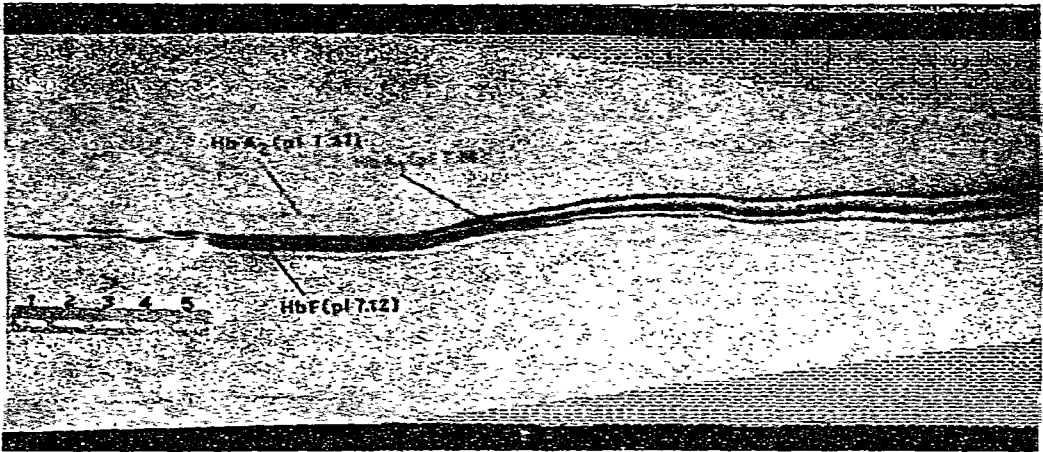
Fig. 16. Continuous flattening of the pH gradient to increase the separation of hemoglobins. The 350-μm thin base gel contained 2% Servalyte pH 6–8. The wedge-like 2-mm thick strips had a width of 2 cm each at their base (right edge of the figure) and covered the shaded areas throughout the total run. Samples (from left to right): 1, 2 and 4 = 15 μl RBC lysate from an adult human individual; 5 = same, but from a human newborn; 3 = 1:1 mixture of 1 and 5; 6 (long band) = 3:1 mixture of samples 1 and 5. Running conditions: 16°C; prerun, 30 min, 7.5 mA (constant current), 1000 V (limit); run, 5 h, 1000 V (constant) (from Altland and Kaempfer[110]).

these silent mutants. A very interesting example comes from a recent article by Whitney III et al.[112]. When working with mouse hemoglobins (Hb), they were able to detect by IEF several neutral substitutions in the α chain. The resolved haplotypes, carrying mutations in three positions (α^{25}, α^{62} and α^{68}), were: Hb_1: Gly^{25} $Val^{62}Asn^{68}$; Hb_2: $Gly^{25}Val^{62}Ser^{68}$; Hb_3: $Gly^{25}Val^{62}Thr^{68}$ and Hb_4: $Val^{25}Ile^{62}Ser^{68}$. In going from Hb_1 to Hb_4 the isoelectric points of the focused, native tetramers, would progressively decrease, while the overall hydrophobicity would concomitantly increase (see Fig. 17). In fact, from the hydrophobicity scale of Nozaki and Tanford[113], we have calculated a $\Delta f_t = -500$ in going from Hb_1 to Hb_2 and a $\Delta f_t = -800$ for the transition Hb_2 to Hb_3 (the overall hydrophobicity of Hb_4 is more difficult to calculate as not enough data are available). We hypothesize that hydrophobicity increments in the α chain bring about pI decrements in the tetramer via loss of positive charges. This is probably not an isolated case, but could be a more general phenomenon. Thus, in horse radish peroxidase, when the prosthetic group protoheme IX binds to the
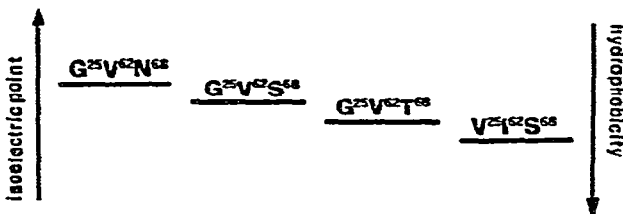

Fig. 17. Separation of rat hemoglobins carrying neutral to neutral spot mutations. The mutants are (from left to right): $Gly^{25}Val^{62}Asn^{68}$; $Gly^{25}Val^{62}Ser^{68}$; $Gly^{25}Val^{62}Thr^{68}$; $Val^{25}Ile^{62}Ser^{68}$. Notice how the isoelectric points decrease as the hydrophobicity of the mutations increase. Drawn from IEF data of Whitney et al.[112].

imidazole of a His residue in the protein chain[114], the pK of this group is lowered from its usual value of six to about zero[115]. When protoporphyrin IX (which contains four imidazoles, of which two are protonatable with a pK of *ca*. 6.5) is dissolved in neutral micelles of Triton X-100, a pK_{app} of *ca*. 0.9 is found[116]. We suggest that, in the native mouse hemoglobin, there could be a positively charged group (a most likely candidate would be Lys) lying within a few angstrom distance from the amino acids in positions 25, 62 and 68 of the α chain. As the hydrophobicity of these amino acids is increased, and the dielectric constant of the environment progressively decreased, concomitantly the pK of the basic group would be lowered, down to a total loss of 1 proton at pH $=$ pI in going from Hb$_1$ to Hb$_4$[117].

That in the native tetramer neutral mutations can produce subtle, but detectable, pI changes, is not surprising. However, we have been able to separate neutral mutants even when working with fully denatured globin chains, where conformational transitions cannot account for pK shifts. We found that out when trying to separate by IEF β- from γ-globin chains, for thalassemia screening[118-121]. When the IEF gels were run in 8 M urea and 3% NP-40, the γ zone was split into two bands, a pI 6.95 chain, corresponding to $^G\gamma$ (glycine) and a lower pI band (pI 6.85) corresponding to $^A\gamma$ (alanine) globin (Fig. 18). These chains differ by having either Gly or Ala at position 136 and are the products of two non-allelic loci, closely linked to α- and β-globin genes. To account for the fact that two neutral mutants, in fully random configuration, do exhibit different pI values in the presence of a neutral detergent, we had suggested[24] that the NP-40 micelle would bind preferentially to the $^A\gamma$ chain, in the hydrophobic stretch of the mutation, going from Met[133] to Leu[141], masking in this process Lys[132], thus producing a charge-shift (loss of one proton unit) in this phenotype. In the model we had proposed[24], we had hypothesized that this peptide segment in the $^A\gamma$ chain had been sorbed in the Stern layer of the micelle,
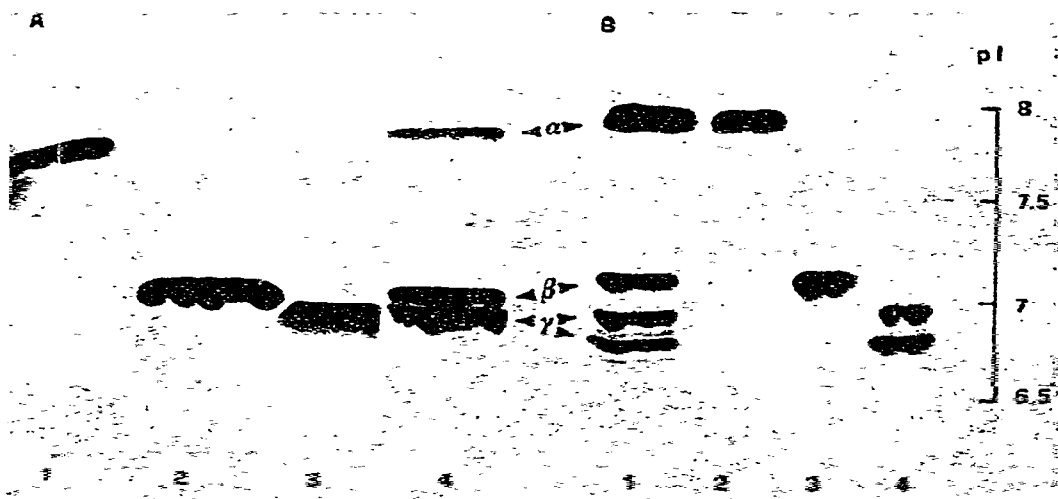

Fig. 18. Separation of human globin chains by IEF in the absence (A) and in the presence (B) of NP-40. Lanes 1, 2 and 3 of A and lanes 2, 3 and 4 of B show chromatographically pure α-, β-, and γ-globin chains, respectively. Lanes 4 of A and 1 of B show unfractionated globins (from Saglio *et al.*[120]).

however an alternative model could depict it embedded in the hydrophobic nucleus of the micelle, as demonstrated in the case of hemin[122] or hydrophobic membrane proteins[123]. The case of $^A\gamma-^G\gamma$ chains does not seem to be a unique phenomenon, as a similar type of charge-shift has been demonstrated even in the case of histones[124]. If similar neutral substitutions prove to be equally detectable in other proteins, then it appears that the use of IEF will enable the detection of twice as many genetic variants as have been found through the use of standard electrophoretic techniques.

6.2. Isoelectric focusing of peptides

Due to its very high resolving power and to the sharpening effect of sample zones in their pI position, IEF appears to be particularly useful for the analysis of peptides, either natural or obtained by protein fragmentation or by synthetic processes, since these substances have a rather high diffusion coefficient as compared with proteins, and usually produce diffused zones in conventional separation techniques. Analytical IEF of peptides was not feasible, however, due to their very close similarity and common reactivity with carrier ampholytes. The classical techniques used for peptide detection (ninhydrin stain, Folin reagent, microbiuret, permanganate oxidation, starch-iodine reaction), in fact work wonders for the detection of focused Ampholine in the gel matrix. Recently, however, successful IEF fractionation of peptides has been achieved through the development of new staining techniques, by which the dye (usually Coomassie Blue G-250) is dispersed, in a leuco form, as colloidal micelles in 12% TCA and 1 N H_2SO_4[61,125]. The focused gel is bathed directly in this solution, whereby the carrier ampholytes are leached out from the gel matrix while the peptides, which are only partly soluble in plain TCA, are further fixed by the dye molecules, which probably act as cross-links over different chains, thus forming a macromolecular aggregate which can be trapped in the random meshwork of gel fibers. By this method, the minimum critical peptide length for fixation and staining appears to be around 15 amino acids (Fig. 19). Dye adsorption onto the peptide is favored by the presence of basic amino acids and by a relatively high content of hydrophobic amino acids. Besides binding to basic groups the dye can interact with hydrophobic residues, particularly if they are in a close sequence[61].

Even though the present method represents an important step forward in peptide analysis, it unfortunately leaves out the class of oligopeptides (from di- to tetradeca- residues) where many biologically active substances are located. By exploiting the ultrathin gels described by Görg et al.[89-93] we have been able to close this gap[62,63] partially. 300-μm thin acrylamide gels are cast, and used as a medium for oligopeptide focusing. Rather high voltages can thus be applied and thin bands obtained. After IEF, the gel is pasted to a sheet of filter paper and immediately dried in an oven at 110°C, whereby the gel layer is reduced to a film of vanishing thickness. The gel-paper sheet can now be sprayed, like a typical paper chromatogram, with any of the specific stain solutions for Arg, Met, Cys, Tyr, Trp and His. In this way, 30% of all the possible amino acids in oligopeptides can be detected, with a sensitivity level of only a few μg of peptides per IEF zone. A method for direct His staining of focused peptides, in a wet, instead of dried gel, has also been independently described[126]. We now have evidence that these developments allow the display of a peptide map, in the single IEF dimension, with great saving of time and materials when compared with the classical, two-dimensional fingerprints[127].
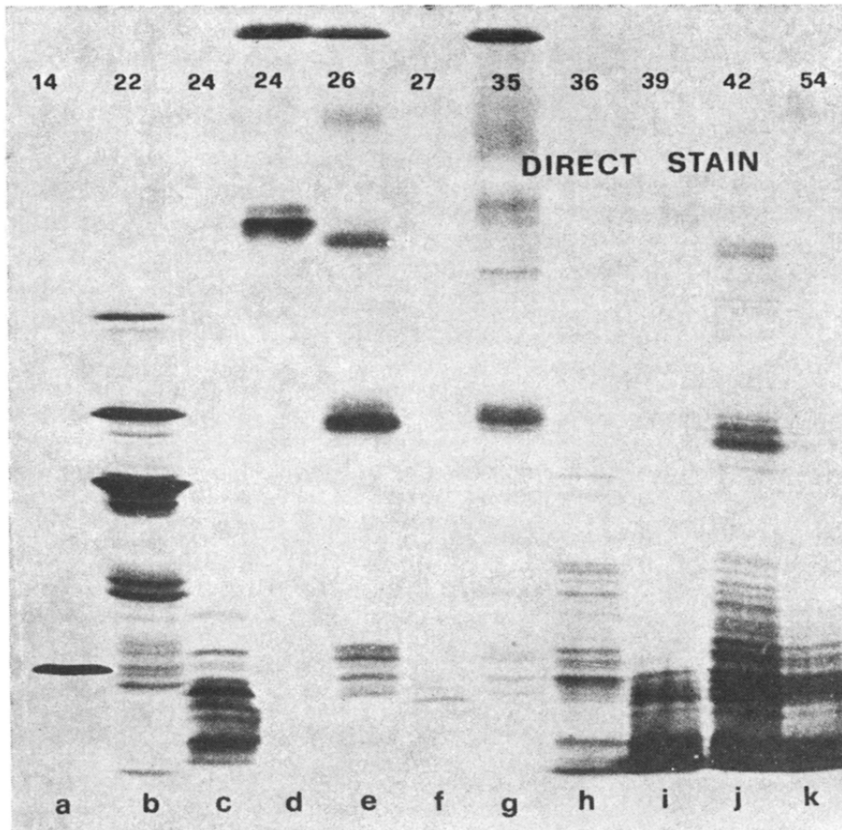
Fig. 19. IEF of peptides in the length range 8–54 amino acids. The gel slab was 0.7 mm thick and contained 7% acrylamide, 2% Ampholine pH 3.5–10 and 8 M urea. 10–15 μl of sample (10 mg/ml) were applied in filter paper strips at the anode after 1 h prefocusing. Total running time, 4 h at 10 W (1000 V at equilibrium). The gel was then dipped in a colloidal dispersion of Coomassie G-250 in 12% TCA. The samples are the following synthetic fragments of the human growth hormone (hGH): a = hGH 31–44; b = hGH 15–36; c = hGH 111–134; d = hGH 1–24; e = hGH 166–191; f = hGH 25–51; g = hGH 157–191; h = hGH 1–36; i = hGH 96–134; j = hGH 115–156 and k = hGH 103–156. Shorter fragments (octa-, nona-, deca- and dodecapeptides) were neither fixed in the gel nor stained (from Righetti and Chillemi[61]).

As a recent development, we have been able to find a general stain[128], independent from peptide length. When a focused and dried ultrathin- layer gel is exposed to iodine vapors in a jar, the carrier ampholytes adsorb the iodine molecules quickly, giving a uniformly brown stain in the gel-paper sheet. By a mechanism still not completely understood, the focused peptides inhibit this adsorption, thus being detected by negative staining, as white zones on a brown background. The staining is fully reversible, since no chemical reaction occurs, and is developed within a few minutes of exposure to iodine. In order to obtain a uniform brown color, rather than a striped background, 4% carrier ampholytes are used in the focusing gel, instead of the usual 2% levels, so that the gaps in between the "stripes" of focused Ampholine are smoothed. A drawback of the present method is its low sensitivity, a minimum of 30 μg peptide/zone being required for detection. However this technique can be

exploited for small scale preparative applications: the peptide can be eluted from the gel in 80% acetic acid with recoveries up to 85%.

6.3. Isoelectric focusing of cells and organelles

IEF of cells was first introduced by Sherbet and co-workers[129-131] in 1972, by using stationary, linear gradients of sucrose (10–55%), glycerol (10–70%) or Ficoll (5–30%) containing 1% Ampholine in a standard LKB 110-ml column. In these early systems, the cells were subjected to the IEF process for as long as 30 h. Subsequently, Just and co-workers[13,132-135] introduced the method of continuous-flow IEF separation of cells, in a modified Hanning apparatus. In this system, the residence time of the cells in the flow-chamber was only between 7–10 min. Cell focusing has also been described in several other reports. Leise and LeSane[136] reported the IEF separation of peripheral lymphocytes of human and rabbit origin in a gradient of dextran-40, while Hirsch and Gray[137] analyzed rat peripheral lymphocytes in dextran gradients in isotonic sucrose. Boltz et al.[138] reported the IEF fractionation of Chinese hamster fibroblasts in a linear Ficoll density gradient made isotonic throughout by sucrose and glucose; Manske et al.[139] have described the IEF analysis of Ehrlich–Lettré mouse ascites tumor cells, as well as rat hepatocytes, in Ficoll-sucrose gradients; even boar[140] as well as bull and rabbit[141] spermatozoa have been isolated by IEF in Ficoll gradients. In the field of procariotic cells, the IEF of bacterial cells has been reported by the Sherbet group[11], and by Langton et al.[142]. According to Talbot[143] and others[144-146], viruses also seem to be amenable to isoelectric fractionation.
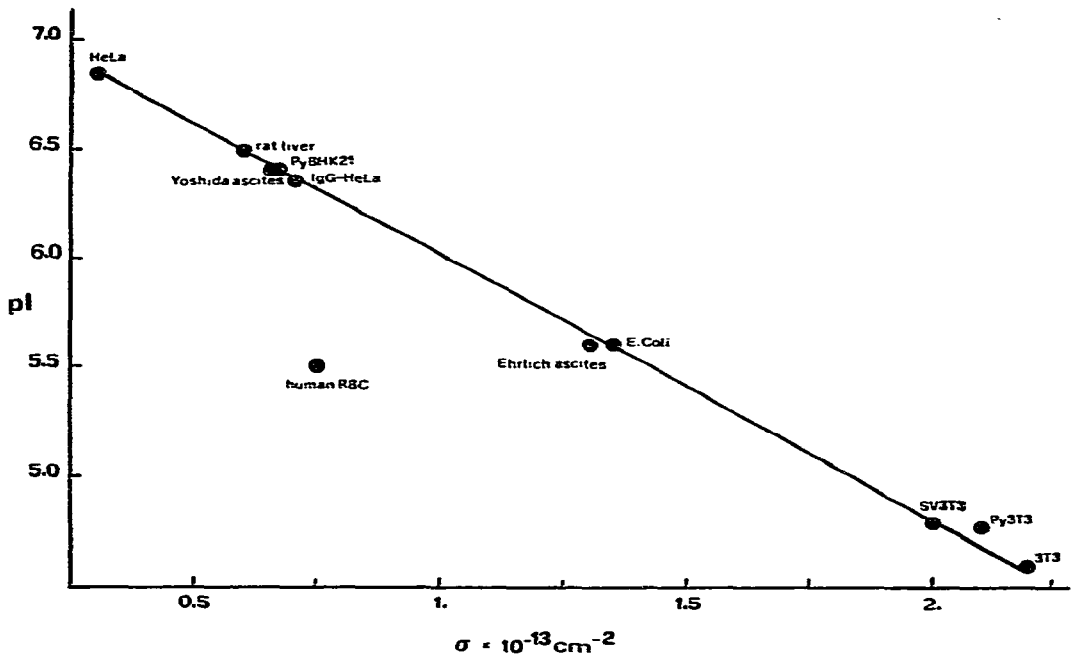

Fig. 20. Plot of isoelectric points (pI) of cells, as determined by IEF, vs. their respective charge densities (σ). Notice the extremely good fit for all cell lines and the huge deviation of red blood cells. Drawn from data tabulated by Sherbet[11].

In an extensive treatise on electrophoresis and isoelectric focusing of cells[11], Sherbet has given evidence that IEF can be used as a probe for characterization of ionizable groups on the cell surface, for measuring cell surface charge densities, pK values of ionizable groups on the cell membrane and for following chemical modifications of charged groups on the cell envelope. According to Sherbet[11], IEF can probe the electrokinetic zone of shear of cells down to a much greater depth than zone electrophoresis. Conventional electrophoresis can probe the surface to a depth of about 1.4 nm, while it appears that the isoelectric zone extends at least five times as much, to a depth of about 6–7 nm below the surface[11]. Moreover, it appears that the isoelectric point (pI) values of cells, as determined by IEF, can be used to calculate their surface charge density (σ), as well as their electrophoretic mobility. In turns, charge densities (expressed as number of charges/cm^2 of cell surface) can be linearly correlated to the electrokinetic potential (ζ potential), provided the latter does not exceed a value of 27 mV. We have tried to plot the values given by Sherbet[11], in order to see how accurate the data are. Fig. 20 shows the correlation between pI and surface charge densities for eleven mammalian cells. The fit is remarkably good, as practically 10 cell lines, ranging in pI values from *ca.* 4.5 to *ca.* 7.0, fall on the linear plot (which gives decrements of σ of $0.85 \cdot 10^{13}$/cm^2 for increments of 1 pI unit). However, there is one cell type, human RBCs, which falls completely out of the linear relationship. Given a pI of 5.5, as reported by Just *et al.*[133], the charge density should be about twice as much. If the σ value is correct, then the pI should be about 6.3. Given the fact that RBCs are perhaps the most extensively characterized mammalian cells, this very poor fit is quite suspicious. If we now plot the ζ potential *vs.* charge density, a linear relationship is again obtained (Fig. 21), however with a considerable scatter of points (50% of the points deviate considerably from linearity).

Notwithstanding this large body of experimental data, IEF of cells still has not been quite established. For instance, Catsimpoolas and Griffith[147], upon focusing mouse spleen lymphocytes, have reported that as soon as the cells are focused they
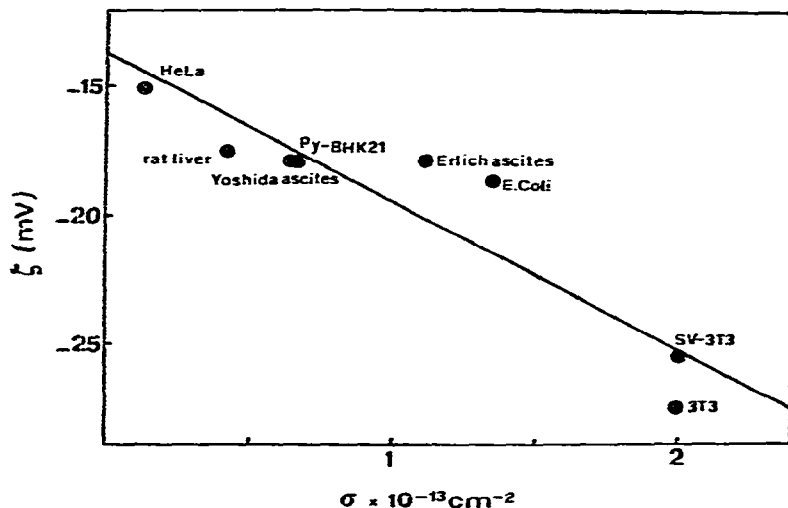
Fig 21. Plot of electrokinetic potential (ζ potential) of different cell lines *vs.* their respective charge densities (σ). Drawn from data tabulated by Sherbet[11].

start to defocus and are finally lysed. They hypothesize that, in the pI region, changes in the membrane occur that could alter the pI towards a more acidic value so that the cells have to seek a new pI position. This may occur repetitively until extensive damage to the membrane causes lysis of the cells. It is a fact that, even though many workers[137,139] have claimed high cell viabilities (up to 90%) after IEF, as measured by dye-exclusion tests, in reality viabilities in terms of plating efficiencies are usually low[138], or recultured cells after IEF remain dormant for very long periods[147]. This has led many workers to suspect that carrier ampholytes might have some degree of cyto-toxicity. Therefore, the question of whether membrane stability and hence cell viability can be maintained under electrofocusing conditions should be critically analyzed. There also remains the question of whether the measured cell pI values are "true" isoelectric points, representing a balance between positive and negative groups in the cell surface. For instance, the pI of *E. coli* is given as 5.6 (ref. 11), practically the same as the pI of RBCs[133] (see Fig. 20). However, in *E. coli*, the ratio of negative to positive charges is 2:1 ($2.899 \cdot 10^{13}$/cm^2 carboxyl groups *versus* $1.4334 \cdot 10^{13}$/cm^2)[11], while in RBCs the ratio of negative to positive groups is 25:1 (ref. 148), *i.e.* enormously higher. How these two cell types can exhibit the same pI remains a mystery.

Additionally, three other parameters that further increase the uncertainty of the data should be considered: (a) at the steady-state, focused carrier ampholytes represent a medium of very low "ionic strength" (now known to be in the range 0.5 to 1 mg ion/l for 1% Ampholine[52]); (b) ampholytes for IEF are capable of chelating doubly positively charged metal ions[45]; (c) they have been demonstrated to form complexes with polyanions, such as nucleic acids[148] and sulphated and carboxylated polysaccharides[149–151], including polyglutamate and polyaspartate[150]. At the light of these data, in fact, McGuire *et al.*[49] have recently demonstrated a pH-dependent binding of carrier ampholytes to the surface of RBCs: the binding is very strong at pH 4, weak but still appreciable at pH 5 and abolished at pH 6 and above. The same phenomena could be reproduced with PEHA, a polyamine which could typically represent the backbone of carrier ampholytes. On the basis of these observations, McGuire *et al.*[49] have drawn a model depicting a segment of an Ampholine, molecule bound, via a stretch of four protonated nitrogens (each two methylene groups apart), to four negative charges (possibly sialic acids or sulphate residues attached to the carbohydrate side chains) on the cell surface (see Fig. 22). The bound Ampholine might change the surface charge characteristics, thus altering the cell stability and contributing to the decrease in cell viability. It is a fact that, even in early studies by the Sherbet group[11], all cells focusing around pH 5 (4.70–5.30) (Yoshida ascites, Ehrlich ascites, Py cells, HeLa cells, normal liver cells), where binding of Ampholine is still appreciable, were in fact all non-viable cells. These data[49] are further corroborated by Hammerstedt *et al.*[141], who found that the apparent isoelectric point (pI_{app}) of sperm cells is not altered when the cells are inserted in a pre-focused column at either pH 7 or pH 8. However, if cells were added to the prefocused gradient at a low pH, the pI_{app} was 2 pH units lower, suggesting either extensive binding of acidic carrier ampholytes, or cell surface damage due to low pH, or peeling-off of basic proteins adsorbed onto the cell glycocalyx region. Actually, binding of Ampholine[49] is more consistent with an increase of pI_{app}, rather than with its decrease, and could explain the rather high pI_{app} of most cells, reported to be in the range 6.0–6.8. On the

other hand, when focusing is performed in citrate buffer[152], most of the cells investigated appeared to have p*I* values in the pH range 3.5–4.7. This type of focusing should be further explored, as citrate allows higher medium ionic strengths, prevents cell aggregation and, being oligoanionic, should not bind to the cell surface[153]. IEF of cells in the presence of "poor" zwitterions, such as glycine, taurine or trimethylaminopropane sulphonate, able to maintain a constant osmolarity in a given pH interval, has also been suggested[49]. Notwithstanding these highly controversial aspects, we feel that IEF, as a method of cell separation and characterization, although still neither thoroughly explored nor well understood, should be further investigated and exploited.
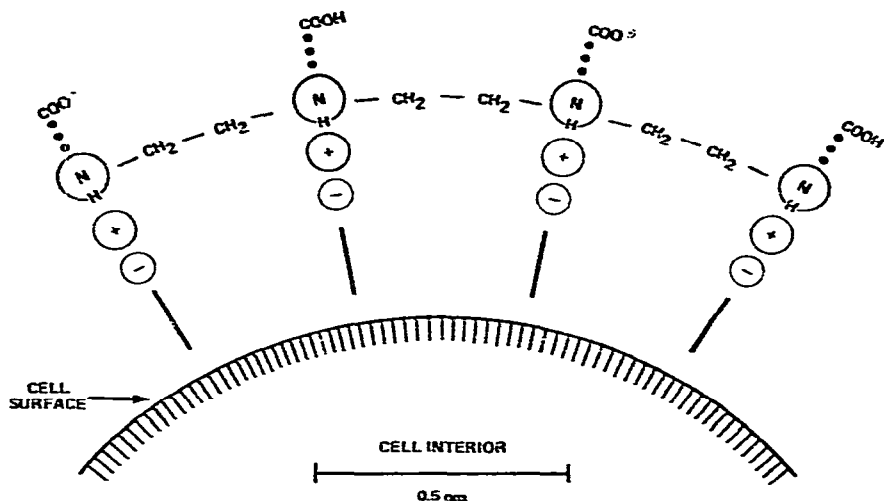

Fig. 22. Hypothetical model of the interaction of one Ampholine species with the cell surface. A segment of an Ampholine molecule, represented by a stretch of four protonated nitrogens, is depicted bound to four negative charges (possibly sialic acid residues) on the cell surface. The carboxyls in the carrier ampholyte are drawn facing away from the plasma membrane. At pH 4, these weak carboxyls (average pK = 4) would be 50% protonated. The curvature of the cell surface, over a distance of 1 nm, has been grossly exaggerated for easy visualization. In reality, over such a short distance, it should be almost planar (from McGuire et al.[49]).

6.4. Titration curves

This is a two-dimensional (2-D) technique, and as such should be treated in the next chapter. However, since it is a rather unique approach to 2-D separations, we feel it deserves a separate description. The first report was presented in 1976, at a meeting in Hamburg, when Rosengren et al.[154] described "a simple method for choosing optimum pH conditions for electrophoresis", which was in fact a direct display in a polyacrylamide gel slab of the titration curves of all the proteins present in a mixture. Fig. 23 shows how these pH–mobility curves are generated. A 2-mm thick polyacrylamide gel slab is cast with a trench in the middle, 10 cm × 1.5 mm × 1 mm, which can be loaded with up to 150 μl of sample. The first dimension consists of sorting electrophoretically the carrier ampholytes contained in the gel, thus generating a stationary pH gradient. No sample is applied at this stage. At this point, the electrode strips, with the respective gel layers underneath are chopped away with a long

knife (Fig. 23B). This step is essential, otherwise much heat will be generated in these regions in the second dimension, due to the presence of 1 M acid and base. New electrodes are then applied perpendicular to the first run and the trench filled with the sample to be analyzed (Fig. 23C). Now electrophoresis perpendicular to the stationary pH gradient is run, usually at 600 V/12.5 cm and for periods of 10 up to 45 min (Fig. 23D). Here are examples of fields of applications of this technique:
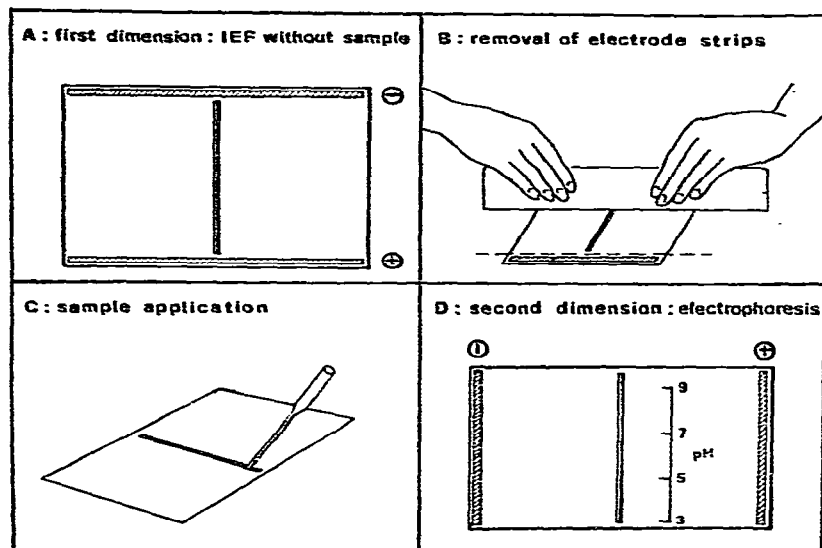

Fig. 23. Experimental procedure for generating titration curves by IEF–electrophoresis. The pH gradient is first formed by focusing the carrier ampholyte mixture (A); the electrode strips are then removed (B); the sample is applied in a trench cut perpendicularly to the pH gradient (C); the second dimension run is started perpendicular to the first dimension axis (D) (from Righetti and Gianazza[156]).

(a) Genetic mutants. It is possible to perform "differential" titration curves by running a protein and its genetic mutants in a mixture. The shape of the respective titration curves should reveal which charged amino acid had been substituted in the mutant phenotype[155]. For instance, in the case of Lys mutants, the two curves should meet around pH 11, while for Glu or Asp mutants the confluence point should be around pH 3. Double charge mutants (*e.g.* Lys→Glu) or same charge replacements (*e.g.* Arg→His) can also be detected. These theoretical titration curves were experimentally verified by running normal human adult hemoglobin (HbA) mixed with any of the following mutants: HbS, HbC, Hb Suresnes and HbG Philadelphia[155,156].

(b) Macromolecule–ligand interaction. Krishnamoorthy *et al.*[157] have been able to run titration curves of met-Hb–inositol hexaphosphate (IHP) and met-Hb–inositol hexasulphate (IHS) complexes. The pH ranges of stability of these liganded states are pH 4.5–6.0 for met-Hb–IHP and pH 3.7–6.0 for met-Hb–IHS. Both complexes appear to have a dissociation constant (K_d) of the order of μM, and exist under these experimental conditions up to 8–10 min of electrophoresis. When the Hb-2,3-diphosphoglycerate complex (known to have a K_d greater than mM) was titrated, only the titration curve of free Hb could be demonstrated within the pH 3–10 range,

indicating that the complex was immediately split by the current. It should be possible, however, to detect very labile intermediates by performing the experiment at −15 to −20°C, as described by Perrella et al.[100]. By using the same 2-D technique, Constans et al.[158] have demonstrated the binding of vitamin D₃, and its derivatives, to the human serum vitamin D-binding protein. It has been shown that the binding of ligand induces conformational changes in the apoprotein. The stoichiometry of the complex, and the pH range of stability of the holoprotein could also be investigated.

(c) *Macromolecule-macromolecule interactions*. Righetti et al.[159] have exploited this 2-D technique to study the interaction between cytochrome b_5(Cyt b_5) and met Hb. While the two proteins, when run singly in the gel, develop the classical sigmoidal shape of a titrated macromolecule, when run in a mixture they exhibit strongly distorted patterns, above the pI in the case of Cyt b_5 and both, above and below the pI for met Hb. The maximum interaction appears to occur in the pH 8.0–8.3 range, and is consistent with a predominant role of Lys residues of met Hb in the binding to acidic amino acids of Cyt b_5[159]. The interaction between hemoglobin and haptoglobin (Hp), as well as the binding of intact α- and β-globin chains to Hp, have also been studied[160]. While Hp–Hb complexes were completely stable in the pH range 3–10, indicating a predominant role of non-ionic interaction, free β chains did not appear to bind to Hp while free α-globins exhibited an intriguing, pH-dependent pattern, indicating a mixed type of interaction (unpublished experiments with R. Krishnamoorthy, D. Labie and M. Waks).

(d) *Titration curves in 8 M urea and detergents*. Since pH–mobility curves of macromolecules, under native conditions, do not allow direct titration of all ionizable groups, but only of surface groups accessible to solvent not engaged in subunit contacts, or other interactions, Righetti et al.[161] have developed electrophoretic titrations in denaturing solvents, such as 8 M urea. In this system, many proteins will exist as random coils, subunits will be split apart, buried groups will be exposed to the solvent and the macromolecule will be stripped free of non-covalently bound ligands or cofactors. When running titration curves of heme-free, α and β globin chains in 8 M urea, a "bird's-eye" view of the total amino acid composition of these two chains could be obtained. In fact, since α and β chains differ mostly in their acidic residues, they come very close below pH 3, where only one positive charge difference is left. Disturbing features are encountered in the fact that they indeed join around pH 3 (while they should not) and that each curve forks both, below and above the pI, probably due to partial precipitation and aggregation in the pI neighbourhood. However, if the titration curves are performed in 8 M urea and 1% NP-40, both of these disturbances disappear since most probably the detergent, by binding to hydrophobic stretches in the polypeptide chain, prevents inter-chain interactions, which would favor flocculation in proximity of the pI[160].

(e) *Titration curves in highly porous matrices*. Bianchi Bosisio et al.[162] have attempted to run titration curves in highly porous media (acrylamide gels containing 15–50% cross-linker, either Bis or diallyltartardiamide (DATD)), which should allow almost unhindered migration of macromolecules in the multimillion MW range. Unfortunately, DATD gels contain up to 80–90% unpolymerized DATD which reacts with proteins and produces gluey and highly stretchable matrices. On the other hand, highly cross-linked Bis gels, at 40 to 50% C levels, are too hydrophobic and produce a collapsed matrix which keeps exuding water. An acceptable compromise are

30% C_{Bis} gels, which are stable and allow practically unhindered migration of globular proteins up to $0.5 \cdot 10^6$ daltons[162].

(f) Direct pK determinations from titration curves. Righetti et al.[163] and Valentini et al.[164] have developed a mathematical theory which would allow direct pK determination of ionizable groups from the shape of the pH–mobility curves. Equations have been derived linking cationic or anionic mobilities to the degree of ionization of simple cation and anions and of uni-uni-valent amphoteric molecules. For non-amphoteric ions, a direct determination of either pK_c or pK_a can be made by measuring the pH ($pH_{\frac{1}{2}}$) corresponding to $\frac{1}{2}$ mobility in the cathodic or anodic directions, respectively. For amphoteric species, the $pH_{\frac{1}{2}}$ values will have to be corrected by a term accounting for the influence of the degree of ionization of the opposite charge ion on the mobility curve of the ion being measured[163,164].

(g) K_d determinations from titration curves. One of the most recent extensions of pH–mobility curves is the possibility of determining dissociations constants (K_d) of ligands to proteins, and their pH dependence. This is achieved by techniques developed for affinity electrophoresis[165]. If the ligand is a macromolecule, it is simply entrapped in the gel matrix, if it is a small molecule , it is covalently bound to the gel fibers. In presence of increasing concentrations of ligand the titration curve of the protein is progressively retarded, in a pH-dependent fashion, and the mobility decrements, when plotted against the ligand molarity in the gel, can be used to calculate K_d values at any pH value. Ek et al.[166,167] have developed this technique for studying the binding of glycogen to phosphorylases *a* and *b*, of blue dextran to several dehydrogenases and of sugars to lectins. An example of these affino-titration curves is given in Fig. 24, which shows binding of *Ricinus communis* seed lectins to allyl-*a*-D-galactose copolymerized in the gel matrix. K_d values can be determined at any pH (usually at 6 different pH values, from pH 4 to 9, in 1 pH unit increments) and then plotted *vs.* pH (see Fig. 25) in order to evaluate the pH-dependence of K_d.

As a general conclusion, it can be stated that titration curves hold a great potential for investigating several physico-chemical parameters of proteins and dynamical aspects of their interactions with ligands.

6.5. Two-dimensional techniques

There are at least six variants utilizing IEF as one of the two dimensions (IEF–SDS gel electrophoresis or IEF–SDS gel gradient electrophoresis; IEF–gel electrophoresis; IEF–gel gradient electrophoresis; IEF–immunoelectrophoresis; IEF–isotachophoresis (ITP) and IEF–electrophoresis in a stationary pH gradient) (see Fig. 26). These techniques have been recently reviewed in detail[12]. The strategy, in a 2-D electrophoretic separation of proteins, would be to discriminate molecules on the basis of net-charge (or free mobility) in the first dimension, followed by fractionation according to size, in the second dimension, or *vice versa*. In this way, one might expect that every region of the 2-D gel slab would be covered by spots. As this aim is very nearly achieved by IEF–SDS runs, we will only survey this technique here, also because it appears to be the most popular. Barrett and Gould[168] and McGilliwray and Rickwood[169] were among the first ones to introduce this method of charge/mass fractionation. However, it was only in 1975, when O'Farrell[170] described a simple method for casting SDS-pore gradient gels and assembling an electrophoretic cell for 2-D runs, and coupled this technique to autoradiography, thus
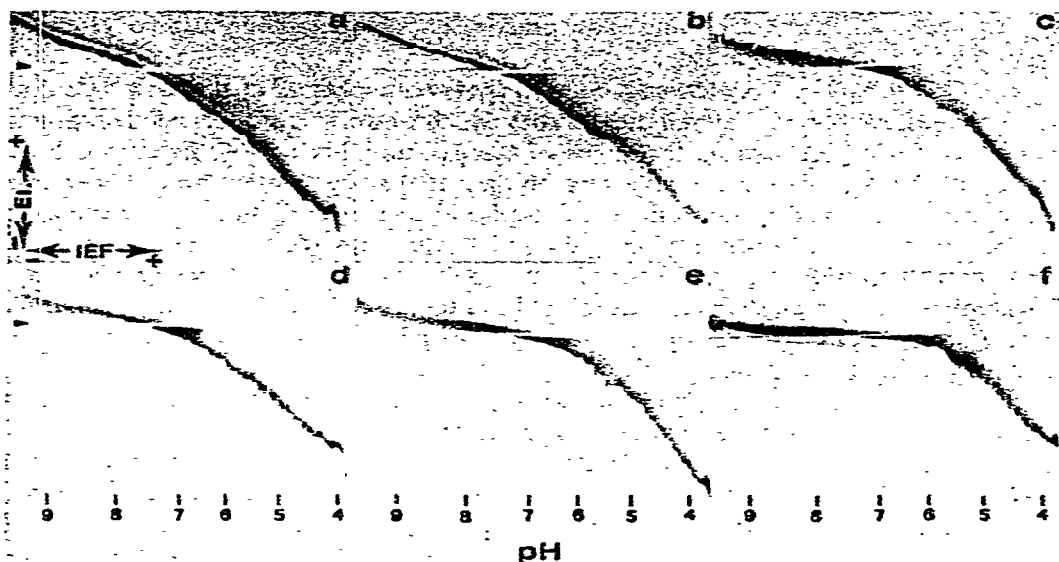
Fig. 24. Affino-titration curves of lectin from *Ricinus communis* seeds. The gel contained 6% T, 4% C_{Bis}, 2% Ampholine pH 3.5–10, and 2 mM Glu, Asp, Lys and Arg. The amount of ally-α-D-galactose copolymerized in the gel matrix was: (a), control, no ligand; (b), $1 \cdot 10^{-5} M$; (c), $4 \cdot 10^{-5} M$; d, $5 \cdot 10^{-5} M$; (e), $7 \cdot 10^{-5} M$ and (f), $10 \cdot 10^{-5} M$. The amount of protein loaded in all cases was 200 μg in 100 μl volume. 1-D, 80 min at 10 W constant. 2-D, 20 min at 700 V constant. In both dimensions the electrolytes were 1 M NaOH at the cathode and 1 M H₃PO₄ at the anode. The gel was cooled at 4°C with a Lauda KR4 thermostat. The two arrow heads in (a) and (d) indicate the sample application trench (zero mobility plane). The two double arrows with positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (El). (from Ek *et al.*[166]).
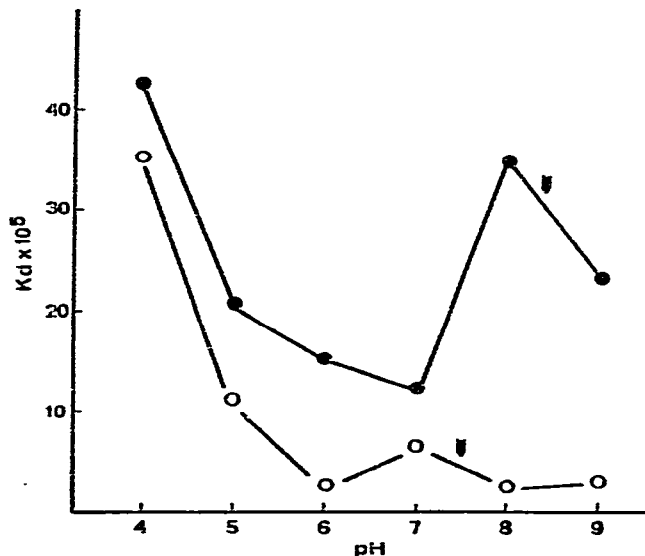

Fig. 25. Variation of the dissociation constants (K_d) as a function of pH for *Ricinus communis* (○) and for *Lens culinaris* (●) lectins. The K_d values reported here have been calculated from the retarded mobilities, at different pH values, in affino-titration curves, such as the ones shown in Fig. 24. The two arrows indicate the p*I* of each lectin (from Ek *et al.*[166]).

revealing more than a thousand spots on the 2-D gel, that 2-D techniques became extremely popular. At the beginning, most 2-D runs were performed in the sequence IEF–SDS[168–172], since it was believed that the anionic detergent SDS would destroy the pH gradient if present in the IEF dimension. It soon became apparent, however, that proteins exposed to SDS could be run in the IEF gel without severely disturbing the IEF separation[173,174]. Thus, recently, two methods have been proposed in which the sequence is reversed into SDS–IEF runs[175,176]. This is not just a mere curiosity, but is in fact quite necessary when extracting whole cells, since boiling SDS favors practically complete solubilization of membrane-bound proteins while decreasing the likehood of alteration of cellular proteins by kinases, proteases or other active enzyms in the extract. In one method[175], the SDS-gel strip is placed on an IEF gel containing 8.5 M urea and 2.5% NP-40: the combined action of urea and non-ionic detergent in the gel results in the splitting of the SDS–protein complex. Best results were obtained with 2.5–3.5% detergent levels in the gel: when NP-40 was lowered below 2% or increased above 4% resolution was impaired. In the other method, the SDS gel is briefly exposed to an interfacing solution comprising 9 M urea, 1% DEAE-cellulose, 5 mM ascorbic acid, 0.2 mM EDTA, 0.12% 2-mercaptoethanol, 10% glycerol in 30 mM Tris, 0.225 M glycine buffer, pH 8.6 (ref. 176). Each component fulfills a given function: urea weakens SDS binding to non-polar regions of proteins; DEAE-cellulose facilitates removal of SDS; ascorbic acid and EDTA significantly increase the solubility of protein components, 2-mercaptoethanol prevents formation of disulfide bridges and glycerol reduces diffusion of low-MW components.
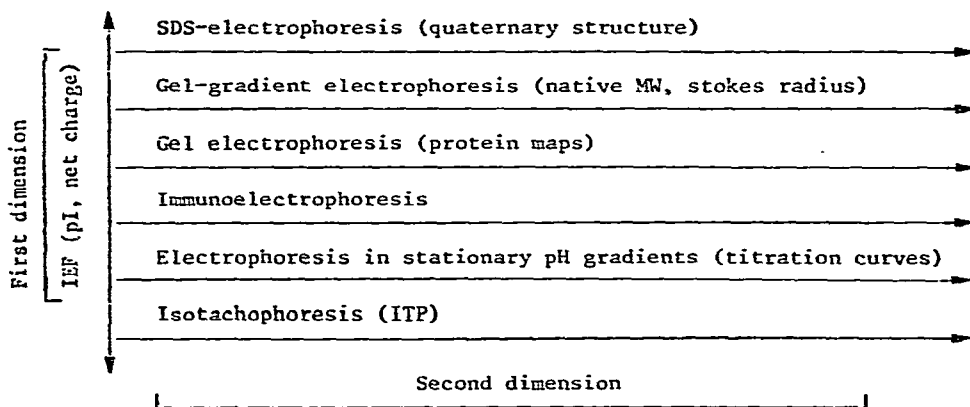

Fig. 26. Diagram illustrating various two-dimensional techniques in which one of the dimensions is IEF (from Gianazza and Righetti[12], pp. 293–311).

A few practical aspects of 2-D runs will be considered here in more details:

(a) Chambers. The basic design remains that described by O'Farrell[170]: the SDS-pore gradient gel is a vertical slab on top of which the IEF gel rod is embedded with the aid of melted agarose. To facilitate the loading of the IEF-gel cylinder, Imada[177] has described a cell in which one of the two glass plates has been bent at an angle of 6.5°. When multiple gel rods have to be cast on a stack of SDS gels, the two systems of Anderson and Anderson[179,179] can be utilized. This set-up for multiple, parallel IEF–SDS runs has been termed ISO-DALT system, to indicate

that separation is based on ISOelectric focusing (charge) in the first dimension and on molecular weight (DALTons) in the second. This has spurred Dean[180] to describe an apparatus for simultaneous processing of eight polyacrylamide gel slabs, complementary to the standard eight-tube disc electrophoretic cell. We are almost tempted to report an icositetra gel slab apparatus for an icositetra gel tube cell... A micro 2-D technique, utilizing gel slabs of approximately stamp size ($ca.$ 1 cm^2) has been developed by Rüchel[181]. Miniaturized systems for pore gradient electrophoresis in slabs have also been adopted by Matsudaira and Burgess[182] and by Ogita and Market [183]. 2-D techniques can also be run in horizontal, ultrathin (240–360 μm) pore gradient slabs, cast onto cellophane sheets[184]. These slabs are compatible with gel strips, instead of gel rods, in the first dimension. In fact, the 1-D gel is simply lowered onto a trench precast in the 2-D slab, and does not have to be sealed to it with agarose.

    (b) Markers. When looking at a 2-D map with more than 2000 spots scattered over it one cannot avoid the impression of being confronted with a starry sky in a clear night. As astronomers have long known, it is imperative to have reference points for orientation. For the IEF dimension, perhaps the simplest and most ingenious procedure is the production of "carbamylation trains" described by Anderson and Hickman[185]. It is known that, when a protein is heated in a solution of urea, its amino groups are progressively carbamylated through the production of cyanate. Since the loss of a free amino group below pH 8.5 results in a unit change in the charge of a molecule, the products of this reaction appear, in the SDS gel, as a row of spots at roughly constant molecular weight, spaced apart by about 0.1 pH units. Carbamylation of human Hb β chain gives a row of 12 such spots corresponding to the blockage of 11 Lys and 1 α-amino group. A carbamylation train of carbonic anhydrase (from bovine erythrocyte) gives a string of 20 spots while a train of creatine phosphokinase (rabbit muscle) is as long as 30 wagons. For the SDS dimension, a series of crosslinked polymers of one protein of known MW could be used as a MW standard[186], however they might exhibit anomalous behaviour in SDS gels. For this reason, Giometti *et al.*[187] have suggested the use of rat heart whole homogenate as an internal reference standard in the DALT dimension. In the SDS run, this homogenate generates 80 lines, of which 12 are major spots distributed at convenient intervals along the gel pattern, covering MW increments from as little as 300 daltons (about 3 amino acid residues) up to 10,000 daltons at the upper end of the gel. This homogenate, when extracted in SDS, dispensed in small aliquots in 0.7% agarose and stored frozen at −20 to −70°C, gives fully reproducible patterns for over 12-month periods of use. As a simple, tracking colored protein for the IEF-SDS gels, cytochrome c has been suggested[188]. An interesting technique, especially useful for comparing 2-D maps generated by two different tissues or cell lines, has been proposed by Choo *et al.*[189]. The two cell lines are grown in two different isotopes, *e.g.* ^{14}C and ^{3}H, then the extracts mixed and analyzed in a single 2-D gel. After fixing and impregnating with scintillator (diphenyloxazole), the dried slab is fluorographed as described by Bonner and Laskey[190]. The spots developed in the fluorographic process are due to the combined photon emission of both ^{14}C and ^{3}H isotopes. After this, the gel slab is covered with black carbon paper (which blocks photons emitted from the gel, as well as the weak β^- radiation of ^{3}H) and now autoradiographed, thus revealing only the strong β^- spots of ^{14}C emission. Comparison of the fluorograph with the autoradiograph allows an accurate comparison of the gene products of the two cell lines. A host of

other specific labelling techniques can be used to reveal in the 2-D map specific groups of proteins. Thus, proteins containing reactive–SH groups can be labelled with [^{14}C]-iodoacetamide; ^{32}P tagging can be used to identify phosphorylated proteins, lectin or antibody binding can reveal few, selected components in the map[191]. It has also been reported that, after the IEF step, the gel can be fixed and stained with Coomassie Blue R-250 and then run in the SDS gel[192]. Apparently, this modification does not
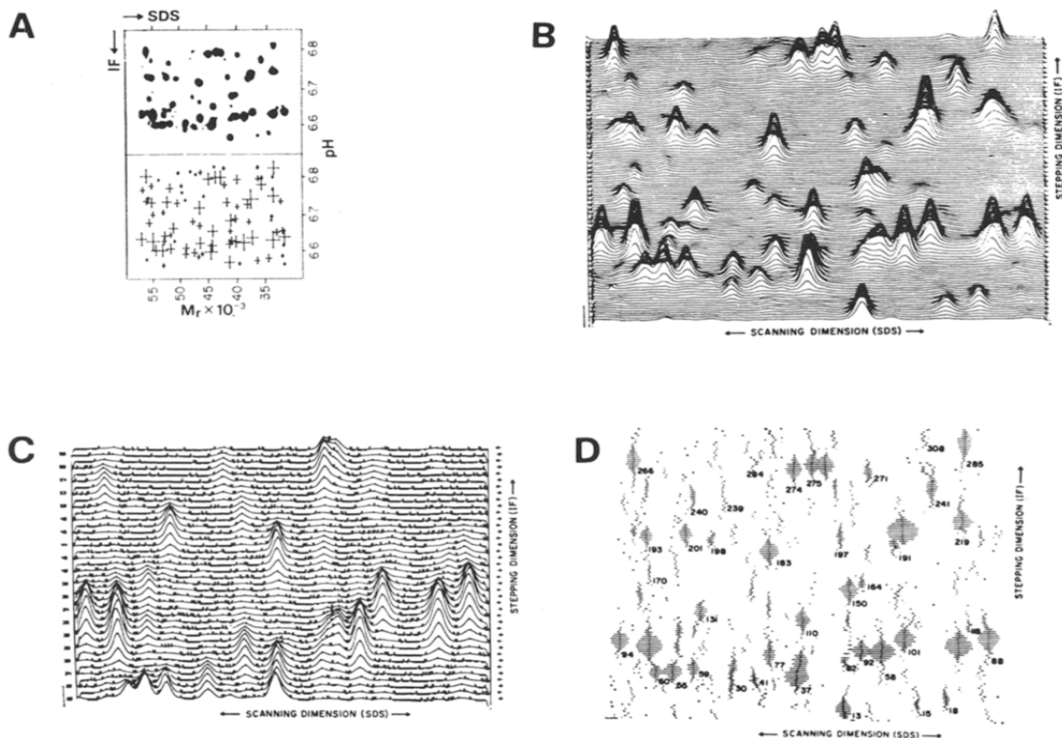

Fig. 27. Example of computer analysis of 2-D electropherograms. (A) The film was taken from a fluorogram representing proteins of confluent B103 cells. The gel was run using pH 6–8 ampholytes in the first dimension and 7.5 acrylamide in the second dimension. The small region shown was 36 × 53 mm in area. Normally an area of 150 × 150 mm is scanned, but for explanatory purposes only this small region was processed. The faintest detectable spot represents about 0.5 cpm. The computer plot shows the spots detected and the area of the cross is proportional to the integrated spot density. (B) Lines of data generated by successive scans across the film shown in A. Each line contains about 380 readings of film density. These data are received and immediately processed by the computer during the line analysis stage of data reduction. The distance between lines represents a 280-μm step in the stepping (pH) dimension. The vertical bar at the left indicates a density of one absorbance unit on the vertical scale. (C) Line analysis. A special program has plotted each line of data with diagnostic marks to indicate exactly how each line was processed. Each peak is enclosed in brackets and its center is marked by vertical lines. The lines shown are lines 20–59 of B, replotted with greater spacing between them. (D) Chain assembly. The integrated peaks detected during line analysis (C) have been assembled into chains in the stepping (pH) dimension. Each horizontal line depicts the peak intensity and its center is marked by a vertical line. Each descending line points to the previous peak of the chain so that the chains can be followed. The numbered chains are then transformed into integrated density peaks, whose relative areas are calculated by the computer (not shown) (from Garrels[194]).

alter the protein patterns obtained, on the contrary it provides sharper spots in the second dimension. In the SDS run, the dye bound to the proteins inside the IEF gel becomes stacked and moves with the bromophenol blue front through the running gel.

(c) Scans. Proper densitometry of 2-D gels is not an easy task, since three coordinate values have to be given to each spot: a pI (x axis), a MW (y axis) and a relative intensity (%). Thus, this task is only a little bit less complicated than astronomers cartography, where a fourth coordinate value is needed. At the turn of the century, the Harvard astronomer E. C. Pickering[193] solved this problem by hiring "computers", ladies hired at 25 cents an hour to fill blank catalogue pages with tiny black ink numbers; no mistakes were permitted. With the Women's Liberation Front on the loose, and the pressure from multinational computer (machines, this time) companies, this approach would be a bit unrealistic today. Several research groups have independently described computer programs for data acquisition and image analysis of 2-D gels[194-201]. In Fig. 27 we give an example of such a computer analysis of 2-D gels. The protein spots in the fluorogram, or autoradiogram, or Coomassie Blue-stained electropherogram, are converted by the computer into crosses (A), whose area is proportional to the integrated spot density. The crosses are transformed into scan lines (B), whose excursions in the cross region are seen as peaks against the line background. A line analysis is then performed (C) and converted into a chain assembly (D). By integrated density analysis of the chain assembly, a densitogram tracing, resembling the scans of 1-D gels, is finally obtained (not shown).

(d) Applications. Given the incredible explosion of 2-D techniques, it would be foolish for us to attempt to summarize this field. We will only briefly survey some recent applications. Extensive sections on 2-D techniques can be found in the proceedings of recent meetings[5-8]. Anderson *et al.*[202-204] have begun the systematic mapping of an estimated 30,000 to 50,000 human protein gene products[202]. They include: mapping of human erythrocyte proteins[203], saliva[204], urinary proteins[205], peripheral blood lymphocytes[206]. Applications in clinical chemistry[207], and to the analysis of human lipoproteins[208], of virus-infected cells[209], of human fibroblasts[210], of liver cytosol proteins[211], of nuclear chromosomal proteins[212], of basic cellular proteins[213,214], of erythrocyte membrane proteins[215] and of mitochondrial ribosomal[216] and microsomal membrane[217] proteins have been described.

7. CONCLUSIONS

How do we end a review like this? It has become a fashion today to give multiple endings (*e.g.* Apocalypse Now, by Francis Ford Coppola) so that the reader can pick up the preferred path. Do you like the happy, the unhappy or the wishy-washy ending? While you think it over, we will simply say *arrivederci* till, if ever, the next review.

8. ACKNOWLEDGEMENTS

to our ignorance in the field. The ideas discussed here represent the personal views of the authors, and are not meant to, nor could they possibly, endorse any official institution or commercial firm.

9. SUMMARY

Recent developments of isoelectric focusing (IEF) are summarized. New approaches to the synthesis of carrier ampholytes are described and some controversial aspects of their properties, especially in regard to their molecular weight, are critically discussed. New aspects of preparative techniques, such as "steady-state rheoelectrolysis" and continuous-flow recycling IEF, are presented. New methodological trends of analytical IEF, such as agarose, cellulose acetate, ultrathin-layer and sub-zero temperature IEF are discussed. Among analytical applications, particular emphasis has been placed on detection of neutral mutations, IEF of peptides, IEF of cells and organelles and on titration curves. The review ends with a tiny chapter on a vast field, the explosive field of two-dimensional techniques.

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