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ISOELECTRIC FOCUSING WITH IMMOBILIZED pH GRADIENTS

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I. Introduction

The technique of isoelectric focusing (IEF) via immobilized pH gradients (IPG) was first announced to the scientific community at a meeting of the International Electrophoresis Society in Athens, April 1982, as the result of an intensive collaborative effort [1]. In five years, the technique has been extensively developed in three fundamental aspects: analytical, preparative and as a first dimension of two-dimensional (2-D) maps. The merits and, recently, the flaws of the IPG technique have been evaluated and recognized, so that at the present writing we feel it is ready for successful introduction in most life-science laboratories.

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It is impossible in the present review to cover extensively the IPG field, as this would require a book: thus we have chosen, after explaining the basics, to give the most recent advances. For covering to a deeper extent methodological and past developments, the reader is referred to two extensive reviews [2, 3] and to proceedings of recent meetings [4-6]. There were many reasons for abandoning conventional IEF in carrier ampholyte (CA) buffers: due to their peculiar distribution along the separation path (interdigitating gaussians) the CA chemicals (a multitude of oligoamino oligocarboxylic acids, buffering in the pH 3-10 interval) create a discontinuous physico-chemical environment, varying in buffering capacity, conductivity and ionic strength within micrometric distances, often noxious to proteins (inducing precipitation, smears, artefactual banding patterns due to CA-protein complexes, even loss of enzymatic activity due to metal chelation) [7]. In order to eliminate these problems, basically due to the discrete distribution of CAs along the electric path, we tried to create a matrix in which the chemical environment would vary in a continuous fashion,

without the discontinuities typical of the steady-state distribution of CAs. This required the ability of performing a continuous titration over any desired pH interval, i.e. abolishing the use of specified buffers able to condense at any given point along the pH scale, as the latter species are in fact the product of an ad hoc titration. Even by adding to the system a great number of such amphoteric buffers, one is still working on the principle of discrete titration, bound to have steps or discontinuities, no matter how minute, among adjacent species. Thus, the thin red line dividing IPGs from CA-IEF is the principle of continuous vs. discontinuous titration, as it can be appreciated from Fig. 1A and B.

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II. The Immobiline chemicals

IPGs are based on the principle that the pH gradient, which exists prior to the IEF run itself, is copolymerized, and thus insolubilized, within the fibres of the polyacrylamide matrix. This is achieved by using, as buffers, a set of seven non-amphoteric, weak acids and bases, having the following general chemical composition: $CH_2=CH-CO-NH-R$, where R denotes either 3 different weak carboxyls, with pKs 3.6, 4.4 and 4.6 or 4 tertiary amino groups, with pKs 6.2, 7.0, 8.5 and 9.3 (Table I). During gel polymerization, these buffering species are efficiently incorporated into the gel (84-86% conversion efficiency at 50°C for 1 hour) [8]. Immobiline-based pH gradients can be cast in the same way as conventional polyacrylamide gradient gels, by using a density gradient to stabilize the Immobiline concentration gradient, with the aid of a standard, two-vessel gradient mixer. As shown in the formula, these buffers are no longer amphoteric, as in conventional IEF, but are bifunctional: at one end of the molecule the buffering group is located,

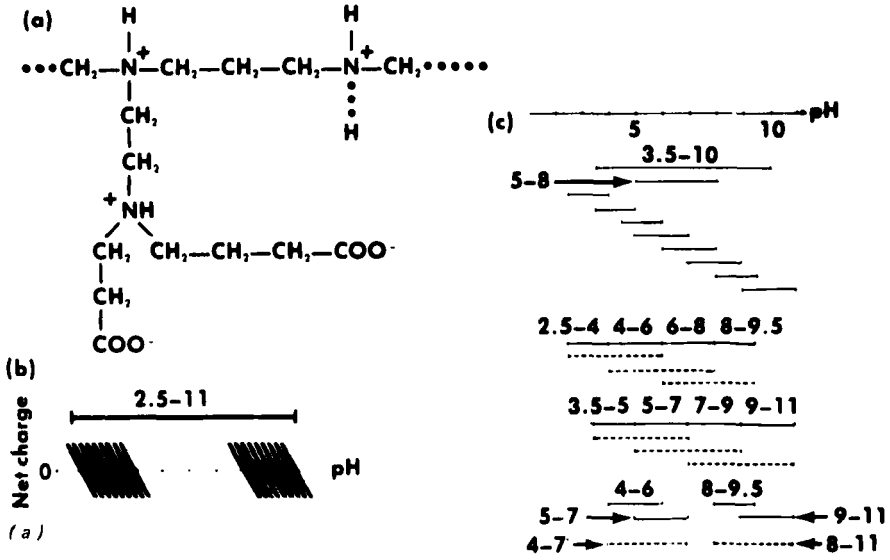

FIGURE 1

The principles of isoelectric focusing in carrier ampholyte buffers (A) and in immobilized pH gradients (B). A: on the left side (a), a representative chemical formula of CAs is shown (aliphatic oligoamino oligocarboxylic acids). On the lower left side (b), portions of hypothetical titration curves of Ampholines are depicted (the steep slope across the pI indicates good buffering capacity and conductivity). Right (c): difference pH cuts for wide and narrow range carrier ampholytes. A typical Ampholine synthesis consists of reacting oligoamines (4 to 6 nitrogen long) with acrylic acid. B: idealized scheme of an Immobiline matrix. The polyacrylamide gel is represented by convoluted threads, whose cross-over points can be taken as the linking of different chains by the cross-linking agent. The Immobiline derivatives are represented as carboxyl and tertiary amino groups, covalently bound to the gel matrix. A given ratio $-COOH/-N-R_2$ in any infinitesimal gel layer defines and maintains in such a stratum a fixed pH value (both figures by courtesy of LKB Produkter AB).

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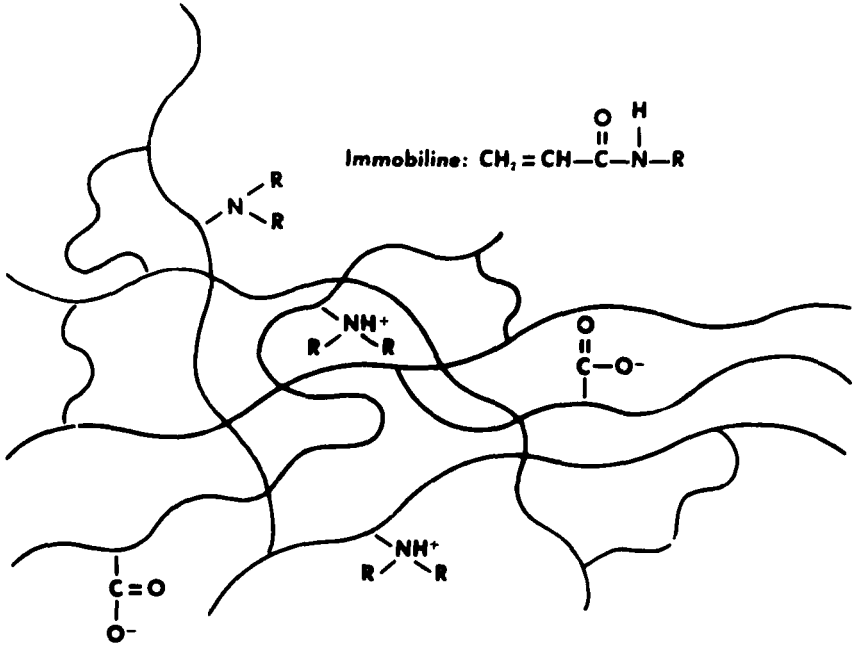

(b)

FIGURE 1 CONTINUED...

and at the other is the acrylic double bond, which will disappear during the grafting process. The three carboxyl Immobilines have rather small temperature coefficients (dpK/dT) in the 10-25°C range, due to their small standard heats of ionization (ca. 1 Kcal/mol) and thus exhibit negligible pK variations in this temperature interval. On the other hand, the four basic Immobilines exhibit rather large ΔpKs (as much as ΔpK=0.44 for the pK 8.5 species) due to their larger heats of ionization (6-12 Kcal/mol). Therefore, for reproducible runs and pH gradient calculations, all the experimental parameters have been fixed at 10°C. Temperature is not the only variable that will affect Immobiline pKs

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TABLE 1

PROPERTIES OF IMMOBILINES™

Apparent pK values [a], $I = 10^{-2}$.

	H_2O		Polyacrylamide gel [b] T=5%, C=3%		Polyacrylamide gel [b] T=5%, C=3% glycerol 25% (w/v)		Physical state at room temperature
	10°C	25°C	10°C	25°C	10°C	25°C	
Acids with carboxyl as buffering group							
Immobiline pK 3.6	3.57	3.58	-	-	3.68 ± 0.02	3.75 ± 0.02	solid
Immobiline pK 4.4	4.39	4.39	4.30 ± 0.02	4.36 ± 0.02	4.40 ± 0.03	4.47 ± 0.03	solid
Immobiline pK 4.6	4.60	4.61	4.51 ± 0.02	4.61 ± 0.02	4.61 ± 0.02	4.71 ± 0.03	solid
Bases with tertiary amine as buffering group							
Immobiline pK 6.2	6.41	6.23	6.21 ± 0.05	6.15 ± 0.03	6.32 ± 0.08	6.24 ± 0.07	solid
Immobiline pK 7.0	7.12	6.97	7.06 ± 0.07	6.96 ± 0.05	7.08 ± 0.07	6.95 ± 0.06	solid
Immobiline pK 8.5	8.96	8.53	8.50 ± 0.06	8.38 ± 0.06	8.66 ± 0.09	8.45 ± 0.07	liquid
Immobiline pK 9.3	9.64	9.28	9.59 ± 0.08	9.31 ± 0.07	9.57 ± 0.06	9.30 ± 0.05	liquid

[a] pK values measured with glass surface electrode without any corrections.

[b] Mean values of 10 determinations. Due to the slow response of the electrode the pK values for the amines are uncertain.

(and therefore the actual pH gradient generated): additives in the gel that will change the water structure (chaotropic agents, such as urea) or will lower its dielectric constant, and the ionic strength itself of the solution, will alter their pK values [9]. Conversely detergents (both non ionic and zwitterionic) do not produce pK shifts, suggesting that the Immobilines do not tend to be incorporated into the surfactant micelle. The list of seven Immobilines is not exhaustive: for extended pH gradients there is often the need of two additional ones, i.e. a strongly acidic (pK<1, sulphated derivative) and a strongly basic (pK>12, quaternary amino acrylamido derivative) compounds with pKs well outside the desired pH range [10], thus able to provide equivalents of acid or base without any buffering action even at the extreme pH values of the titration. Recently, an additional Immobiline, with pK 10.3, has been synthesized, which has proven very useful for generating strongly alkaline pH gradients (pH 10-11) able to focus such alkaline species as cytochrome C, elastase and trypsin [11, 12]. In reality, we have omitted from the list the most important and most abundant 'Immobiline' in the system, so visible and obvious as to be completely ignored: water, which is always present in all formulations in non-negligible amounts (55.56 M) as compared with the average 10 mM concentration of a buffering Immobiline. As we will see in section VII, water strongly interferes with IPG formulations below pH 4 and above pH 10.

Since they are copolymerized within the matrix, the Immobiline buffers no longer migrate in the electric field: this means that the pH gradient is stable indefinitely, though this condition has to be established before the onset of polymerization, and can only be destroyed if and when the polyacrylamide gel is hydrolized. At conventional matrix concentrations (5%T) and at the standard Immobiline

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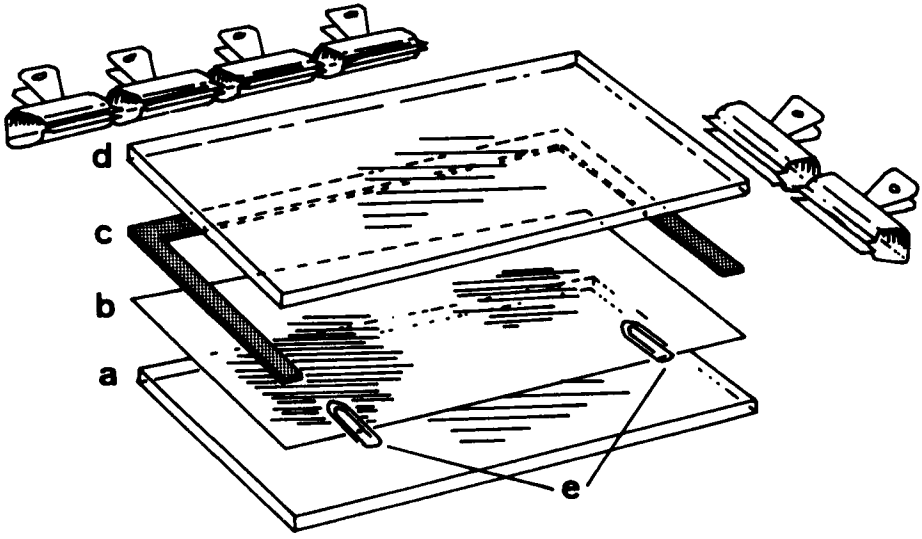

FIGURE 2

Assembly of a gel cassette for casting IPGs. A: (a) Glass base plate (3 mm thick); (b) Gel Bond PAG foil; (c) U-gasket (in general silicone or rubber, often glued to the glass cover plate; usually 0.5 mm thick); (d) Template: 3 mm thick glass plate with pieces of self-adhesive Dymo tape (250 μm thick) for moulding of gel slots; (e) paper clips as temporary, additional spacers (from Görg et al., ref. 13). B: preparation of the cover plate with a strip of Dymo tape and cutting-out of pocket-forming slots (by courtesy of Drs. A. Görg and R. Westermeier).

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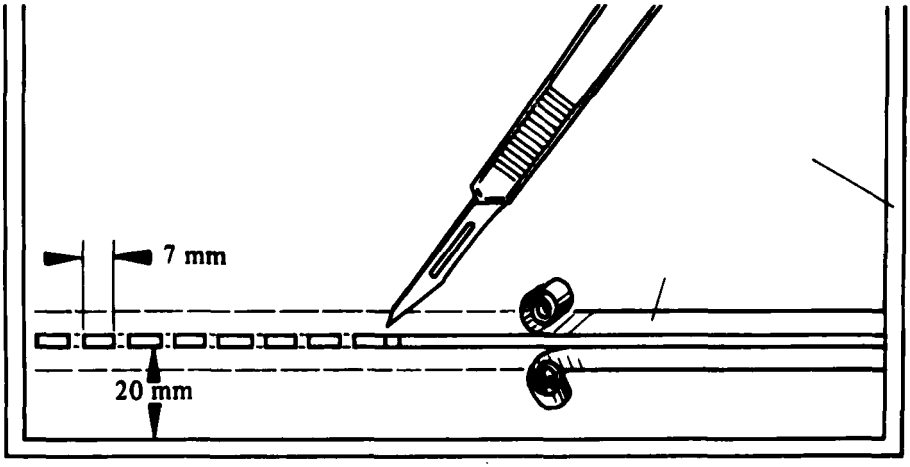

FIGURE 2 CONTINUED...

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levels (ca, 10 mM buffering ion) there is about 1 buffering group (or titrant) every 47 acrylamide residues, which means that, statistically, two Immobiline species are about 94 carbon atoms apart.

III. Methodology

-Gel casting with a two vessel gradient mixer

For the cassette, the basic assembly is just as depicted in Fig. 2A (as Immobiline gels usually have to be washed, it was not feasible, till recently, to cast them in glass cylinders) [13]. To the cover glass, we suggest binding pocket-forming strips (up to 20), cut out of adhesive embossing tape (Dymo), for applying the sample in a free liquid layer. Then gluing the intact length of tape and cutting out and removing 3 mm wide segments, perpendicular to it, so as to leave glued to the glass separate rectangles of tape (Fig. 2B). The depressions formed by the

Dymo tape in the gel layer are about 250 μm deep, and can usually accomodate volumes up to 10-12 μl. A double volume can be arranged by gluing two embossing strips on top of each other. It is always best, however, to make sure that, after opening the cassette, the pockets are sealed at the bottom by a thin film of polyacrylamide gel.

The final assembly is shown in Fig. 3: notice that the two upper clamps on the top of the cassette are removed and that two paper clips are inserted instead [14]. This greatly facilitates the insertion of the plastic tubing conveying the solution from the mixer to the chamber by forcing the top rims of the glass slabs to diverge and thus widen the 0.5 mm gap. However, cover glasses with three indentations at the top are now available, so that the tubing can be inserted directly into any of these 3 V-shaped channels without widening the top rim with paper clips as shown in Fig. 3. The gradient mixer is positioned about 5-8 cm from the chamber top (the liquid will flow down by gravity) and is filled with the acidic and basic solutions. One should remember to fill only one chamber first, and then to remove any air from the channel connecting the two chambers by gently opening the central valve. The gel chamber stands vertically on a levelling table and the capillary tubing is inserted in the middle. At this point stirring is started (500 rpm), the catalysts added (TEMED and persulphate, in this order), both valves are open and the density (and pH) gradient is allowed to flow in the gel cassette. There are ca. 10 min before the onset of polymerization at 10°C, but in a hot room in summer (and in 8M urea) the reaction will be faster (2-3 min at temperatures > 30° C). Once the whole gradient has been poured, the clips are quickly removed and the two upper clamps fastened in their position.

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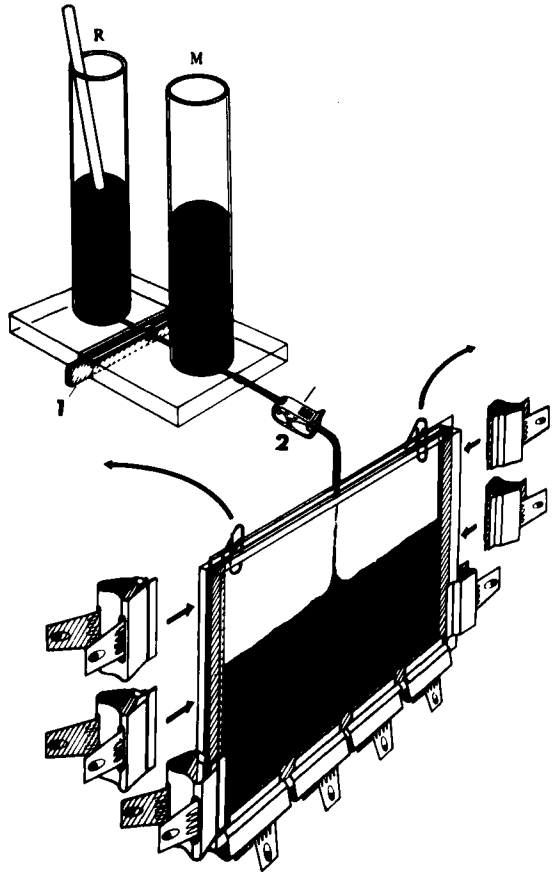

FIGURE 3

Gel cassette and gradient mixer for casting an Immobiline gel. The basic, light solution is pipetted into the reservoir (R) while valves (1) and (2) are closed. The connecting tube is filled by opening (1) for a brief moment. The acidic, heavy solution is pipetted into the mixing chamber (M). Stirring is started in both vessels and the catalysts (TEMED and persulphate, in this order) added. Valves 1 and 2 are simultaneously openend and the gel cassette filled by gravity feeding. When the liquid has reached about two-thirds of the cassette height, the paper clips (temporary spacers) are removed and the four remaining upper clamps inserted in place, so that the liquid rises to fill up the chamber (courtesy of Drs. A. Görg and R. Westermeier, ref. 14).

After standard polymerization (1 h, 50°C) the gel cassette is removed from the oven, the eight clamps unfastened and the supporting glass plate gently pried open with the tip of a spatula. The Gel Bond PAG foil can now be lifted from one corner and gently peeled off, with the bound polyacrylamide gel layer, from the other plate closing the cassette. The first step at this point is weighing (after blotting any traces of liquid around the ridges of the foil, if needed), as the gel has to be washed and will swell in water during this procedure. It is well to mark the weight of the gel on the plastic backing. The gel is now washed in 1 L of distilled water for 30-40 min for 0.5 mm gels (double this time for 1 mm gels). The washing step is essential: TEMED, persulphate and 12-16% unpolymerized Immobilines must be removed, otherwise large plateaux of free acid and free base will form at the anode and cathode, respectively, and will prevent the protein from focusing. After washing, the gel should be blotted with soft tissue and then, with the aid of a fan, reduced to its original weight.

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-Reswelling of dried Immobiline gels

For routine applications, is often convenient to cast gels by a batch procedure and then store them dry for subsequent use. Gelfi and Righetti [15] studied the IPG gel swelling kinetics as a function of the following variables: (a) solvent composition (plain water, 8M urea, 2% Nonidet P-40 and their mixture); (b) gel composition (varying %T at fixed %C); (c) temperature (in the range 20-60°C); (d) gel thickness (from 0.5 to 2 mm; Fig. 20); (e) operative pH interval (from the extreme acidic, pH 3.5-5, to the extreme alkaline, pH 8.5-10, ranges) under isoionic conditions. The following practical guidelines for gel drying should be observed: (i) the gel should have a low matrix content (e.g.

3%T); (ii) they should have reduced thickness (0.5, max. 1 mm; for greater thicknesses, the swelling exponential becomes too steep); (iii) alkaline gels should be dried in vacuo over silica, to prevent CO_2 adsorption; (iv) the gels should be extensively washed prior to drying. In regard to the last point, it is better, in the last washing, to equilibrate the gel in 1% glycerol, which will keep moisture in the dried gel and will 'lubricate' the polyacrylamide coils, thus preventing an irreversible collapse of the gel matrix. Precast, dried Immobiline gels encompassing a few acidic ranges, are now available from LKB Produkter AB: they all contain 4%T and they span the following pH ranges: pH 4-7; pH 4.2-4.) (e.g., for α_1-antitrypsin analysis); pH 4.5-5.4 (e.g., for Gc screening) pH 5.0-6.0 (e.g., for transferrin analysis) and pH 5.6-6.6 (e.g., for phosphoglucomutase screening). Precast, dried IPG gels in the alkaline region have not been introduced as yet, possibly because at higher pHs the hydrolysis of both the gel matrix and the Immobiline chemicals bound to it is much more pronounced. There could be two ways to overcome this problem: (a) wash the alkaline gel, prior to drying, in traces of formic acid (the lower pH will greatly extend their life span); (b) store the dried gels at -20°C [16]. It has been found that the diffusion of water through Immobiline gels does not follow a simple Fick's law of passive transport from high (the water phase) to zero (the dried gel phase) concentration regions, but it is an active phenomenon: even under isoionic conditions, acidic ranges cause swelling 4-5 times faster than alkaline ones. Given these findings, it is preferable to reswell dried Immobiline gels in a cassette similar to the one for casting the IPG gel. Fig. 4 shows the reswelling system produced by LKB: the dried gel is inserted in the cassette, which is clamped and allowed to stand on the short side; via a

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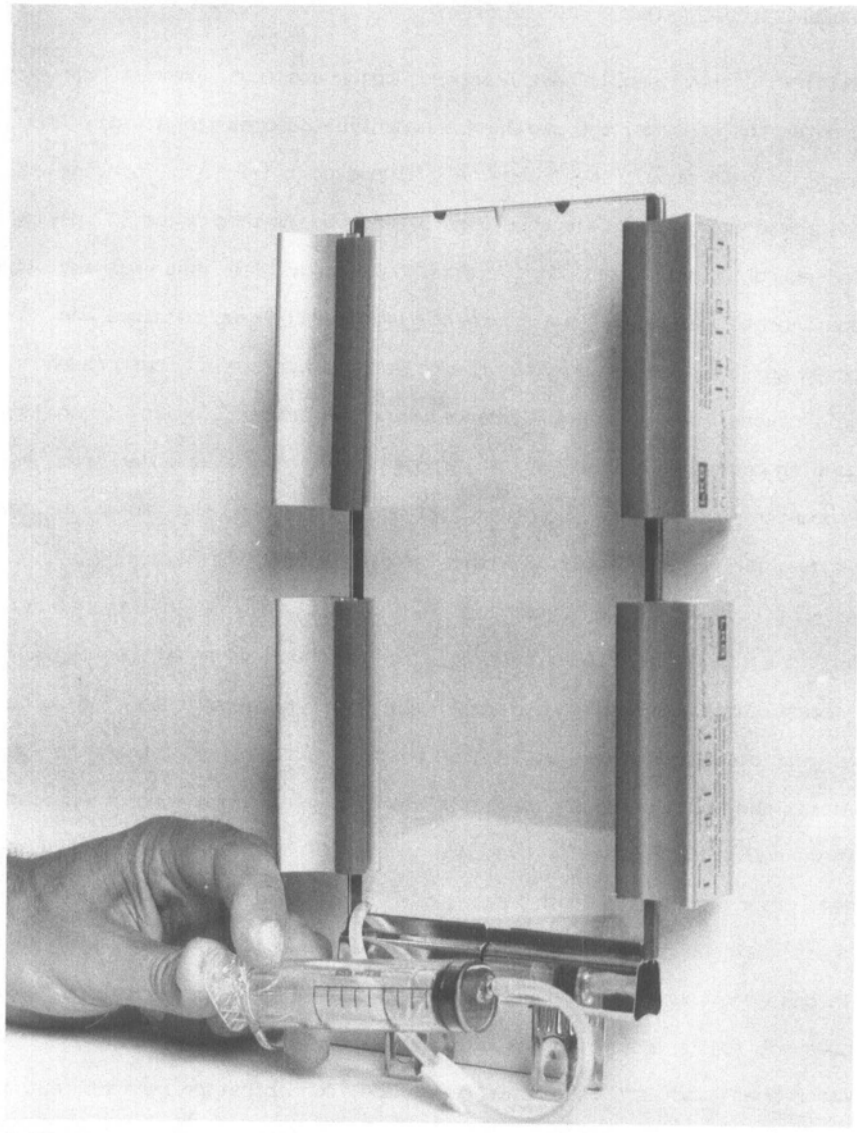

FIGURE 4

Reswelling cassette for IPG gels. After washing and drying, the Gel-Bond-pasted IPG matrix is inserted in the cassette, which is clamped and allowed to stand on its short side. On the lower left corner of the cover glass, a 3 mm diam. hole with a short tubing connection allows the filling of the reswelling solution via a syringe.

small hole in the lower left side and a cannula, the reswelling solution is gently injected in the chamber, till complete filling. As the system is volume-controlled, it can be left to reswell overnight, if needed. Gel drying and reswelling is the preferred procedure when an IPG gel containing additives is needed: in this case it is always best to cast an 'empty' gel, wash it, dry it and then reconstitute it in presence of the desired additive (e.g., urea, alkyl ureas, detergents, carrier ampholytes and mixtures thereof).

-The use of plateaux

Plateaux, added at one or both ends of the IPG gels, introduce a new dimension in the Immobiline technique, as they greatly enhance its versatility. Several types of plateaux can be utilized, either alone or in combination: (a) pH plateaux; (b) porosity plateaux (c) affinity ligand plateaux. pH plateaux were first reported by Ek et al. [17], their main purpose being salt removal from the IPG matrix. It is known that IPGs, being covalently bound to the gel matrix, are in principle unaffected by salt ions. There are, however, some practical limits to the amount of salt that can be tolerated even in IPGs, and they are due to: a) initial conductivity of the IPG gel; b) generation of strongly acidic and strongly basic gel zones in proximity of the electrodes as a consequence of the physical separation of the salt ions. With respect to the first point, different amounts of salts can be tolerated in different pH ranges: at acidic pH (pH 3.5-5) as much as 10-15 μmoles NaCl/ml gel solution can be tolerated, the corresponding amount in the pH range 9-10 being 3-4 μmoles NaCl/ml gel solution (it should be remembered that the free proton mobility is 350.10^{-5} cm^2 V^{-1} sec^{-1} while the corresponding $-OH^-$ mobility is only 85.10^{-5} cm^2 V^{-1} sec^{-1} at 25° C).

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However in the pH range 5.5 to 9, where the conductivity of the system is at a minimum, and the contribution of free H^+ and $-OH^-$ quite small, the maximum tolerable amount is barely 0.5 μmoles NaCl/ml gel solution.

The second problem (collection of free cations and anions at the two electrodes, with formation of plateaux with extreme pH values) can be solved by elongating the gel with pH plateaux at the two extremes, where the ion components of the salt can collect just outside the Immobiline pH gradient. In this case, it is best to polymerize a 50% longer gel (e.g. 15 cm instead of the 10 cm standard length). Enough of the acidic, dense solution, is injected in the chamber to form a 2.5 cm long plateau, which is allowed barely to polymerize with the aid of a hot fan; over this is cast the desired Immobiline gradient, of standard 10 cm length. Over the latter, the remaining light, basic solution is now gently floated to form a 2.5 cm long cathodic plateau. The entire assembly is then polymerized under standard conditions, in the oven at 50 C for 60 min. During the run, the anionic and cationic components of the salts in the sample will be seen as refractive lines moving out of the separation gel and collecting in the two plateaux at the electrodes. At this point, one can remove them by excision and repositioning of the electrode wires, thus continuing the experiment in a salt-free environment. Another reason for using pH plateaux, in addition to salt collection, is for loading the sample at a favorable pH, especially when using ultranarrow pH gradients. E.g., when running very alkaline or acidic pH gradients, the sample is loaded in a lower , or higher, pH plateau, respectively, so as to minimize sample loss due to harsh pH environments and to increase its net charge for rapid migration into the real separation gel [11, 12].

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Porosity plateaux were first described by Righetti and Gelfi [18] for strenghtening the walls of the sample application trench during preparative runs in 'soft' polyacrylamide gels. As described in section IX, low %T gels (e.g. 2.5 to 3%T) afford much higher sample loads than standard 5 or 6%T matrices. However, in such soft and thick gels, the wall of the trench will collapse inhibiting the sample application to the matrix. Thus, all the gel layer around the trench was cast as a more robust, 5%T zone, which also contained a more acidic pH plateau and a double ionic strength plateau to enhance the protein mobility in the loading zone. Even though porosity plateaux have been described only for this purpose, they can be utilized as sieves to eliminate some unwanted, higher Mr species, present in the sample mixture: under these conditions, a run with two simultaneous separation criteria, i.e. a mass coupled to a charge fractionation, would be carried out.

Affinity ligand plateaux have also been recently described [19]: they are very useful in coupling two independent separation parameters, i.e. an affinity pre-purification step, based on a specific biological activity, followed by a charge fractionation in the IPG matrix proper. We have used such affinity plateaux to specifically sequester human albumin in serum analysis by trapping Blue Dextran in the cathodic pH shelf containing the sample application pocket. At 1% concentration, practically all albumin is sequestered from the sample under analysis. In this example, the immobilization of the ligands is simple, due to their entrapment in the gel fibers, caused by their extremely large size; in other cases, the ligand will have to be bonded to the 'affinity shelf', just like the Immobiline chemicals are.

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IV. Narrow and ultranarrow pH gradients

We define these as gradients from a minimum of 0.1 to a maximum of 1 pH unit. Within these limits, in general, we work on a 'tandem' principle, i.e. we choose a 'buffering' Immobiline (e.g. a base or an acid), having its pK within the pH interval we want to generate, and a 'non-buffering' Immobiline (e.g. an acid or a base, respectively), having its pK at least 2 pH units removed from either pHmin or pHmax of our pH range, which will provide equivalents of acid or base, respectively, to titrate the buffering group but will not itself buffer in the desired pH interval. For these calculations, we used to resort to modified Henderson-Hasselbalch equations [1] and to rather complex nomograms found in the LKB Application note No. 321. Today, LKB Note No. 324 lists 58, 1 pH unit wide gradients, starting with the pH 3.8-4.8 interval and ending with the pH 9.5-10.5 span, separated by 0.1 pH unit increments. In Table II are the relative recipes giving the Immobiline volumes (for 15 ml of mixture) needed in the acidic (mixing) chamber to obtain pHmin and the corresponding volumes for the basic (reservoir) chamber of the gradient mixer needed to generate pHmax of the desired pH interval. Only between pH 4.6-5.1 (pHmin) and 7.2-7.4 (pHmin) we work on the troika principle, as in these regions there are wide gaps in the pKs of neighbouring Immobilines. Let us take the pH 4.6-5.6 interval: there are no available Immobilines with pKs within this pH region, so the nearest species, pKs 4.6 and 6.2 will act both as partial buffers and partial titrants and then a third Immobiline is needed in each vessel, a true titrant which will bring the pH to the desired value (for pHmin we will use pK 3.6 and for pHmax pK 9.3 as titrant, respectively).

If a narrower pH gradient is needed, this can be derived from any of the 58 pH intervals tabulated by a simple linear interpolation of

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TABLE II

1-pH Unit Gradients: Volumes of Immobiline for 15 ml Each of Starting Solution (2 gels)

Control pH at 20°C	Volume (µl) 0.2 M Immobiline pK Acidic dense solution 3.6	4.4	4.6	6.2	7.0	8.5	9.3	pH range	mid point	Control pH at 20°C	Volume (µl) 0.2 M Immobiline pK Basic light solution 3.6	4.4	4.6	6.2	7.0	8.5	9.3
3.84±0.03	–	750	–	–	–	–	159	3.8– 4.8	4.3	4.95±0.06	–	750	–	–	–	–	591
3.94±0.03	–	710	–	–	–	–	180	3.9– 4.9	4.4	5.04±0.07	–	810	–	–	–	–	667
4.03±0.03	–	–	755	–	–	–	157	4.0– 5.0	4.5	5.14±0.06	–	–	745	–	–	–	584
4.13±0.03	–	–	713	–	–	–	177	4.1– 5.1	4.6	5.23±0.07	–	–	803	–	–	–	659
4.22±0.03	–	–	689	–	–	–	203	4.2– 5.2	4.7	5.33±0.08	–	–	884	–	–	–	753
4.32±0.03	–	–	682	–	–	–	235	4.3– 5.3	4.8	5.42±0.10	–	–	992	–	–	–	871
4.42±0.03	–	–	691	–	–	–	275	4.4– 5.4	4.9	5.52±0.12	–	–	1133	–	–	–	1021
4.51±0.04	–	–	716	–	–	–	325	4.5– 5.5	5.0	5.61±0.14	–	–	1314	–	–	–	1208
4.64±0.05	562	–	600	863	–	–	–	4.6– 5.6	5.1	5.69±0.04	–	–	863	863	–	–	105
4.75±0.05	458	–	675	863	–	–	–	4.7– 5.7	5.2	5.79±0.04	–	–	863	863	–	–	150
4.86±0.04	352	–	750	863	–	–	–	4.8– 5.8	5.3	5.90±0.04	–	–	863	863	–	–	202
4.96±0.03	218	–	863	863	–	–	–	4.9– 5.9	5.4	5.99±0.03	–	–	863	863	–	–	248
5.07±0.03	158	–	863	863	–	–	–	5.0– 6.0	5.5	6.09±0.04	–	–	863	803	–	–	338
5.17±0.04	113	–	863	863	–	–	–	5.1– 6.1	5.6	6.20±0.04	–	–	863	713	–	–	443
5.24±0.18	1251	–	–	1355	–	–	–	5.2– 6.2	5.7	6.34±0.04	337	–	–	724	–	–	–
5.33±0.12	1055	–	–	1165	–	–	–	5.3– 6.3	5.8	6.43±0.03	284	–	–	694	–	–	–
5.43±0.12	899	–	–	1017	–	–	–	5.4– 6.4	5.9	6.53±0.03	242	–	–	682	–	–	–
5.52±0.09	775	–	–	903	–	–	–	5.5– 6.5	6.0	6.63±0.03	209	–	–	686	–	–	–
5.62±0.07	676	–	–	817	–	–	–	5.6– 6.6	6.1	6.73±0.03	182	–	–	707	–	–	–
5.71±0.06	598	–	–	755	–	–	–	5.7– 6.7	6.2	6.82±0.03	161	–	–	745	–	–	–
5.81±0.06	536	–	–	713	–	–	–	5.8– 6.8	6.3	6.92±0.03	144	–	–	803	–	–	–
5.91±0.05	486	–	–	689	–	–	–	5.9– 6.9	6.4	7.02±0.03	131	–	–	884	–	–	–
6.01±0.05	447	–	–	682	–	–	–	6.0– 7.0	6.5	7.12±0.03	120	–	–	992	–	–	–
6.10±0.04	416	–	–	691	–	–	–	6.1– 7.1	6.6	7.22±0.03	112	–	–	1133	–	–	–
6.11±0.11	972	–	–	–	1086	–	–	6.2– 7.2	6.7	7.21±0.03	262	–	–	–	686	–	–
6.21±0.09	833	–	–	–	956	–	–	6.3– 7.3	6.8	7.31±0.03	224	–	–	–	682	–	–
6.30±0.08	722	–	–	–	857	–	–	6.4– 7.4	6.9	7.41±0.03	195	–	–	–	694	–	–
6.40±0.07	635	–	–	–	783	–	–	6.5– 7.5	7.0	7.50±0.03	171	–	–	–	724	–	–
6.49±0.06	565	–	–	–	732	–	–	6.6– 7.6	7.1	7.60±0.03	152	–	–	–	771	–	–
6.59±0.05	509	–	–	–	699	–	–	6.7– 7.7	7.2	7.70±0.03	137	–	–	–	840	–	–
6.69±0.05	465	–	–	–	683	–	–	6.8– 7.8	7.3	7.80±0.03	125	–	–	–	934	–	–
6.78±0.04	430	–	–	–	684	–	–	6.9– 7.9	7.4	7.90±0.03	116	–	–	–	1058	–	–
6.88±0.04	403	–	–	–	701	–	–	7.0– 8.0	7.5	8.00±0.03	108	–	–	–	1217	–	–
6.98±0.04	381	–	–	–	736	–	–	7.1– 8.1	7.6	8.09±0.03	103	–	–	–	1422	–	–
7.21±0.06	1028	–	–	–	750	750	–	7.2– 8.2	7.7	8.36±0.05	548	–	–	–	750	750	–
7.31±0.06	983	–	–	–	750	750	–	7.3– 8.3	7.8	8.46±0.05	503	–	–	–	750	750	–
7.41±0.05	938	–	–	–	750	750	–	7.4– 8.4	7.9	8.56±0.05	458	–	–	–	750	750	–
7.66±0.15	1230	–	–	–	–	1334	–	7.5– 8.5	8.0	8.76±0.04	331	–	–	–	–	720	–
7.75±0.12	1037	–	–	–	–	1149	–	7.6– 8.6	8.1	8.85±0.03	279	–	–	–	–	692	–
7.85±0.10	885	–	–	–	–	1004	–	7.7– 8.7	8.2	8.95±0.03	238	–	–	–	–	682	–
7.94±0.08	764	–	–	–	–	893	–	7.8– 8.8	8.3	9.05±0.06	206	–	–	–	–	687	–
8.04±0.07	667	–	–	–	–	810	–	7.9– 8.9	8.4	9.14±0.06	180	–	–	–	–	710	–
8.13±0.06	591	–	–	–	–	750	–	8.0– 9.0	8.5	9.24±0.06	159	–	–	–	–	750	–
8.23±0.06	530	–	–	–	–	710	–	8.1– 9.1	8.6	9.34±0.06	143	–	–	–	–	810	–
8.33±0.05	482	–	–	–	–	687	–	8.2– 9.2	8.7	9.44±0.06	130	–	–	–	–	893	–
8.43±0.04	443	–	–	–	–	682	–	8.3– 9.3	8.8	9.54±0.06	119	–	–	–	–	1004	–
8.52±0.04	413	–	–	–	–	692	–	8.4– 9.4	8.9	9.64±0.06	111	–	–	–	–	1149	–
8.62±0.04	389	–	–	–	–	720	–	8.5– 9.5	9.0	9.74±0.06	105	–	–	–	–	1334	–
8.40±0.14	1208	–	–	–	–	–	1314	8.6– 9.6	9.1	9.50±0.06	325	–	–	–	–	–	716
8.49±0.12	1021	–	–	–	–	–	1133	8.7– 9.7	9.2	9.59±0.06	275	–	–	–	–	–	691
8.59±0.10	871	–	–	–	–	–	992	8.8– 9.8	9.3	9.69±0.06	235	–	–	–	–	–	682
8.68±0.08	753	–	–	–	–	–	884	8.9– 9.9	9.4	9.79±0.06	203	–	–	–	–	–	689
8.78±0.07	659	–	–	–	–	–	803	9.0–10.0	9.5	9.88±0.06	177	–	–	–	–	–	713
8.87±0.06	584	–	–	–	–	–	745	9.1–10.1	9.6	9.98±0.06	157	–	–	–	–	–	755
8.97±0.05	525	–	–	–	–	–	707	9.2–10.2	9.7	10.08±0.06	141	–	–	–	–	–	817
9.07±0.04	478	–	–	–	–	–	686	9.3–10.3	9.8	10.18±0.06	129	–	–	–	–	–	903
9.16±0.07	440	–	–	–	–	–	682	9.4–10.4	9.9	10.28±0.06	119	–	–	–	–	–	1017
9.26±0.07	410	–	–	–	–	–	694	9.5–10.5	10.0	10.38±0.06	111	–	–	–	–	–	1165

From LKB Application Note No. 324 (1984). The pH range (given in the middle two columns) is the one existing in the gel during the run at 20°C. For controlling the pH of the starting solutions, the values (control pH) are given at 20°C.

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intermediate Immobiline molarities. Suppose that from a pH 6.8-7.8 range [excellent for most hemoglobin (Hb) analyses] we want to obtain a pH gradient of 7.1 to 7.5 (this will resolve neutral mutants which cofocus with HbA) [20]. Fig. 5 shows the graphic method: the limiting molarities of the two Immobilines in the 1 pH unit interval are joined by a straight line (because the pouring of the gradient is done linearly) and then the new pH interval is defined according to experimental needs (in our case, pH 7.1-7.5). Two lines are drawn from the two new limits of the pH interval, parallel to the ordinates (broken vertical lines). Where they intersect the two sloping lines defining the two Immobiline molarities, four new lines (dashed) are drawn parallel to the abscissa and four new molarities of the Immobilines defining the new pH interval are read directly on the ordinates. This process can be repeated for any desired pH interval down to ranges as narrow as 0.1 pH units. From this example, it is clear that the number of ultranarrow pH gradients which can be created is almost endless, as any starting and ending point can be choosen along the pH scale. This sharply contrasts with CA-IEF, where barely a hadful of prepared pH cuts are available: the reason lies again in the principle of continuous titration, operative in IPGs, vs. the discontinuous chemical synthesis of buffers obtainable in CA-IEF. Thus, it is only with IPGs that a true 'pH-gradient-engineering' could be achieved and made available to the scientific community to tailor to any possible separation problem based on surface charge differences.

V. Extended pH gradients: linear and non-linear formulations

For creating extended pH intervals, several buffering species have to be mixed, and the situation becomes considerably more complex. This has been solved with the aid of computer programs designed specifically for

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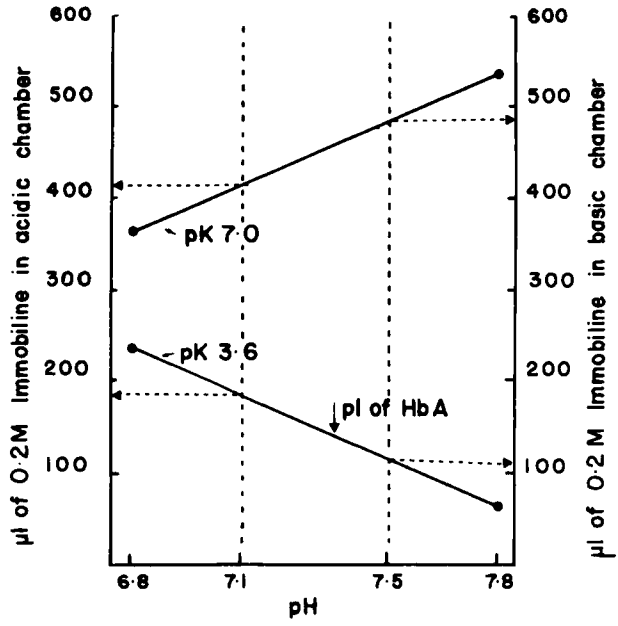

FIGURE 5

Graphic representation of the preparation of narrow (up to 1 pH unit) IPG gradients on the 'tandem' principle. The limiting molarities of pK 7.0 (buffering species) and pK 3.6 (titrant) Immobilines needed to generate a pH 6.8-7.8 interval, as obtained directly from Table II, are plotted in a graph. These points are joined by straight lines and the new molarities needed to generate any narrower pH gradient within the stated pH intervals are then obtained by simple linear interpolation (broken vertical and horizontal lines). In this example, a narrow pH 7.1-7.5 gradient is graphically derived (from Rochette et al., ref. 20).

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the purpose [21-23]. The basic findings are: a) for generating a linear pH gradient the buffering power has to be constant throughout the desired pH interval; b) to avoid deviations from linearity, the titrants should have pKs well outside pHmin and pHmax of the wanted pH range (in general, at least 2 pH units removed from the limits of the pH interval). As a consequence of this, for pH ranges wider than 3 pH units, two additional Immobilines are preferred as titrants: one strongly acidic (pK<1) and one strongly basic (pK>12), as discussed in Section II.

In Table III are recipes for 2- and 3-pH unit wide gradients whose intervals can be generated without the aid of strongly acidic and basic titrants. We had then computed the remaining 4, 5 and 6-pH unit wide IPG gradients needed to complete these formulations. However, these recipes, in order to obtain the best possible linearity, contained the two strongly dissociated titrants, which are not commercially available. In order to avoid confusion, we have recalculated and listed in Table IV (A to F) all gradients between 4 and 6 pH units, with formulations which exclude the two strong titrants and utilize only 6 commercially available Immobilines (out of seven, since the pK 4.4 and 4.6 species should never be mixed together in extended IPG recipes, as they will locally alter the pH profile by providing too much buffering power; in all these recipes, only the pK 4.6 has been used). In entries G and H, formulations are given for the widest possible IPG span, a pH 3-10 interval, prepared with a two chamber mixer either according to the 'same concentration' principle (G, by that we mean that all the six buffering Immobilines are present in the same molarities in the two chambers) or according to the 'different concentration' approach (H, where, in addition to the titrants, also the six buffering Immobilines

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TABLE III

Broad pH Gradients: Volumes of Immobiline for 15 ml of Each Starting Solution

CONTROL pH AT 20°C	VOLUME (µl) 0.2 *M* IMMOBILINE pK ACIDIC DENSE SOLUTION							pH RANGE	MID POINT	CONTROL pH AT 20°C	VOLUME (µl) 0.2 *M* IMMOBILINE pK BASIC LIGHT SOLUTION						
	3.6	4.4	4.6	6.2	7.0	8.5	9.3				3.6	4.4	4.6	6.2	7.0	8.5	9.3
3.53 ± 0.06	299	—	223	157	—	—	—	3.5– 5.0	4.25	5.06 ± 0.07	212	—	310	465	—	—	—
4.00 ± 0.06	569	—	99	439	—	—	—	4.0– 6.0	5.0	6.09 ± 0.14	390	—	521	276	—	—	722
4.54 ± 0.06	415	—	240	499	—	—	—	4.5– 6.5	5.5	6.53 ± 0.05	—	—	570	244	235	—	297
5.08 ± 0.03	69	—	428	414	—	—	—	5.0– 7.0	6.0	7.01 ± 0.06	—	—	474	270	219	—	320
5.56 ± 0.03	—	—	450	354	113	—	—	5.5– 7.5	6.5	7.51 ± 0.09	347	—	—	236	287	284	—
6.06 ± 0.08	435	—	—	323	208	44	—	6.0– 8.0	7.0	8.11 ± 0.09	286	—	—	174	325	329	—
6.56 ± 0.13	771	—	—	276	185	538	—	6.5– 8.5	7.5	8.66 ± 0.06	192	—	—	153	278	362	—
7.03 ± 0.24	1349	—	—	—	272	372	845	7.0– 9.0	8.0	8.94 ± 0.07	484	—	—	—	232	189	546
7.50 ± 0.11	668	—	—	—	445	226	348	7.5– 9.5	8.5	9.37 ± 0.06	207	—	—	—	925	139	346
8.10 ± 0.07	399	—	—	—	364	355	94	8.0–10.0	9.0	9.89 ± 0.05	91	—	—	—	329	366	289
4.01 ± 0.05	578	—	110	450	—	—	—	4.0– 7.0	5.5	7.02 ± 0.14	302	—	738	151	269	—	876
5.03 ± 0.12	702	—	254	416	133	346	—	5.0– 8.0	6.5	8.12 ± 0.07	175	—	123	131	345	346	—
6.04 ± 0.14	779	—	—	402	93	364	80	6.0– 9.0	7.5	9.01 ± 0.06	241	—	—	161	449	237	225
6.98 ± 0.07	542	—	—	—	378	351	—	7.0–10.0	8.5	9.88 ± 0.05	90	—	—	—	324	350	280

From LKB Application Note No. 324 (1984). See also footnote to Table II.

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4,5 and 6-pH-Unit-Wide Gradients Prepared with Only Six Available Immobilines

A 4–8, NO TITRANTS

Initial pH : *4.090*
Final pH : *8.010*
Notes : *pH in solution at 25°C: 4.12–7.97*
Buffer concentrations

pK	3.57	Cham 1	7.850	Cham 2	0.000
pK	4.51	Cham 1	3.380	Cham 2	7.384
pK	6.21	Cham 1	3.136	Cham 2	4.792
pK	7.06	Cham 1	1.564	Cham 2	1.894
pK	8.50	Cham 1	2.261	Cham 2	4.454
pK	9.59	Cham 1	0.000	Cham 2	3.838

B 5–9, NO TITRANTS

Initial pH : *5.060*
Final pH : *9.040*
Notes : *pH in solution at 25°C: 5.13–8.91*
Buffer concentrations

pK	3.57	Cham 1	11.059	Cham 2	0.000
pK	4.51	Cham 1	7.759	Cham 2	3.317
pK	6.21	Cham 1	2.903	Cham 2	3.510
pK	7.06	Cham 1	1.835	Cham 2	2.833
pK	8.50	Cham 1	10.601	Cham 2	3.892
pK	9.59	Cham 1	1.630	Cham 2	3.071

C 6–10, NO TITRANTS

Initial pH : *5.980*
Final pH : *10.000*
Notes : *pH in solution at 25°C: 5.97–9.72*
Buffer concentrations

pK	3.57	Cham 1	12.546	Cham 2	1.329
pK	6.21	Cham 1	3.634	Cham 2	4.446
pK	7.06	Cham 1	3.237	Cham 2	4.813
pK	8.50	Cham 1	3.471	Cham 2	3.185
pK	9.59	Cham 1	3.761	Cham 2	4.341

D 4–9, NO TITRANTS

Initial pH : *4.140*
Final pH : *8.920*
Notes : *pH in solution at 25°C: 4.18–8.65*
Buffer concentrations

pK	3.57	Cham 1	11.048	Cham 2	1.964
pK	4.51	Cham 1	3.138	Cham 2	5.656
pK	6.21	Cham 1	3.092	Cham 2	4.801
pK	7.06	Cham 1	0.298	Cham 2	3.952
pK	8.50	Cham 1	3.336	Cham 2	0.942
pK	9.59	Cham 1	2.951	Cham 2	8.834

E 5–10, NO TITRANTS

Initial pH : *5.040*
Final pH : *10.040*
Notes : *pH in solution at 25°C: 5.11–9.79*
Buffer concentrations

pK	3.57	Cham 1	7.505	Cham 2	0.286
pK	4.51	Cham 1	6.178	Cham 2	0.788
pK	6.21	Cham 1	3.970	Cham 2	0.447
pK	7.06	Cham 1	3.646	Cham 2	5.593
pK	8.50	Cham 1	3.027	Cham 2	4.138
pK	9.59	Cham 1	1.689	Cham 2	3.637

F 4–10, NO TITRANTS

Initial pH : *4.150*
Final pH : *9.950*
Notes : *pH in solution at 25°C: 4.16–9.66*
Buffer concentrations

pK	3.57	Cham 1	14.699	Cham 2	0.000
pK	4.51	Cham 1	0.000	Cham 2	1.523
pK	6.21	Cham 1	6.073	Cham 2	0.667
pK	7.06	Cham 1	1.187	Cham 2	6.503
pK	8.50	Cham 1	4.452	Cham 2	2.089
pK	9.59	Cham 1	0.000	Cham 2	4.763

G 3–10, SAME CONCENTRATION

Initial pH : *3.000*
Final pH : *10.000*
Notes : *pH in solution at 25°C: 3.02–9.75 (mixture: 7.76)*
Buffer concentrations

pK	0.80	Cham 1	14.211	Cham 2	0.000
pK	3.57	Cham 1	4.137	Cham 2	4.137
pK	4.51	Cham 1	3.764	Cham 2	3.764
pK	6.21	Cham 1	4.017	Cham 2	4.017
pK	7.06	Cham 1	3.041	Cham 2	3.041
pK	8.50	Cham 1	3.780	Cham 2	3.780
pK	9.59	Cham 1	4.278	Cham 2	4.278
pK	12.00	Cham 1	0.000	Cham 2	6.647

H 5–10, DIFFERENT CONCENTRATIONS

Initial pH : *2.970*
Final pH : *9.960*
Notes : *pH in solution at 25°C: 3.00–9.66*
Buffer concentrations

pK	0.80	Cham 1	17.083	Cham 2	0.000
pK	3.57	Cham 1	2.252	Cham 2	0.000
pK	4.51	Cham 1	5.013	Cham 2	2.596
pK	6.21	Cham 1	6.688	Cham 2	0.000
pK	7.06	Cham 1	0.000	Cham 2	7.115
pK	8.50	Cham 1	6.931	Cham 2	2.072
pK	9.59	Cham 1	3.946	Cham 2	5.899
pK	12.00	Cham 1	0.000	Cham 2	0.747

Nonlinear 4–10 Immobilized pH Gradient

"IDEAL" 4–10					
Initial pH			: *4.190*		
Final pH			: *9.980*		
Notes			: *pH in solution at 25°C: 4.24–9.70*		
Buffer concentrations			*(no titrants)*		
pK	3.57	Cham 1	9.321	Cham 2	0.577
pK	4.51	Cham 1	4.327	Cham 2	0.000
pK	6.21	Cham 1	8.943	Cham 2	2.596
pK	7.06	Cham 1	0.000	Cham 2	3.173
pK	8.50	Cham 1	0.000	Cham 2	1.154
pK	9.59	Cham 1	0.000	Cham 2	1.846
"IDEAL" 4–10					
Initial pH			: *4.040*		
Final pH			: *9.880*		
Notes			: *pH in solution at 25°C: 4.13–9.61*		
Buffer concentrations			*(with acidic titrant)*		
pK	0.80	Cham 1	7.028	Cham 2	0.910
pK	4.51	Cham 1	7.659	Cham 2	0.000
pK	6.21	Cham 1	9.010	Cham 2	2.703
pK	7.06	Cham 1	0.000	Cham 2	3.604
pK	8.50	Cham 1	0.000	Cham 2	1.352
pK	9.59	Cham 1	0.000	Cham 2	2.523

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are present in totally different molarity ratios in the two chambers). It should be noted, however, that in neither case could such a wide pH range be created without resorting to strong titrants.

Up to now, IPG formulations have been given only in terms of rigorously linear pH gradients. While this has been the only solution adopted so far, it might not be the optimal one in some cases. Altering the pH slope in some portions of the gradient might be required in those pH regions overcrowded with proteins. Table V gives two examples of a non-linear, pH 4–10 interval, obtained either without titrants (upper part) or with only the strongly acidic titrant (lower part). This has been calculated for a general case, e.g. the separation of proteins in a complex mixture, such as a cell lysate. Gianazza and Righetti [24] have computed the statistical distribution of the pI's of water-soluble proteins, given in the histogram shown in Fig. 6 (insert). With the

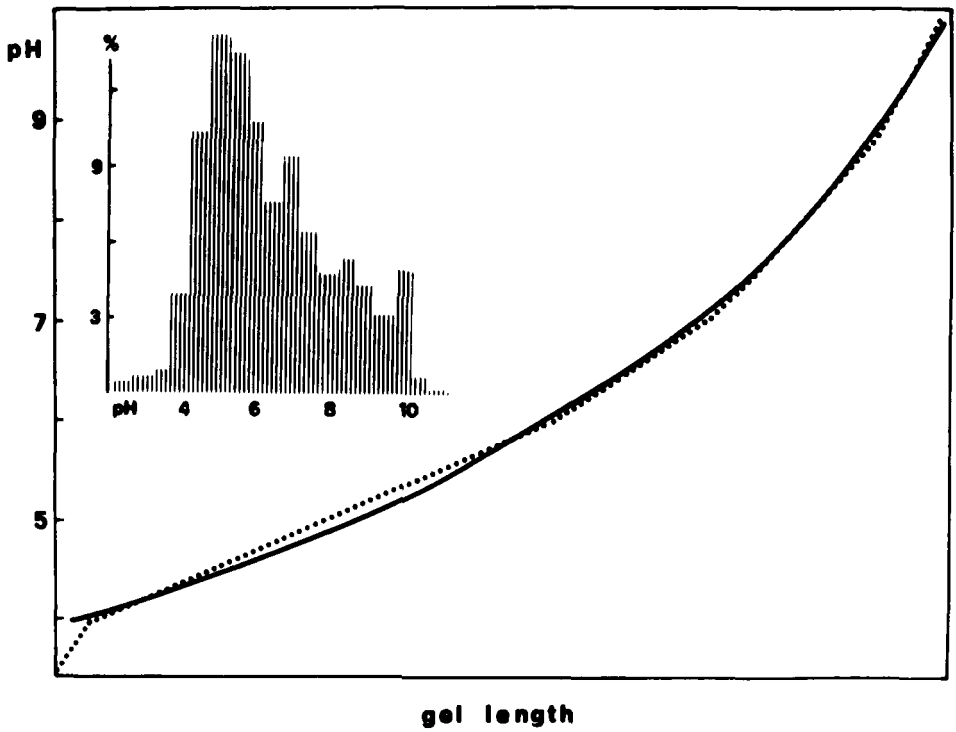

FIGURE 6

Non-linear 4-10 pH gradient: "ideal" (.....) and actual (------, formulation D, including acidic titrant from Table VI) courses. The shape for the "ideal" profile was computed from data on the statistical distribution of protein pIs (Gianazza and Righetti, ref. 24); the relevant histogram is redrawn in the figure inset (from Gianazza et al., ref. 39).

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relative abundance of different species, it is clear that an optimally resolving pH gradient should have a gentler slope in the acidic portion, and a steeper course in the alkaline region. Such a general course has been calculated by assigning to each 0.5 pH unit interval in the pH 3.5-10 region a slope inversely proportional to the relative abundance of proteins in that interval: by such a procedure, the ideal (dotted) curve, Fig. 6 was obtained. Of the two formulations given in Table V, the one including the strongly acidic titrant followed most closely (solid line) the theoretically predicted course. In a separation of a crude lysate of Klebsiella pneumoniae, a great improvement in resolution for the acidic cluster of bands, without loss of the basic portion of the pattern, was obtained [25]. What is also important here is the establishment of a new principle in IPG technology, namely that the pH gradient and the density gradient stabilizing it need not be co-linear, the possibility existing of modulating the former, by localized flattening of pH gradients for increased resolution, while leaving the latter unaltered. Clearly, such non-linear pH gradients are extremely useful as a first dimension of 2-D fractionations, especially when analyzing complex mixtures (e.g., total cell lysates).

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VI. Mixed-bed, Immobiline-carrier ampholyte gels

In 1985, we [26] and others [27, 28] reported the use of mixed-bed, CA-IPG gels, by which the primary, immobilized pH gradients, is admixed with a secondary, soluble carrier ampholyte driven pH gradient. It sounds strange that, given the problems connected with the CA buffers (discontinuities along the electrophoretic path, pH gradient decay, etc.), which the IPG technique was supposed to solve, one should resurrect this past methodology. The reason given to this approach was

for overcoming conductivity minima in IPGs, centred around pH 7, which were supposed to hamper protein focusing [27, 28]. As we were working with membrane proteins [26] from Streptococcus cremoris and later with microvillar hydrolases, partly embedded in biological membranes [29], we gave a completely different explanation, namely that addition of CAs to the sample and IPG gel would increment protein solubility, possibly by forming mixed-micelles with the detergent used for membrane solubilization [26] or by directly complexing with the protein itself [29]. It is a fact that, in the absence of CAs, these same proteins would essentially fail to enter the gel and mostly precipitate or give elongated smears around the application site (in general cathodic sample loading). As the same phenomenon recently happened even with ferritin, a cytoplasmic, freely soluble protein, we were forced to explore in more detail the effect of CAs on IPG matrices and to broaden the meaning of our first explanation [30]. It was found that, on a relative hydrophobicity scale, the four basic Immobilines (pKs 6.2, 7.0, 8.5 and 9.3) are decidedly more hydrophobic that their acidic counterparts (pKs 3.6, 4.4 and 4.6): upon incorporation in the gel matrix, the phenomenon becomes cooperative and could lead to formation of hydrophobic patches on the surface of such a hydrophilic gel as polyacrylamide. As the strength of a hydrophobic interaction is directly proportional to the product of the cavity area times its surface tension, it is clear that experimental conditions which lead to a decrement of molecular contact area axiomatically weaken such interactions. Thus, our original idea of CAs as solubilizing ions in IPG matrics has been extended to the hypothesis of CAs as shielding molecules coating, on the one side, the polyacrylamide matrix studded with Immobilines (especially the basic ones) and, on the other side, the protein itself. This strongly quenches

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the direct hydrophobic protein-IPG matrix interaction, effectively detaches the protein from the surrounding polymer coils and allows good focusing into sharp bands. This phenomenon can be appreciated in Fig. 7: when horse-spleen ferritin is focused alone in an IPG gel, it gives poor smears in the proximity of the application site; however, upon addition of 3 to 4% CAs (1.5 to 2% actual gel concentration, as the IPG matrix covers 1 pH unit interval while the CAs span 2 pH units) a sharp array of ferritin bands is developed in the gel, with essentially no protein remaining in the pocket. For this to happen, the CA shielding species should already be impregnated in the Immobiline gel and present in the sample solution as well. If added afterwards, e.g. by electrophoretic migration from the electrode strips, they will be ineffective as, once the hydrophobic protein-matrix interaction has occurred, the surface which the CAs were supposed to mask will not be available any longer for such shielding action (in other words, CAs can only prevent the phenomenon, cannot cure it a posteriori). The users of the mixed CA-IPG technique should be aware of another fundamental fact: the shielding mechanism is most effective in the unfocused state while, if the protein is applied to a prefocused CA bed, severe precipitation and poor protein migration and banding patterns ensue (see Fig. 8). Two possible explanations to this phenomenon are: (i) in the unfocused state, both CAs and proteins have a maximum of charge: this means a local increase in ionic strength which could shift the equilibrium towards the bound state (CAs coating hydrophobic particles on the protein surface) as higher ionic strengths favor hydrophobic interactions; (ii) prior to focusing, the IPG matrix is still impregnated by salts, which act as counterions to the bound charges, and which cannot be eliminated during the washing step. These salts also increase the local ionic strength and

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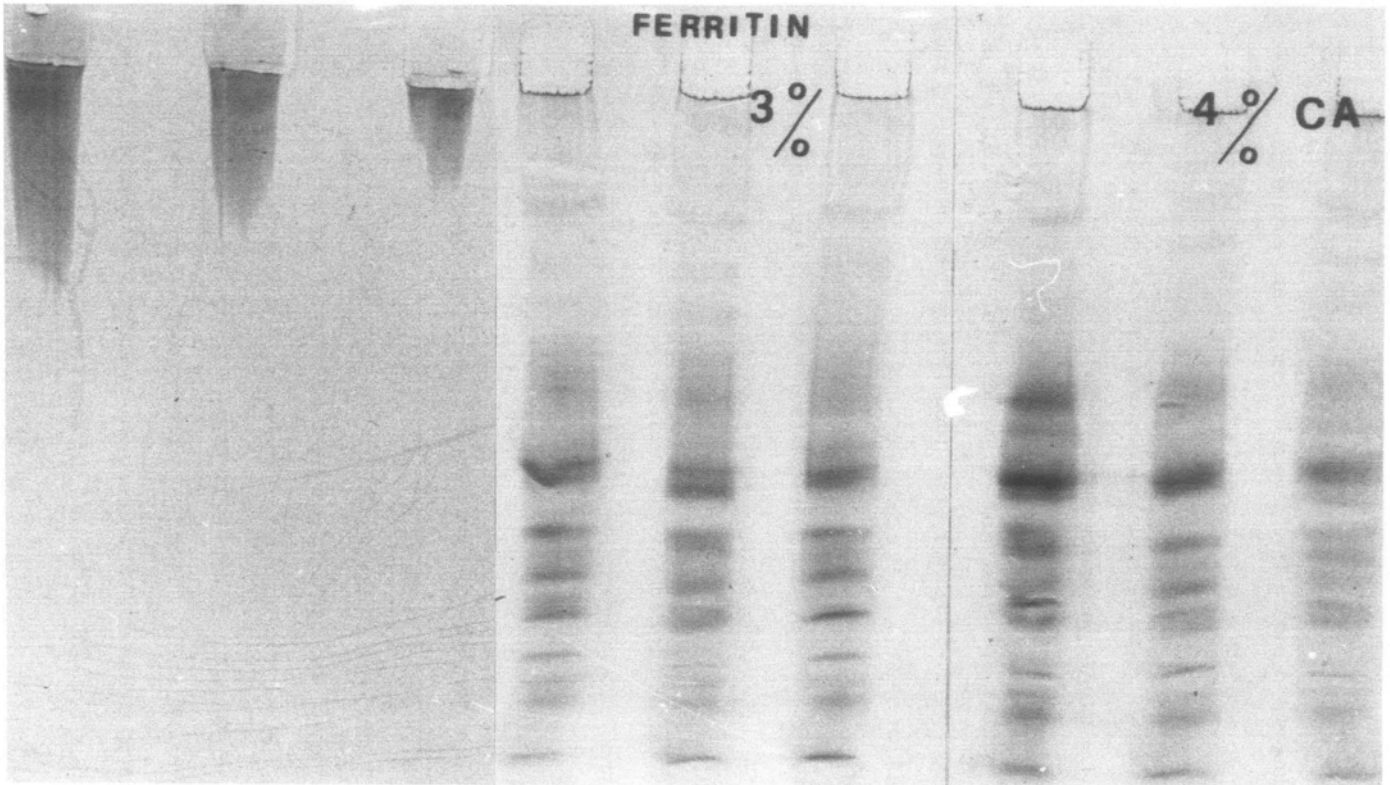

FIGURE 7

Effect of addition of carrier ampholytes on the hydrophobic protein-IPG matrix interaction. (A) 4%T polyacrylamide gel containing a pH 4-6 immobilized gradient in the absence of CAs; (B) 4%T gel containing a pH 4-5 IPG, cut into two halves and impregnated with 3% and 4% carrier ampholytes (CA) in the pH 406 range. Sample load (from left to right in each gel); 200, 150, 100 ug horse spleen ferritin. Focusing overnight at 2000V (final) and 10°C. Staining with Coomassie Blue R-250 (from Righetti et al., see ref. 59.)

could drive the equilibrium towards the formation of CA-protein complexes. Most lickely, the two mechanisms operate simultaneously [31].

A note of caution should be put on the undiscriminate use of the CA-IPG technique. At high CA leves (>1%) and high voltages (>1000 V/10 cm) these gels start exuding water with dissolved carrier ampholytes, with severe risks of short-circuits, sparks and burning on the gel surface. The reason for this is shown in Fig. 9: the array of focused carrier ampholyte species acts like a chain of mini-water pumps, with water uptake upon diffusion away from the pI and water discharge at the pI zone, due to a regain of the state of zero net chage. This results in accumulation of water droplets at the Ampholine pI zone and dehydration on either side of the peak. The phenomenon is minimized by chaotropes (e.g., 8M urea) by polyols (e.g., 30% sucrose) and by lowering the CA molarity in the gel [32]. As an answer to the basic question: when and how much CAs to add, we suggest: (a) if your sample focuses well as such, ignore the mixed-bed technique (which presumably will be mostly needed with hydrophobic proteins and in alkaline pH ranges); (b) add only the minimum amount of CAs (in general around 1%) needed for avoiding sample precipitation in the pocket and for producing sharply focused bands. As a last remark, while the hypothesis of hydrophobic protein-IPG matrix interaction seems the most likely (there are really no conductivity problems in IPG gels!) it cannot be excluded that, for some samples, added CAs might act simply as buffering ions in the bulk water solution preventing protein denaturation due to abrupt pH changes in the sample layer as salt components are split by the current into a strongly alkaline (towards the cathode) and a strongly acidic (towards the anode) boundary.

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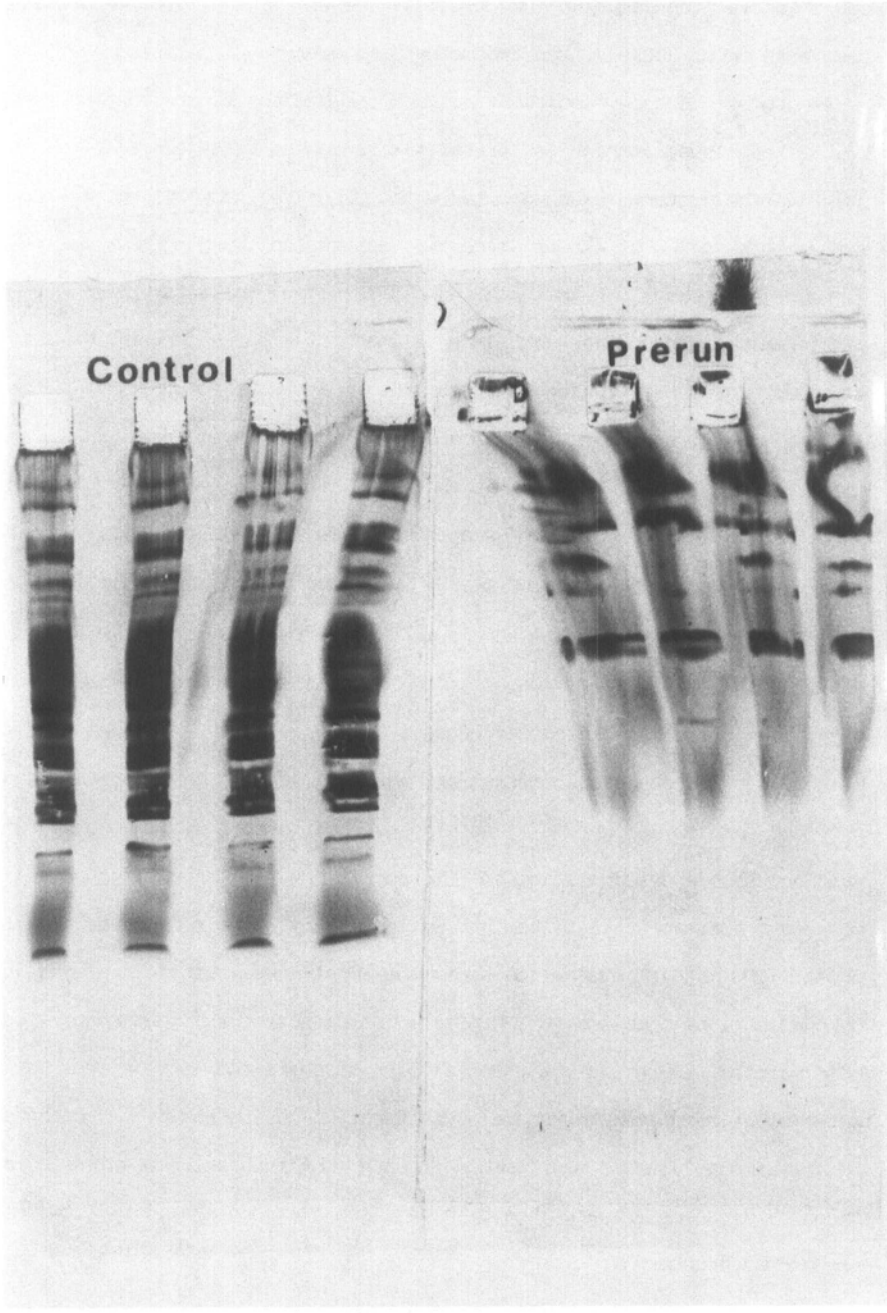

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VII. Focusing at extreme alkaline and acidic pH values

As stated in Section II, when trying to formulate IPG recipes outside the pH 4-10 range, e.g., a more acidic (pH 3-4) or more alkaline (pH 10-11) IPG intervals, one is faced with severe problems, as two new 'Immobilines' become operative, a weak base with a pK=-1.74 and a weak acid with a pK=15.74. They are the H^+ and OH^- ions in equilibrium with undissociated water. It might be argued that their pKs are so remote from the extremes of even the most extended IPG interval (pH 3-11) that they should not alter these pH values. They do, in fact, as there are constantly 55.56 moles of water present throughout the system (hence the provocative concept of water as an Immobiline) vs. 3-10 mM of each buffering Immobiline. The effects of water on the system can be appreciated in Fig. 10: it is seen that already at pH 3 and pH 11 the buffering power of water is non-negligible, while outside these limits it rises so sharply as to nullify any attempt of using IPGs in these regions. In addition to the problem of the buffering power of water,

FIGURE 8

Effect of prefocusing a mixed-bed, CA-IPG gel. The IPG gel contained a pH 4-5 gradient (made with Immobilines of pKs 4.6 and 9.3). 100 µg of ferritin were applied to a prefocused (right side, prerun) or to a control gel in which the protein was focused simultaneously with the background CAs (in the IPG gel at 2% concentration). Note the poor focusing, smearing and sample precipitation in the prefocused gel. More than 50% of the protein sample (loaded in the same amount as the control in the left panel) was lost by precipitation in the pocket, suggesting that the solubilizing power and shielding effect of CAs is maximal only in the unfocused state (from Rabilloud et al., ref. 31).

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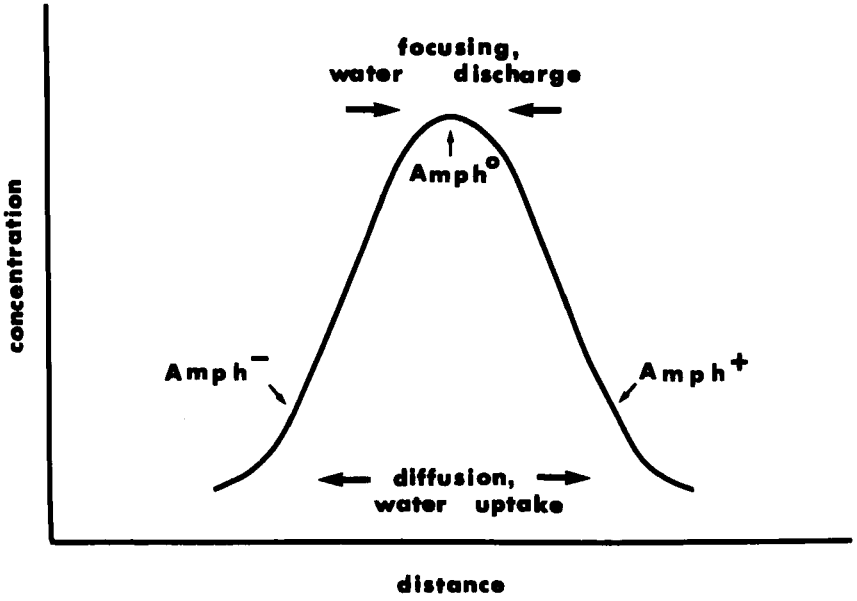

FIGURE 9

Hypothetical model of the concentration distribution of a focused carrier ampholyte. The peak is subjected to two opposite forces: a focusing process, driven by the electric field, and a diffusion process, driven by the absolute CA concentration in the zone. The diffusion event is depicted as a hydration step, as the anions ($Amph^-$) and cations ($Amph^+$) in equilibrium with the isoelectric species ($Amph^\bullet$) acquire more hydration water. The focusing event is represented as a water deposition step at the pI zone (from Astrua-Testori and Righetti, see ref. 32).

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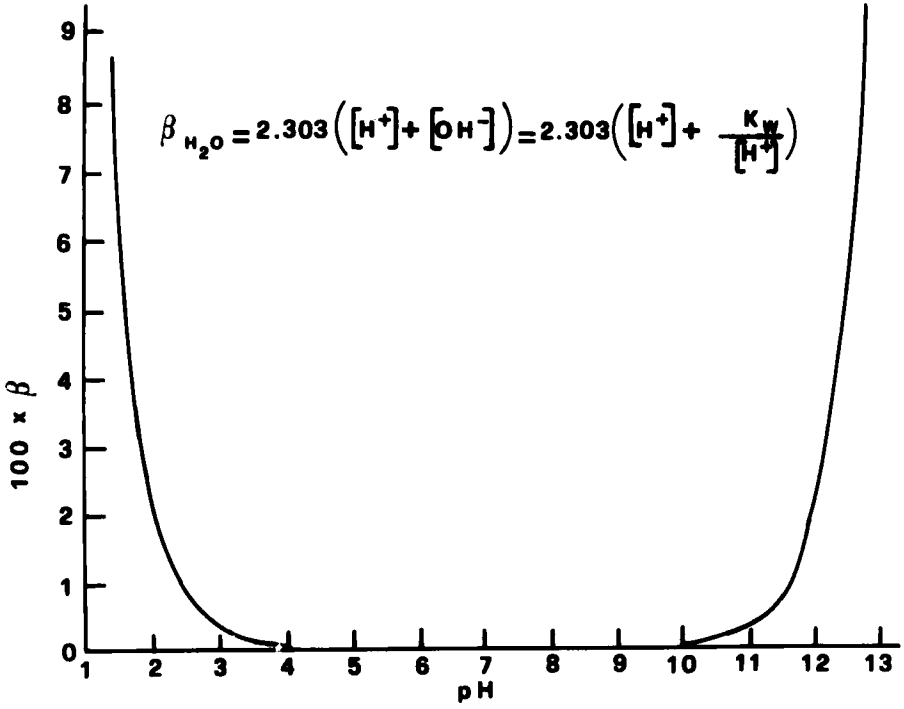

FIGURE 10

Buffering power of water along the pH axis. For calculations, we have taken as pK of the base H^+ the value of -1.74 and as pK of the acid OH^- the value of 15.74 (the molarity of water being 55.56). Kw is the ionic product of water. It is seen that water does not have any appreciable buffering power in the pH 4-10 interval.

there is another serious drawback at using IPGs at these pH extremes: the matrix acquires a net charge, negative at low pH, positive at high pH. E.g., at pH 3 the Immobiline matrix must contain 1 mM extra carboxyls to neutralize the 1 mM protons defining the pH value in the bulk water; at pH 11 it will bear an extra 1 mM positively charged amino

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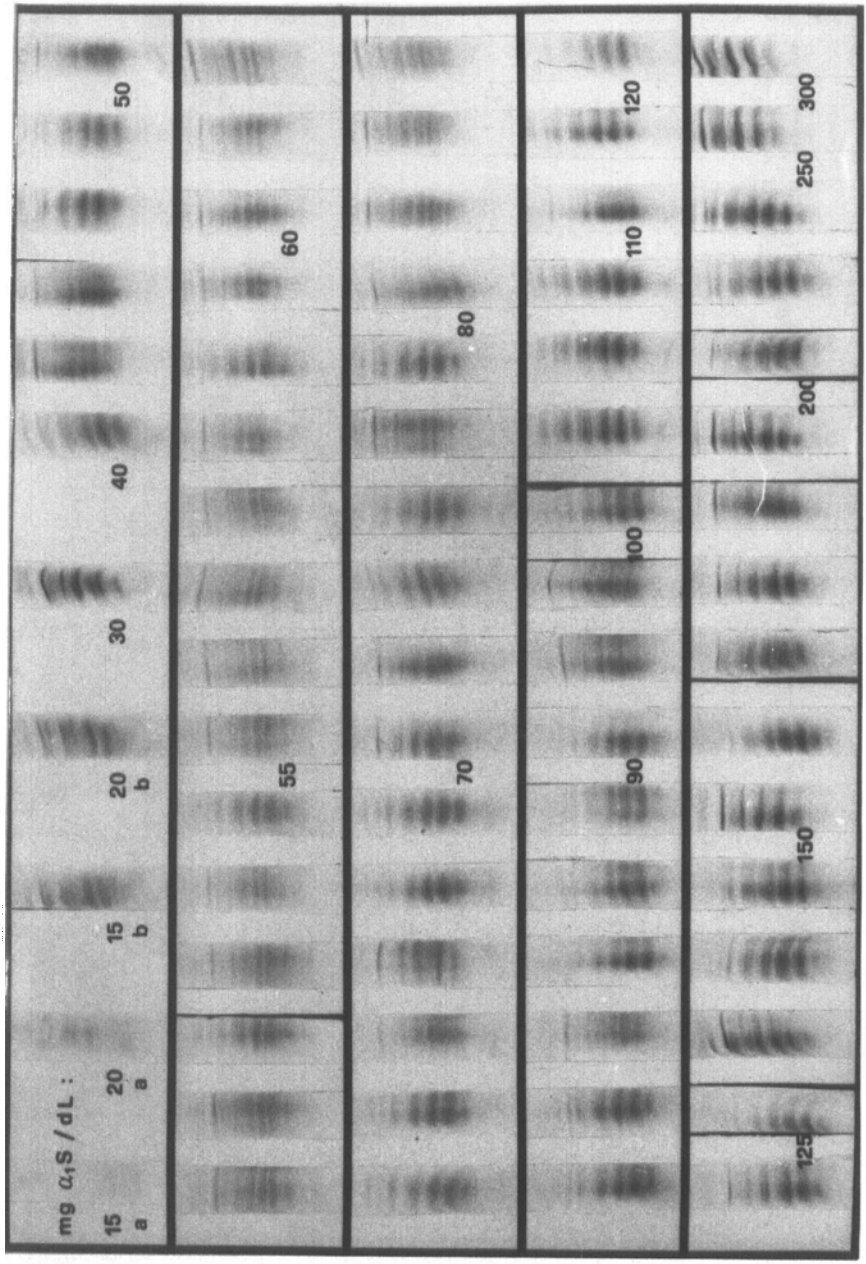

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FIGURE 11

α_1-acidic glycoprotein patterns on 2.8-4.5 IPGs for 73 serum samples, ordered according to their increasing protein titer. The gel was a 3%T, 4%T polyacrylamide matrix, the average buffering power of grafted Immobilines being 9 mequiv./(L.pH). Run: overnight at 200V, then 90 min at 1500V, at 6°C. Staining with Coomassie Brilliant Blue (from Gianazza et al., see ref. 35).

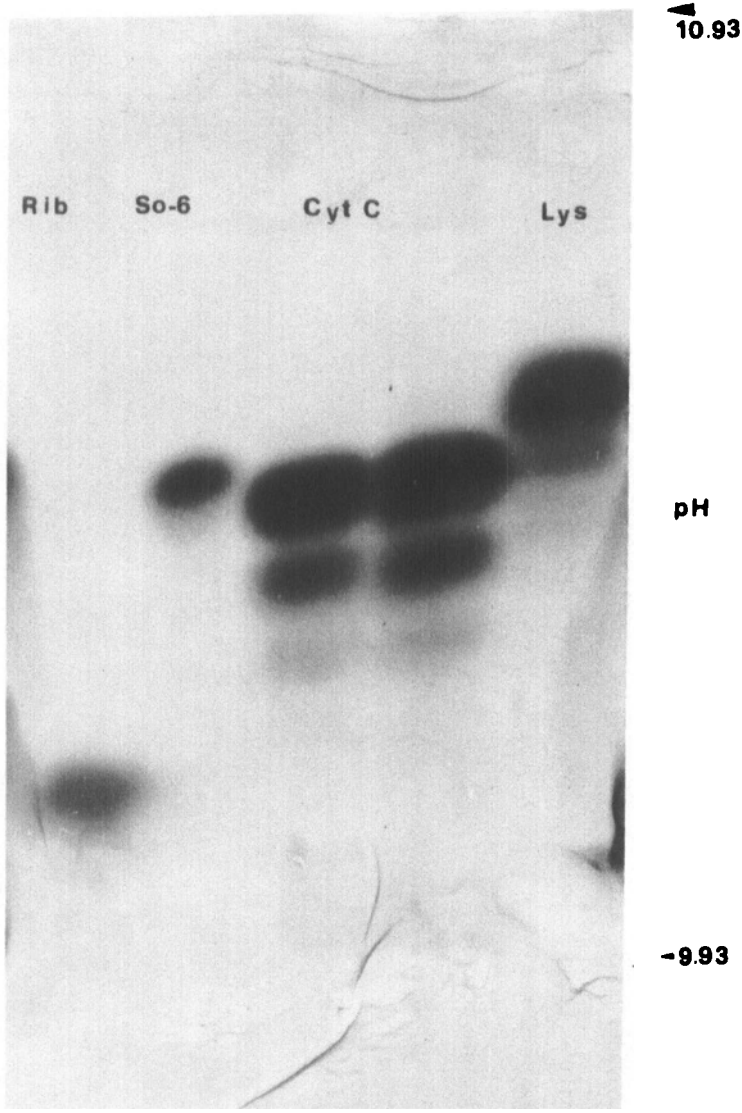

FIGURE 12

IPG separations in an alkaline, pH 10-11, range. A: focusing of alkaline proteins and staining with Coomassie Blue. Rib: ribonuclease; So-6: soluble leaf protein purified from Saponaria officinalis; CytC: cytochrome C; Lys: lysozyme. B: focusing of a proteolytic enzyme

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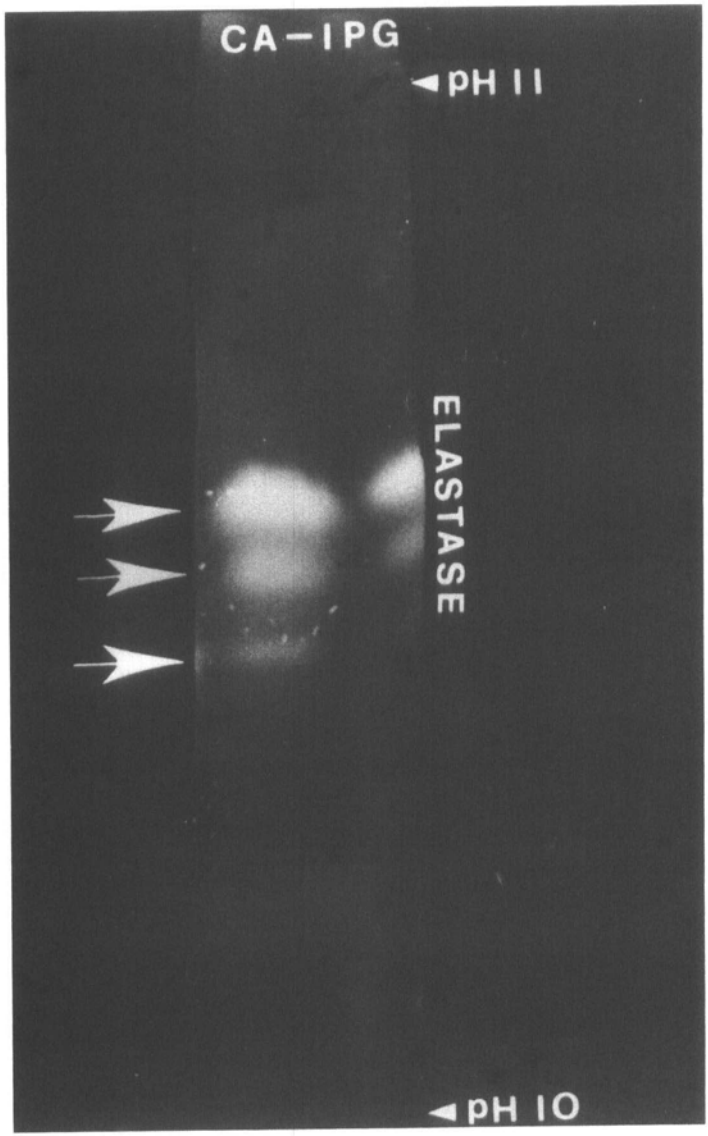

(elastase) followed by *in situ* zymogramming with a cellulose acetate overlay impregnated with a substrate giving a fluorescent product upon hydrolysis. In both cases, the sample was applied in a constant-pH (pH 8) plateau at the anodic side (from Gelfi *et al.*, ref.11 for A and from Sinha and Righetti, ref. 12 for B).

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groups for balancing the 1 mM oxydryls in solution. This excess bound charges will produce a strong electrosmotic flow which will eventually dry out the cathodic and anodic extremes, with consequent burning of the gel matrix. Given these premises, focusing with IPGs at pH extremes would appear unfeasible. On the contrary, we have obtained excellent separations in the pH 3-4 and pH 10-11 ranges by resorting to viscosity gradients (which act as quenchers of conductivity and electrosmosis) incorporated in the gel matrix. These viscosity gradients (in general sucrose or sorbitol) will be oriented so as to have the denser part in the most conducting region of the gel [33, 34]. Fig. 11 shows the separation of α_1-acidic glycoprotein, a human serum protein strongly glycosylated, in patients having normal (40-80 mg/100 ml) or abnormal (100-300 mg/100 ml) levels of this acute phase reactant: 6 to 8 isoforms are well resolved, with different quali- and/or quantitative patterns, with a pI distribution in the pH 3.0-3.8 range [35]. At the other extreme, in the pH 10-11 range, it has been possible to obtain sharply resolved bands of such alkaline proteins as, e.g., cytchrome C and lysozyme, which are in general lost in the cathodic compartment in conventional IEF [11] (Fig. 12A). In addition, it has even been possible to perform zymograms of proteases focused at such alkaline extreme: as shown in Fig. 12B, elastase has been resolved into 3, fully active components, the most alkaline band having a pI=10.70 [12].

VIII. Two dimensional maps

According to Giddings [36], 'two-dimensional (2-D) separations are those techniques in which a sample is subjected to two displacement processes oriented at right angles to one another. The displacement steps carry components from their point of application out into a 2-D

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Type of displacement	Abbreviation		Property controlling displacement
Bulk displacement	BLK	N	Nonselective
Flow	FLO	N	Nonselective
Chromatographic	CHR	K	Partition coefficient
Field flow fractionation	FFF		Field interaction parameter
Electrophoretic	ELP	u	Electrical mobility
Isoelectric	IEL	I	Isoelectric point
Isotachophoretic	ITP	u	Electrical mobility
Dielectrophoretic	DEL	k	Dielectric constant
Sedimentation	SED	s	Sedimentation coefficient
Isopycnic sedimentation	IPY		Density
Magnetic gradient	MAG		Magnetic susceptibility
Thermal diffusion	THD	D'	Thermal diffusion coefficient
Thermogravitational	THG		Thermal diffusion factor
Diffusophoretic	DIF		Interfacial energy
Photophoretic	PHO		Photophoretic mobility

1) From Giddings, see ref. 1.

One-dimensional displacements which might serve as building blocks for 2D separation techniques[1]

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bed where there is more space for their resolution that in one-dimensional (1-D) separation systems'. The high intrinsic resolving power of 2-D separations holds great promise for resolving complex biological samples, for which 1-D methods are not powerful enough. In fact the maximum separation power of 2-D systems is described approximately by the multiplicative law:

$$n_2 \sim n_y n_x \sim n_1^2$$

where the subscripts 2 and 1 refer to 2-D and 1-D values, respectively, and n is the peak capacity, i.e. the maximum number of peaks or zones that will fit into the available separation space. According to Giddings [36], there are at least 15 primary displacements for separation of micro- and macromolecules, based on their different physico-chemical properties: they are listed in Table VI. If we note that there are N^2 combinations for N displacements, this means that the 15 primary separation techniques in Table VI can originate 225 different ways of

performing 2-D maps. Impressive, isn't it? Yes and not. Giddings notices that this is a much too large number and yet it is much too small since many of the 15 primary 1-D principles can be fractionated into sets of subtechniques, which in turn could generate novel 2-D fractionation principles. Thus Giddings thinks of assembling a grand matrix representing as many as 10^4 to 10^6 distinguishable 2-D techniques. The reader will then excuse us if, out of this sesquipedal number, we dare to fish out only a single example, 2-D maps based on charge fractionation (focusing) in the first dimension, followed by a size-dependent analysis (sodium dodecyl sulphate, SDS, electrophoresis) in the second, orthogonal dimension. Such technique, first reported by Klose [37] (IEF-SDS), was subsequently highly refined by the Andersons [38], under the acronym ISO-DALT. In fact, the method we are here outlining is yet another variant, utilizing immobilized pH gradients in the first dimension, and thus publicized under the name IPG-DALT: there are three approaches to this technique, as describd below.

The variant adopted in our lab is illustrated in Fig. 13: the IPG gel slab is usually cut into separate lanes before sample application. This prevents lateral spreading and cross-contamination even when running samples with widely different protein concentration or buffer composition. After the IPG run, the separate gel strips are cut while still bound to the polyester foil: this ensures dimensional stability and thus helps improving spot reproducibility in the 2-D plane. After incubation in the SDS denaturing solution, the vertical gel slab for the 2nd-D (we use a disc buffer, stacking gel and a porosity gradient in the running gel, e.g. 5-17%) is overlaid with 1% melted agarose and the IPG strip immediately transferred and sealed in situ by the gelling agarose. This mixed horizontal-vertical operation mode allows us to run 6-12 SDS

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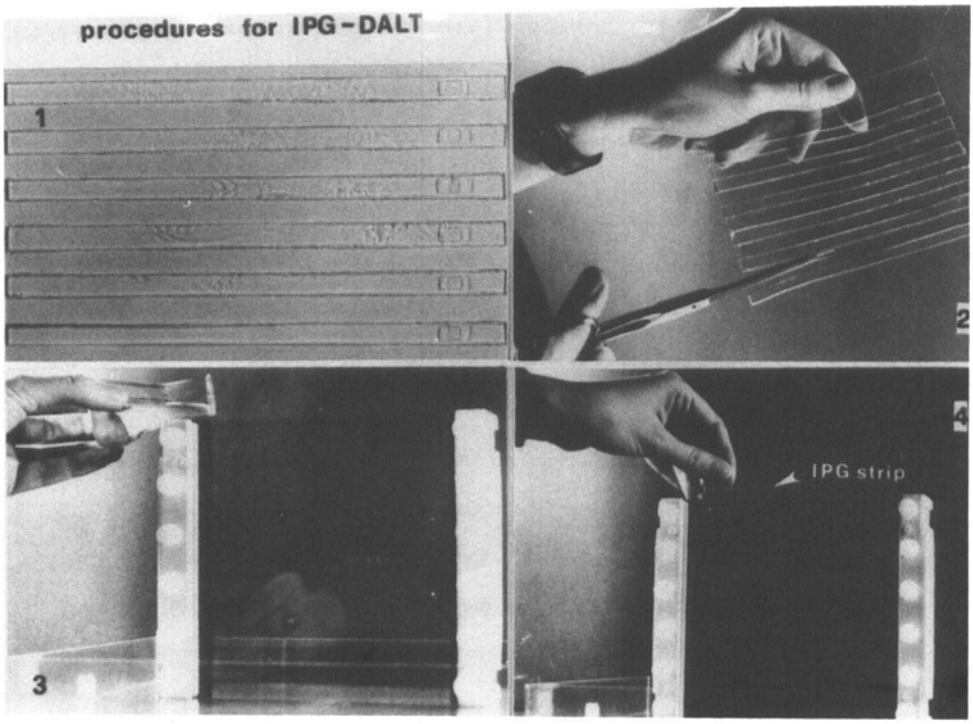

FIGURE 13

Horizontal to vertical transfer in 2-D maps. Upper left: IPG gel slab with pre-cut lanes onto a single plastic (Gel Bond PAG) support; upper right: cutting individual sample strips after the IPg run (the plastic on the contact side with the SDS gel must be cut flash with the supported IPG strip); lower left: pouring melted agarose on the top of the 2nd-D SDS slab; lower right: the equilibrated IPG strip is lowered in situ on the stacking SDS gel before onset of agarose gelation (from ref. 3).

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slabs in the 2nd-D by utilizing large tanks (e.g. the Bio Rad Protean cell). We have utilized this approach for the analysis of serum proteins [39, 41], of myofibrillar proteins [42], of bacterial plasma membranes [25] and of rat erythrocyte ghosts, kidney microvilli and heart biopsies [43]. Two important methodological points: (a) in the first run, for a good transfer of focused proteins to the SDS slab, the IPG matrix should not exceed the standard molarity value of 3 mequiv/(LxpH); (b) complex protein mixtures are best applied in a pocket at the anodic side, so as to minimize hydrophobic interaction with the matrix and severe streaking (see also section VI).

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Fig. 14 shows an alternate protocol developed by Görg's group: the IPG gel on plastic backing is sliced into individual gel strips prior to the run (panel a). The IEF run is thus performed already in separated, individual strips (panel b). After the IPG run and SDS equilibration, the IPG strip is applied to the surface of the horizontal SDS gel without size alteration and no agarose overlay (panel c) [44]. In urea-detergent gels, for avoiding spot streaking, Görg et al. [45] have proposed lowering the amount of Nonidet P-40 from 2% to only 0.5%: this probably minimizes the formation of mixed detergent micelles and helps the detachment of the Nonidet P-40 from the protein coil and its substitution for SDS. In a recent approach, Görg et al. [46], again for minimizing problems of streakings appearing during the silvering procedure, have suggested to add iodoacetoamide to the DTT contained in the SDS-interfacing buffer: the former will react with the excess of thiol-reducing agent, thus eliminating potentially hazardous free -SH groups, which appear to act as units for micro-deposition of silver.

The third approach, adopted by Hochstrasser's group [47] is shown in Fig. 15: it consists in casting IPG gels in narrow glass tubes, instead

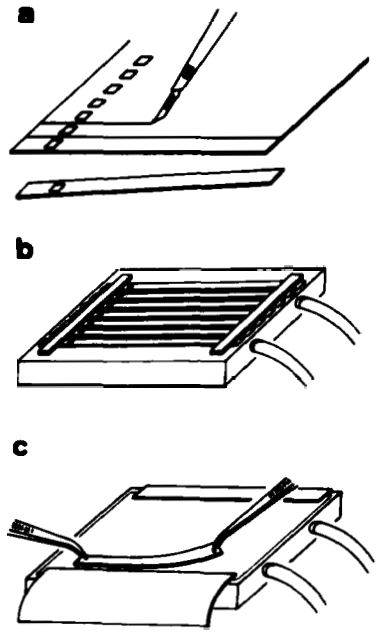

FIGURE 14

Horizontal 2-D electrophoresis with IPGs in the first dimension. The IPG gel on plastic backing is sliced into individual gel strips prior to IEF (a). During the IEF run, single IPG strips are aligned on the cooling block (b). After IEF, the IPG strip is equilibrated and applied to the surface of the horizontal SDS gel with no agarose overlay (c) (from ref. 44).

of the standard flat slab geometry. This approach has been made possible by the discovery of mixed-bed, CA-IPG gels, as reported in Section VI [26]. The mixed-bed method eliminates the need for washing the IPG gel (which, in a cylindrical configuration, could not be washed anyhow as it would have to be extruded from the supporting glass tube); moreover, it

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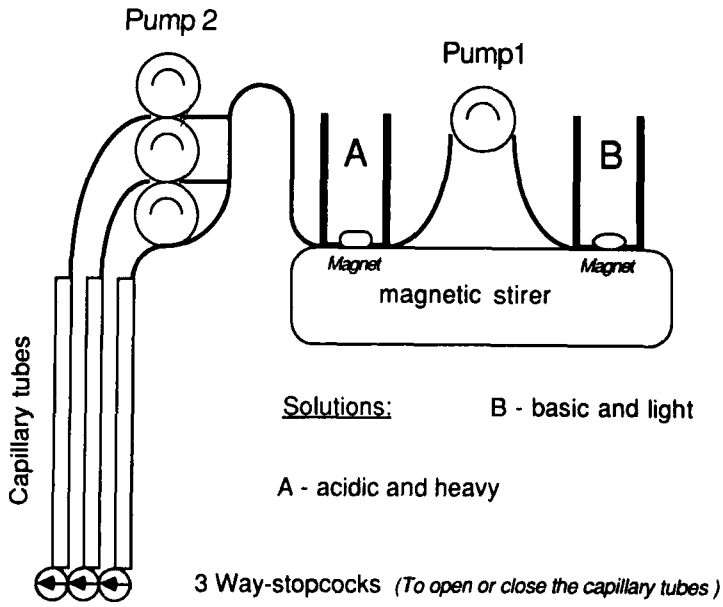

FIGURE 15

Preparation of cylindrical IPG gels. The solution from the reservoir (B) is transferred by pump 1 to the mixing chamber (A). A multichannel pump (Ismatec, Zurich) conveys the gradient solution simultaneously to the top of 20 glass tubes (200 x 1.5 mm i.d.) standing vertically (from ref. 47).

strongly quanches hydrophobic interactions between proteins and the IPG matrix [30, 31]. The resulting serum patterns are quite impressive: according to those authors [47], a properly stained 2-D gel reveals ca. 600 spots from human serum. Fig. 15 shows how the technique is performed: owing to the small volume of each gel (250 µl), 20 tubes have to be filled simultaneously with a multi-channel pump delivering individually the required volume to each tube, from a two-chamber mixing

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device containing only 2.5 ml of each limiting solution. However, it must be underlined that, in this last method [47], due to the fact that the gel is not firmly bound to the glass walls, it tends to stretch or to shrink in presence of increasing amounts of carrier ampholytes, thus loosing the dimensional stability which gives the highly reproducible 2-D patterns so typical of IPG runs.

IX. Preparative aspects

Just as, analytically, IPGs appear to have a resolving power (ΔpI, in pH units) about one order of magnitude higher than conventional IEF (ΔpI=0.001 vs. a ΔpI=0.01 pH unit in CA-IEF) so, preparatively, IPGs seem to afford a comparably higher loading capacity, approaching or even passing the load limit of isotachophoresis [17, 48]. There are a few reasons for that: one is given in Fig. 16: it is seen that the solubility of an isoionic protein, plotted against pH near the isoionic point, is a parabola, with a fairly narrow minimum at relatively high ionic strengths ($\underline{I}$) but with progressively wider minima, on the pH axis, at decreasing $\underline{I}$ values. At very low $\underline{I}$ values (e.g., 1 mequiv./L, as typical of conventional IEF) two phenomena are apparent: (a) the minimum of solubility for β-lactoglobumin spans a 0.4 pH unit interval, centred on the pI value; (b) the amount of protein which can be brought in solution is fairly low, only 2 mg/ml. At higher $\underline{I}$ values (e.g., 20 mequiv./L, values which can be easily obtained in an IPG matrix) the same protein has an increment of solubility at the pI of a factor of 8, and its solubility minimum is confined to a narrow pH span (barely 0.05 pH units). Thus, we can equate IPG gels to 'salting in' media and CA-IEF gels to 'salting out' milieux. The reason for this is that a protein in an IPG matrix is isoelectric but not isoionic, in the sense that it

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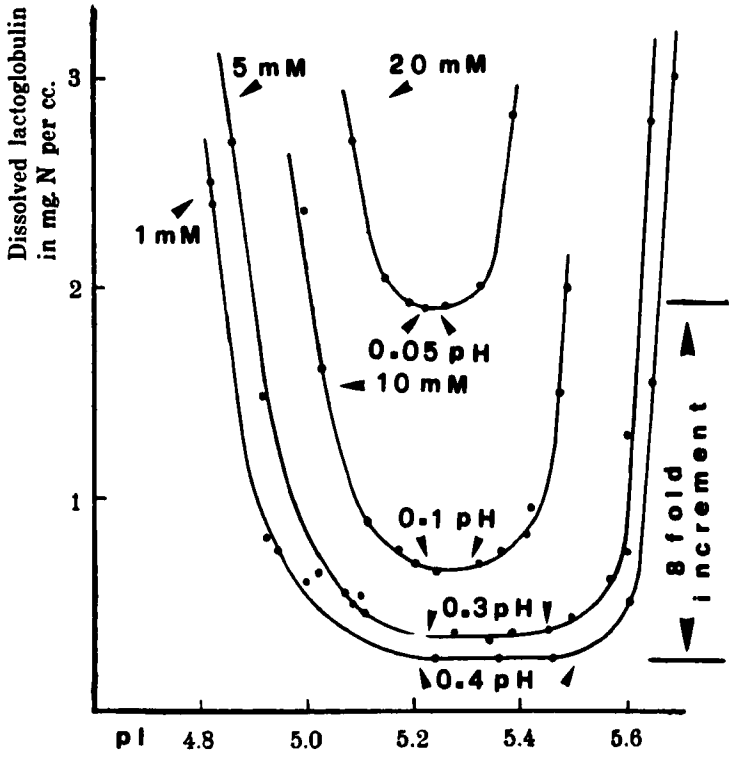

FIGURE 16

Solubility of β-lactoglobulin in the pH range 4.8-5.6 as a function of the ionic strength of the environment, from 1 mM to 20 mM NaCl. The amount of protein dissolved has been determined as Kjeldahl nitrogen. The pH values on the parabolas give the pH spans of the solubility minima, centred on a theoretical pI of 5.3 (modified from Grönwall, see ref. 61).

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forms a salt with the surrounding ions bound to the polyacrylamide coils, a sort of a 'protein-Immobilinate' or 'Immobiline-proteinate', depending on which species act as a carboxyl donor. Thus the protein solubility at the pI is substantially increased due to the fact that the Immobiline matrix provides counter-ions (different from protons) to the isoelectric protein and allows for salt formation. Such a phenomenon was recently demonstrated by focusing proteins and measuring their diffusion coefficient in the absence of the electric field: it was seen that, in IPG matrices of increasing Immobiline molarities, the diffusion coefficient was progressively and strongly diminished. The opposite was true in CA-IEF gels, suggesting that proteins focus in the boundaries between adjacent Ampholine peaks: as these boundaries quickly diffuse when interrupting the electric field, the protein diffusional transport is incremented too [49].

A second cause for incremented protein loads can be seen in Fig. 17: it is seen that the protein load is strongly dependent on the amount of polyacrylamide (%T) utilized for preparing the supporting gel. At a relatively high matrix content (6%T) the maximum tolerated protein load in an isoelectric zone is only 30 mg/ml gel volume whereas, in highly diluted gels (2.5%T), this upper limit is strongly increased, up to 90 mg protein/ml gel volume [18]. This has been interpreted as a competition for the available water between the two polymers, the polyacrylamide coils and the protein invading them. They both sequester and coordinate water in their hydration shell, however in high %T gels the matrix coils, being the most abundant species, sequester most of the available water, leaving preciously little liquid volume for the protein to be dissolved in. Such highly diluted gels have two additional advantages: (a) by diluting the matrix, while keeping constant the

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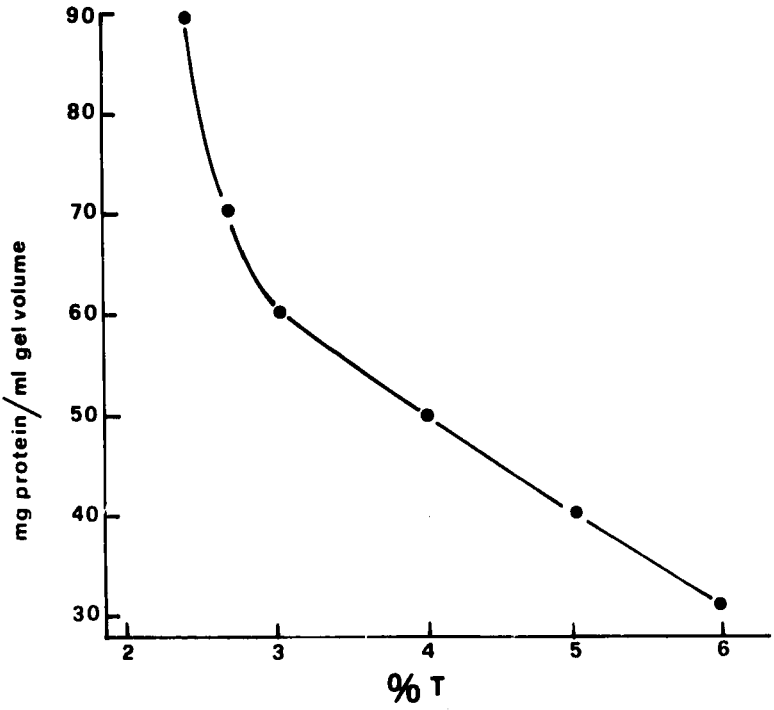

FIGURE 17

Relation between loading capacity (in terms of mg protein/ ml gel volume) and %T (T=grams acrylamide + cross linker per 100 ml gel volume) value of the gel matrix. Notice that, while in the range 3% to 6%T the protein load decreases linearly, in softer gels (<3%T) it increases exponentially (from Righetti and Gelfi, see ref. 18).

amount of Immobiline (the conventional ca. 10 mM buffering ion), the charge density on the polymer coil is in fact increased, and this results in sharper protein zones and increased protein loading capacity; (b) below 3%T, the visco-elastic forces of the gel are weakened, allowing the osmotic forces in the protein zone to predominate and draw

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more water from surrounding gel regions: this results in a further increment in load ability within a given protein zone due to local gel swelling and concomitant increase in cross-sectional area [18].

On the basis of the above observations, we have carried the preparative IPG technique to what could be the ultimate evolutionary step. Fig. 17 suggests that, if the gel matrix were totally abolished, possibly much greater protein loads could be tolerated. In addition, as discussed in Section VI, hydrophobic proteins are not compatible at all with high levels of Immobilines, as they are bound to the matrix by hydrophobic interaction and are poorly extracted after the fractionation process. Both observations lead to only one possible conclusion: why not try to perform a preparative IPG run in the absence of both, gel matrix and Immobilines (quite a paradox, isn't it)?. This concept has materialized in the novel technique, called 'segmented Immobilized pH gradients' as exemplified in Fig. 18: the IPG gel is composed of two (or more) segments separated by liquid interlayers, which function as recycling chambers. The floor and the ceiling of such flow chambers are IPG gel extremities, arranged so as to have pIs just lower (in the anodic side) and just higher (in the cathodic side) than the protein of interest to be purified. The electric field and the liquid flow are orthogonally coupled, and the protein feed kept in a large, thermostated and stirred reservoir. As the protein solution is continuously recycled, only the protein of interest is maintained isoelectric, by a continuous titration process, by the two IPG walls, while all the non-isoelectric impurities are swept away and collect either in the anodic or cathodic IPG segments. This new process (patent pending) has quite a few advantages: (a) it can handle very large liquid volumes; (b) it can tolerate very large protein amounts, as only a fraction of it enters the

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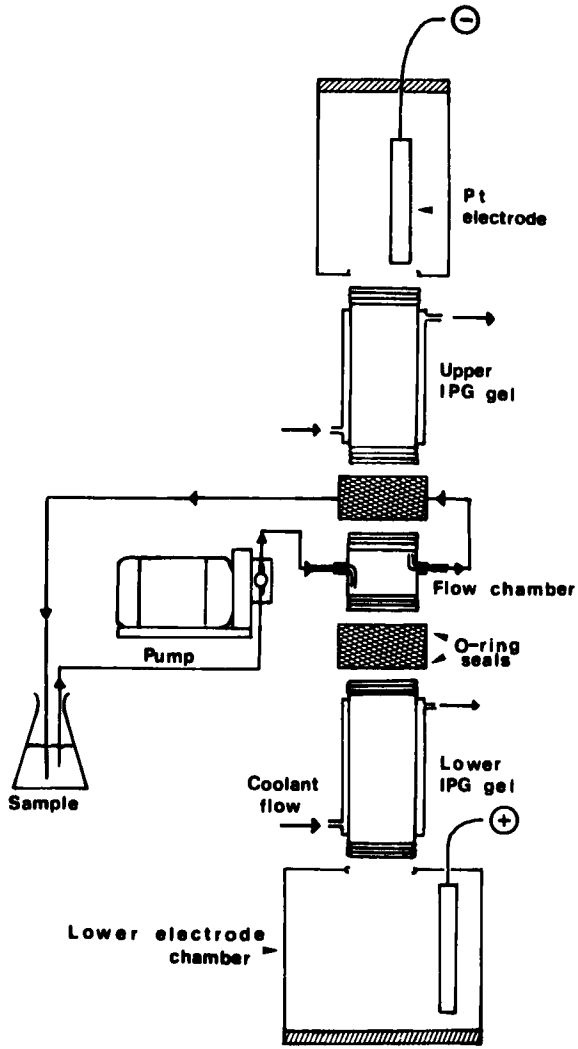

IPG gel; (c) it allows very high protein recoveries, as the component of interest never enters the IPG gel and thus does not have to be extracted from it. The principle of this novel approach is further explained in Fig. 19: the extremities of the two IPG gel segments delimiting the recycling chamber can be envisaged as isoelectric membranes, endowed with a strong buffering capacity, having pI value on either side and in proximity of the pI of the component to be purified. The latter thus, by a continuous titration process, stays isoelectric for the duration of the experiment and is therefore unable to leave the sample reservoir. Initially, we had performed these preparative runs in vertical chambers delimited by segments of Immobiline gradients [50, 51]; subsequently we adopted an horizontal configuration and utilized simply isoelectric Immobiline membranes to face the flow chamber, instead of true pH gradients [52, 53]. The latter aprroach has the advantage that the impurities can cross such isoelectric membranes and collect in the

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FIGURE 18

Sketch of the recycling, segmented IPG apparatus. A central flow-chamber is coupled, via two symmetric O-ring seals, to an upper and lower glass cylinders (2 cm diam., 8 cm in height) which serve as containers of cathodic and anodic segments of a polyacrylamide gel containing immobilized pH gradients of appropriate pH intervals. The upper electrode chamber is connected to the upper IPG gel segment via a water-tight O-ring seal, while the bottom segment (here depicted with a screw-on connection) can in reality be plunged directly in the anolyte solution. The sample is recycled from a refrigerated reservoir by a peristaltic pump, in general operated at maximum speed (5 ml/min) (from Faupel et al., see ref. 50).

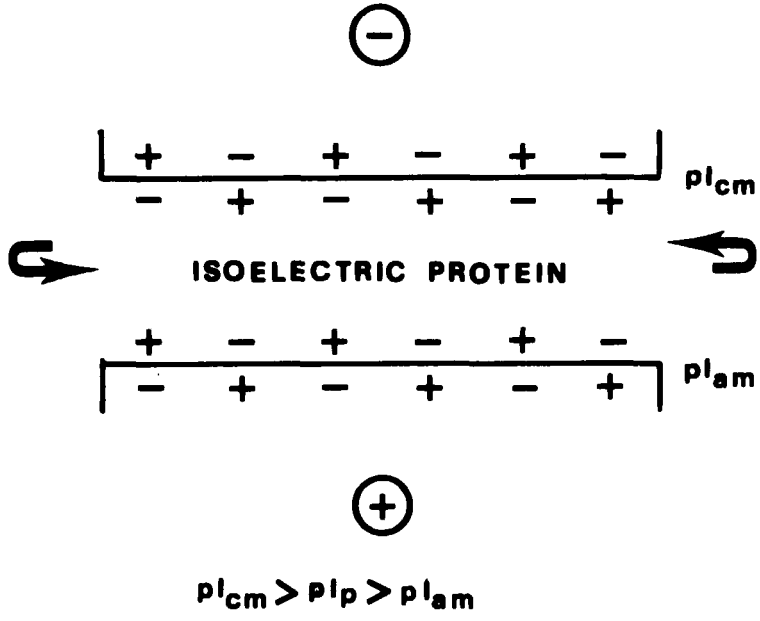

FIGURE 19

The concept of isoelectric IPG membranes. It is hypothesized that the two IPG gel extremities facing the recycling chamber in Fig. 18 act like two isoelectric membranes titrating the protein of interest to its isoelectric point (pI), thus keeping its mobility constant and equal to zero all throughout the desalting process. For this to occur, it is necessary that pIcm > pIp > pIam, where the subfixes indicate cathodic membrane, protein and anodic membrane, respectively. In addition, the two Immobiline 'membranes' satisfy the condition of having high buffering capacity at their pI value. The curved arrows indicate protein recycling in the desalting chamber (from Righetti et al., ref. 51).

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electrolyte compartments, without clogging the IPG gel. The idea of amphoteric, isoelectric Immobilines membranes, to be used in multicompartment electrolyzers, had in fact been explored recently by us in collaboration with the Institute of Chemical Engineering in Lausanne [54].

As we have given here only the latest developments, we will summarize now the salient features of preparative IPG runs: (1) in Ek et al. [17] general guidelines are given for preparative IPG fractionations (protein detection, band excision, electrophoretic elution in hydroxyapatite beads, equations for calculating maximum protein loads); (2) in Gelfi and Righetti [48], optimization of experimental parameters is studied (gel thickness, ionic strength and pH gradient width), while in Righetti and Gelfi [18] the load capacity of soft gels is evaluated; (3) in Casero et al. [55] recovery of proteins from Immobiline matrices into ion-exchange resins (CM- for basic, DEAE-Sephadex for acidic proteins) is studied; (4) in Righetti et al. [56] a direct recovery of proteins from an Immobiline gel into a free liquid phase is evaluated; (5) finally, in Bartels and Bock [57] a simultaneous fractionation-recovery technique in a mixed-bed, Immobiline gel containing Sephadex-filled trenches, is discussed.

X. The flaws of IPGs

After reading all these wonders, quite a few of our readers might hardly wait for the want to plunge in the IPG technique. Well, one of the most disappointing aspect of Science is that new techniques, which are developed just to overcome the flaws of existing ones, generate new, unexpected problems on their own, often more severe than the ones of already established methodologies. Luckily this is not the case with

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IPGs, but there are problems one should be aware off, while waiting for their solution. There are three defects afflicting the IPG technique: (a) hydrolysis of Immobilines; (b) spontaneous auto-polymerization and (c) relative hydrophobicity of basic Immobilines. Problem (a) is quite a nuisance because, upon hydrolysis, only acrylic acid is incorporated into the IPG matrix, with a strong acidification of the calculated pH gradient. Hydrolysis is an auto-catalyzed process for the basic Immobilines, as it is pH-dependent [16]: for the pK 8.5 and 9.3 species, such a cleavage reaction on the amido bond can occur even in the frozen state, at a rate of about 20% per year [58]. Auto-polymerization, a recently discovered accident [30, 31], is also quite deleterious for the IPG technique. Again, this reaction occurs particularly to alkaline Immobilines, and is purely auto-catalytical, as it is greatly accellerated by deprotonated amino groups [31]: oligomers and n-mers are formed, which stay in solution and can even be incorporated into the IPG gel, as in principle they still contain a double bond at one extremity (unless they anneal to form a ring). As shown in Fig. 20, upon analysis on a Bio Gel P-2 column, these autopolymerization products are seen to range in size from simple dimers and trimers to molecules having the same elution volume of a 64000 dalton protein, like hemoglobin. Analysis of the pK 9.3 Immobiline stored frozen revealed, after more than 6 months of storage, the presence of ca. 20% polymer (Fig. 21). These products of autopolymerization, when added to proteins in solution, are able to bridge them via two unlike binding surfaces; a lattice is formed and the proteins (especially larger ones, like ferritin, α_2-macroglobulin, thyroglobulin) are precipitated out of solution. This precipitation power is quite strong, and begins already at the level of short oligomers (>decamer) [31]. There is an easy test to check for the

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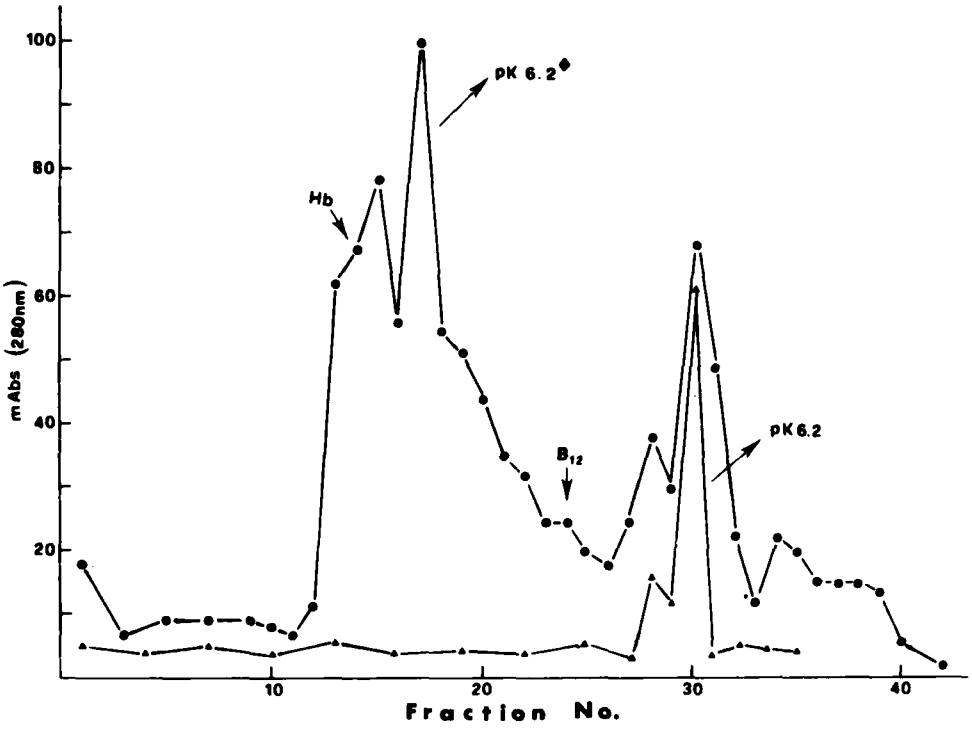

FIGURE 20

Measurement of the polydispersity of Immobiline chemicals. A Bio Gel P-2 column (0.8 x 45 cm) equilibrated with 50 mM phosphate buffer, pH 7.0, was loaded with 50 µl of 50 mM solutions of pK 6.2 Immobiline. The column was eluted at a rate of 3 ml/h and 0.8 ml aliquots collected. The absorbance (mAbs) was read at 280 nm. Hb: elution position of hemoglobin (void volume); B12: elution position of vitamin B12. Elution profile with triangles: control pK 6.2 Immobiline (non-precipitating); elution profile with solid circles: pK 6.2 Immobiline able to precipitate ferritin (from Rabilloud et al., see ref. 31).

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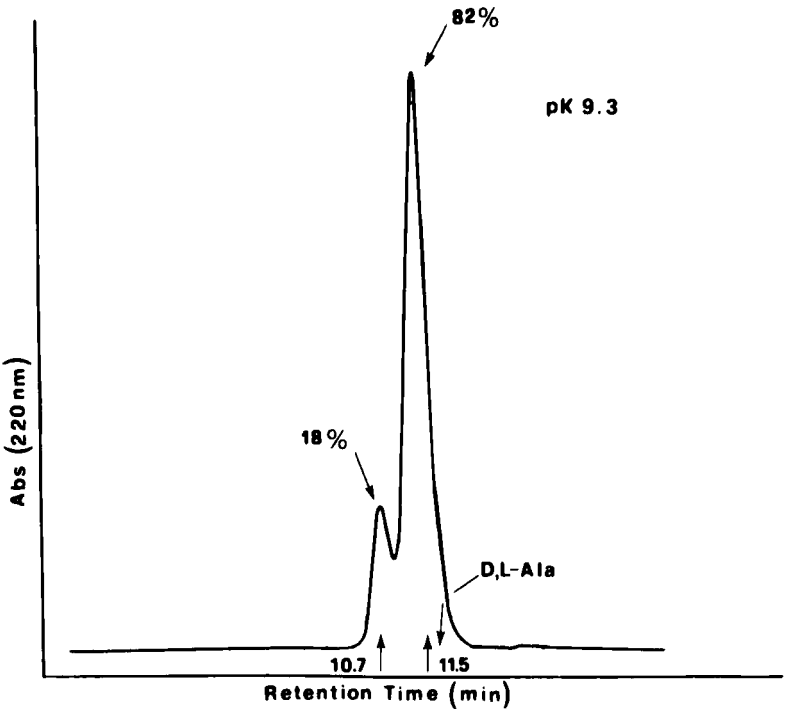

FIGURE 21

Control of the degree of self-polymerization of Immobiline chemicals. Analysis performed by gel filtration on HPLC in a Bio Gel TSK 30 column. Samples and column were buffered at pH 6.8 with 20 mM phosphate. 10 µl of 50 mM pK 9.3 Immobiline were injected and eluted at 1 ml/min flow rate. The eluate was monitored at 220 nm and automatically integrated. Note the large amount of polymeric material (18%) present in the sample. D,L-Ala: elution position of alanine, marker of the column total volume (from Rabilloud et al., see ref. 31).

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presence of polymers, called 'ferritin precipitation test' [30]; as a short-term remedy, we have described an easy method for oligomer removal, based on adsorption onto hydrophobic polymer phases (e.g., the XAD-2 polymer or a C18-bonded phase) [59, 60].

In reality, the drawbacks outlined in (a) and (b) will soon disappear, as they are an accident linked to the storage of the chemicals. In collaboration with other scientists (Gaveby et al., in preparation) we are finishing a long-term experiment on a suitable solvent which will completely abolish both degradation pathways. There remains, however, a third, most severe problem, i.e., the relative hydrophobicity of basic Immobilines, which will require a long-term approach, namely the chemical synthesis of alkaline species with more hydrophilic substituents. As discussed in Section VI, hydrophobic interaction with an IPG matrix can be quite disastrous, as no remedy will be available once such interaction has already occurred [26, 29-31]. As a short-term remedy, we suggest the following: (a) impregnate the IPG matrix with CAs; (b) add CAs also to the protein sample; (c) do not prefocus the IPG gel; (d) use fairly high CA levels (typically in the 1-2% range) and (e) try to lower the Immobiline level in the gel (e.g., to 1/2 the standard concentration given in published recipes). If all the above fails, bury your failures in a bottle of Moet-Chandon (we tried also a Dom Perignon and it seems to work with the same efficiency: there are rumors that even a Mercier will do it).

XI. Conclusions. Future trends.

We would not render justice to the IPG technique if we neglected to conclude that, notwithstanding some shortcomings, IPGs have performed wonders and have amply solved the problems they were supposed to

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overcome: notably an indefinite stabilization of the pH gradient, an astonishing reproducibility from run to run, abolition of discontinuities in the gel matrix (thanks to the principle of continuous titration). Among other notable features of IPGs: (a) their remarkable performance at acidic and alkaline pH extremes (truly unexpected) and (b) their adaptability to any separation problem by their intrinsic property of engineering any desired pH interval, no matter how minute or how extended. We feel we can veritably conclude, without exaggerating, that IPGs represent the technique for antonomasia among all electrokinetic processes. What future trends can we envisage? We dare to suggest the following : (a) the synthesis of a second generation of basic Immobilines, hydrophilic enough to overcome the problems of hydrophobic interaction with proteins (see sections VI and X); (b) a bunch of legionaries (possibly with a strong background in organic chemistry) willing to spend a few years of their life grafting Immobilines to agarose matrices and perhaps to acetate foils; (3) a major effort towards development and exploitation of large-scale IPG runs, e.g. with the recently discovered 'segmented Immobiline' gradients.

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