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Isoelectric focusing of histones in extremely alkaline immobilized pH gradients: comparison with capillary electrophoresis

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Abstract

Various classes of calf thymus histones (fractions II-AS, VI-S, VII-S and VIII-S) were separated to the steady state in an extremely alkaline immobilized pH gradient, covering non-linearly the pH 10–12 interval. Successful separations were obtained in 5%T, 4%C polyacrylamide matrices, reswollen in 8 *M* urea, 1.5% Tween 20, 1.5% Nonidet P-40 and 0.5% Ampholine pH 9–11. Additionally, in order to quench the very high conductivity of the gel region on the cathodic side, the reswelling solution contained a 0–10% (anode to cathode) sorbitol gradient. The best focusing was obtained by running the gel at 17°C, instead of the customary 10°C. All major histone components had p*I* values between pH 11 and 12 and only minor components (possibly acetylated and phosphorylated forms) focused below pH 11. By summing up all bands in Arg- and Lys-rich fractions, eight to ten major components and at least twelve minor zones are clearly resolved. In contrast, capillary zone electrophoresis (in a coated capillary, 7 *M* urea, 50 m*M* Tris–acetate buffer, pH 8.0) can only resolve six major fractions and two minor, broad zones.

1. Introduction

Histones are extremely alkaline proteins, unusually rich in lysine (Lys) and arginine (Arg), which act as counter ions to the high negative charge of nucleic acids and have thus a major role in the organization of chromatin structure. In the nuclei of all eukaryotic cells DNA is packed basically in the same way: two each of histones H2A, H2B, H3 and H4 form the fundamental chromatin subunit, the nucleosome, whereas histone H1 is associated with the chromosomal linker region. Four of these five histone classes each consist of several variants, differing to some extention their amino acid sequence [1,2]. The rest arises from various post-translational modifications which affect principally the charge, in the case of acetylation, phosphorylation, ADP-ribosylation, or the mass, in the case of ubiquination, of the parent histone molecule.

There are three basic electrophoretic methods for histone fractionation. The first is electrophoresis in acetic acid–urea gels, according to Panyim and Chalkley [3], which separates molecules, to a large extent, on the basis of charge. The second technique is electrophoresis in Tri-

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ton–acetic acid–urea (TAU) gels for separating the various subtypes of H2A, H2B and H3 and their modified forms [4–6]. The third is electrophoresis in sodium dodecyl sulphate (SDS) gels, which separates principally on the basis of mass and, in a few instances, also on the basis of different extents of phosphorylation [7]. As an additional variant, capillary zone electrophoresis (CZE) has been successfully applied to histone typing. CZE is performed in a free phase, in an uncoated capillary, in 110 m*M* phosphate buffer (pH 2.0) containing 0.03% (w/v) hydroxypropylmethyl (HPM)-cellulose [8]. By CZE, rat liver core histones were separated into five major and four minor peaks.

Ideally, isoelectric focusing (IEF), owing to its very high resolving power, could be well suited for histone analysis, provided that a strongly alkaline pH gradient can be established [9]. Indeed, there are only a few reports on IEF of histones in soluble, carrier ampholyte buffers. In one, the following subfractions have been isolated and reported to have p*I* 10.5 10.5 (H1), 10.5 (H2A), 11.0 (H2B) and 11.0 (H3) [10]. Similar data have also been obtained by Kopelovich et al. [11]. In reality, conventional IEF of histones to steady-state conditions seems to be an impossible proposition, owing to the well known fact that [especially in two-dimensional (2-D) maps] it is almost impossible to obtain stable pH gradients above pH 8 [12]. In fact, for most alkaline proteins, one had to resort to non-equilibrium pH gradient electrophoresis [13]. Moreover, in one of the few instances in which such 2-D separations were tried, it was reported that histones gave pronounced artefacts resulting from their interaction with another group of nuclear proteins, the so called "high mobility group" (HMG) non-histone proteins [14].

With the advent of immobilized pH gradients (IPG) [12], owing to the possibility of extending the fractionation interval to pH 10 and above, focusing of histones seemed a more realistic proposition. In fact, early attempts were disastrous. Righetti et al. [15] reported in 1983 that IPG matrices interacted strongly with at least two classes of proteins, histones and the histone-like, HMG-chromatin proteins, forming insoluble complexes. By preparing soluble strings of "carboxyl-surface" homopolymers of p*K* 3.6 and 4.6 Immobilines, sparse along the neutral polyacrylamide coil (5%T, 0%C linear polymers containing 10 m*M* total Immobilines), it was shown that these would complex by ionic interaction with HGMs and precipitate out of solution [15]. Later, when novel Immobiline compounds with a very high p*K* could be synthesized, it was shown to be possible to create stable pH gradients in the pH 10–11 interval, where a number of very alkaline proteins (e.g., lysozyme, cytochrome *c*) [16] and enzymes (e.g., elastase) [17] could be successfully fractionated. However, even in this last case, focusing of histones was not feasible [16].

Based on their amino acid composition, theoretical p*I* calculations predict that most histones should have p*I* values in the pH 11–12 range, and even higher. At such high pH values, Righetti et al. [15] had shown that interaction between histones and the Immobiline matrix should cease (see Fig. 5 in ref. [15]). Thus, in principle, focusing of histones should be feasible provided that one can create such extremely alkaline pH gradients. In this work, we modelled IPG intervals in the pH 10–12 range and achieved some unique separations of histones under true steady-state conditions. To our knowledge, this has not been reported previously.

2. Experimental

2.1. Materials

All IPG experiments were performed in an LKB 2117 Multiphor II horizontal electrophoresis system together with an LKB 2297 Macrodrive 5 power supply and Multitemp II thermostat. IPG gel casting was carried out by using an LKB 2117-903 2-D gradient and Immobiline gel kit. Acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED), Repel-Silane, Gel Bond PAG film, agarose and Ampholine (pH 9–11) were pur-

chased from Pharmacia–LKB Biotechnology (Uppsala, Sweden). The ten acrylamido buffer kit (p*I* Select) was purchased from Fluka (Buchs, Switzerland). Cytochrome *c* protein standard, the histone preparations II-AS, VI-S (Arg rich, slightly Lys rich), VII-S (slightly Lys rich) and VIII-S (Arg rich, subgroup F), all from calf thymus, and the two classes of detergent used (Tween 20 and Nonidet P-40) were obtained from Sigma (St. Louis, MO, USA) and sorbitol from Merck (Darmstadt, Germany).

2.2. Analytical IPGs

The technique has been described in detail previously [12]. Briefly, an IPG pH 10–12 interval was calculated and optimized with the computer program of Giaffreda et al. [18] (available from Hoefer Scientific, San Francisco, CA, USA). It was immobilized on to a 5%T, 4%C polyacrylamide matrix, typically having a size of 10 × 11 cm with a 0.5 mm thickness, bound to a polyester foil (Gel Bond PAG). After casting and polymerization, the gels were extensively washed in distilled water, dried and then reconstituted to their original mass by reswelling in a cassette in the presence of different amounts of additives. The optimum reswelling cocktail, able to maintain histones in solution, was found to be 8 *M* urea–1.5% Tween 20–1.5% Nonidet P-40–0.5% Ampholine covering a 2 pH unit interval (pH 9–11) and a 0–10% (anode to cathode) sorbitol gradient. In addition, all gels were run under light paraffin oil, so as to prevent CO_2 adsorption [19]. Protein samples (generally 1–2 mg/ml) were usually loaded (in a 30–50-μl volume) in plastic troughs at the anodic gel side, after a brief prefocusing step (1 h) at low voltage gradients (500 V). Analytical IPGs were run at 10 and 17°C, 1300 V, for a maximum of 4 h. Staining was effected with Coomassie Blue R-250 in 10% acetic acid–30% ethanol in the presence of 0.1% copper sulphate [20].

2.2. Capillary zone electrophoresis

CZE was performed on a Waters Quanta 4000E Ion Analyzer, equipped with Millennium software. The fused-silica capillary was purchased form Polymicro Technologies (Phoenix, AZ, USA) and coated with a covalently bound layer composed of the novel, highly hydrolysis-resistant, highly hydrophilic monomer N-acryloylaminoethoxyethanol (AAEE) (6%T at 0%C) [21] so as to completely suppress electroendosmosis and prevent histone binding to the wall. A mixture of all available histones (II-AS, VI-S, VII-S and VIII-S), at a concentration of 5 mg/ml, was dissolved in 110 m*M* phosphate buffer (pH 2.0) in the presence of 0.03% HPM-cellulose. The capillary (37 cm × 100 μm I.D.) was equilibrated in 50 m*M* Tris–acetate buffer (pH 8.0) in 7 *M* urea. Sample injection was for 8 s at 15 kV and the run was at 15 kV, 95 μA, 25°C, in the cathodic direction. Detection was at 214 nm.

3. Results

Knowing a priori that histones should have p*I* values in the pH 11–12 range, and perhaps even higher, attempts were made to create an extremely alkaline range, encompassing the pH 10–12 interval, with the IPG technique. In principle, such a gradient should not perform well, owing to the strong electrosmotic flow created by the net positive charge of the matrix, forcing a strong solvent flow directed towards the anodic gel extremity. The best gradient we could achieve with existing Immobiline buffers is shown in Fig. 1. The recipe was 10.8 m*M* of p*K* 10.3 with 7.5 m*M* of p*K* 4.6 Immobilines at the acidic extremity and just 10 m*M* of p*K* >13 quaternary base at the cathodic gel end. It should be noted that in fact this gradient has a marked sigmoidal profile. This is due to the paucity of Immobiline buffers in this pH region and because, in the cathodic chamber, the p*K* >13 Immobiline has to act simultaneously as a buffering and titrant ion. By chemical laws, such a gradient could never be linear [22]. Note additionally that the quaternary Immobiline (denoted p*K* >13 species) is used as a free ion to drive the pH in the gel matrix up to 12 (presumably it dictates alone the pH course in the

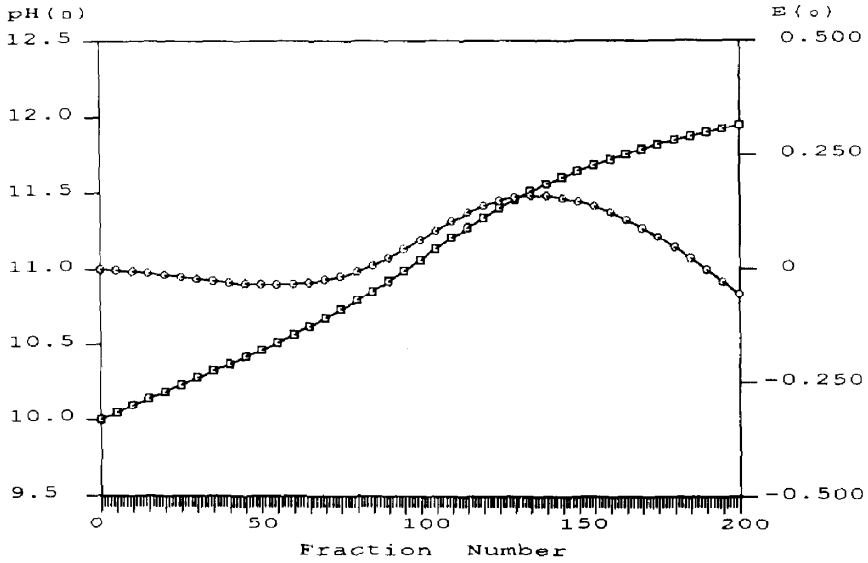

Fig. 1. Profile of a pH 10–12 immobilized gradient. □ = pH gradient; ○ = deviation from linearity. The average buffering power of the gradient is 8 mequiv./l·pH. The gradient was calculated and optimized with the program of Giaffreda et al. [18].

pH 11–12 interval). As it cannot violate the law of electroneutrality, it will then have as a counter ion the free OH^- groups generated by dissociation of water. Note in fact that, in order to reach pH 12, precisely 10 m*M* of p*K* >13 Immobiline are needed, which will be matched by 10 mmol of free OH^- which have to be present in the bulk solvent, by definition, at pH 12.

Fig. 2A shows the separation, in the pH 10–12 IPG gradient outlined above, of two different histone families, the VIII-S (tracks 1–3) and II-AS (tracks 4–6) groups. The separation was performed in 7 *M* urea–1.5% Nonidet P-40–0.5% Ampholine pH 9–11 at 10°C. Although still not optimum, this separation is nevertheless unique in that it displays five major histone bands, focused in a steady-state position in the upper gel region (pH 11–12 interval), i.e., precisely where histones are supposed to band. Moreover, the spectrum of components seems to reflect the amino acid composition of these proteins, since samples 1–3 (VIII-S, Arg-rich) have a much heavier band distribution in the more alkaline positions than samples 4–6 (II-AS, a mixed population of Arg- and Lys-rich), which are more abundant in the lower p*I* positions. It is also clear that fraction VIII-S must contain some components with p*I* 12 and higher, as some bands (see track 3) are seen to reach the cathodic gel extremity and collect just under the electrodic compartment. Fig. 2A in fact does not do justice to the high quality of the separation. Although not visible here, a number of minor components are also sharply focused in the pH 10–11 interval, which appears here as an empty region. Fig. 2B shows an expansion of this region, developed about 20 times more (combined product of time and lens opening). A large number of minor components (more than a dozen, sharply focused bands) are now seen focusing in this region (perhaps representing acetylated and phosphorylated forms). At the bottom of the photograph (ca. pH 10) is seen the imprint of the sample application surface well, of rectangular profile, showing that indeed there is almost no protein precipitation at the sample port.

We further tried a number of experimental modifications in order to improve the pattern of Fig. 2A and B. The final set-up employed consisted in reswelling the gels in 8 *M* urea, 1.5% Nonidet P-40, 1.5% Tween 20 and 0.5% Ampholine (pH 9–11 range) against a 0–10% (anode to cathode) gradient of sorbitol and

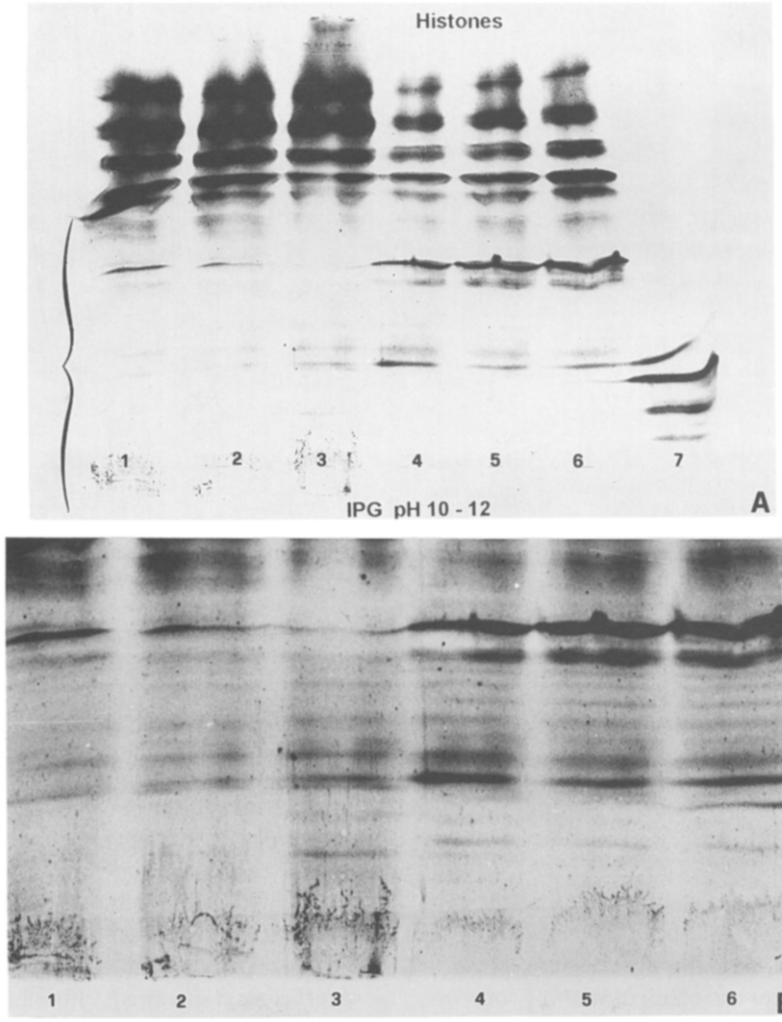

Fig. 2. Focusing of histones in the pH 10–12 interval of Fig. 1. (A) gel 5%T, 4%C polyacrylamide matrix, containing a 10–12 IPG, reswollen in 7 *M* urea, 1.5% Nonidet P-40 and 0.5% Ampholine pH 9–11. The gel was run at 10°C under a layer of light paraffin oil at 500 V for the first hour, followed by increasing voltage gradients, after sample penetration, up to 1300 V for a total of 4 h. The samples (2 mg/ml; 50 μl seeded) were loaded in plastic wells at the anodic gel surface. Staining with Coomassie Brilliant Blue R-250 in Cu^{++}. Tracks 1–3 = VIII-S histones; 4–6 = II-AS histones; 7 = cytochrome *c* (the main upper band has a p*I* of 10.6). (B) Expansion of the bracketted gel region in (A), developed with a twentyfold increase in exposure (time × lens opening). Note the fine spectrum of sharp bands (more than a dozen) focusing in the pH 10–11 region.

running the gels at 17°C instead of the customary 10°C. The results are shown in Fig. 3, which we believe is the first true equilibrium focusing of histones to be reported. With the markedly improved performance of this gradient, we can now see at least ten major histone fractions (combined among all the different populations) truly focusing in the pH 11–12 range. Moreover, the banding pattern clearly follows the inherent amino acid composition of each fraction: 1 = VII-S, Lys-rich, 3 = mixed-population of Arg- and Lys-rich and 4 = VIII-S, Arg-rich. Although not visible here, the lower gel portion (pH 10–11 interval) again shows the large number of minor components visible in the over-exposed photograph in Fig. 2B.

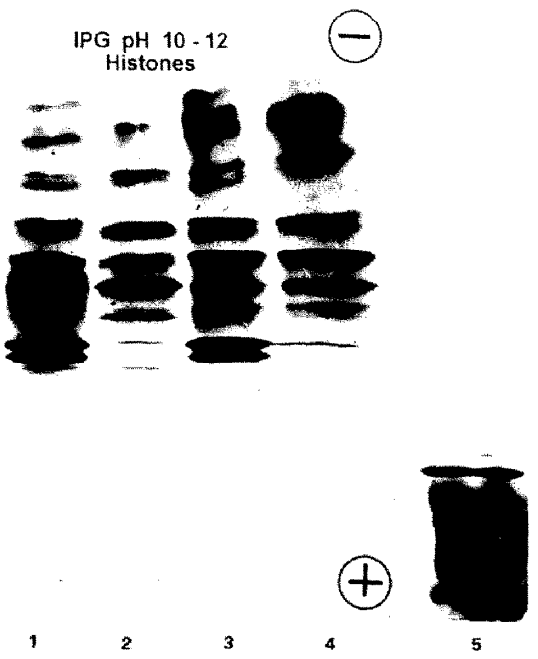

Fig. 3. Focusing of histones in the pH 10–12 interval of Fig. 1. All conditions as in Fig. 2, except that the gel was reswollen in 8 *M* urea–1.5% Nonidet P-40–1.5% Tween 20–0.5% Ampholine pH 9–11 and a 0–10% (anode to cathode) gradient of sorbitol and run at 17°C instead of 10°C as in Fig. 2. Histone samples: 1 = VII-S; 2 = VI-S; 3 = II-AS; 4 = VIII-S. The p*I* 10.6 marker (cytochrome *c*) is in track 5.

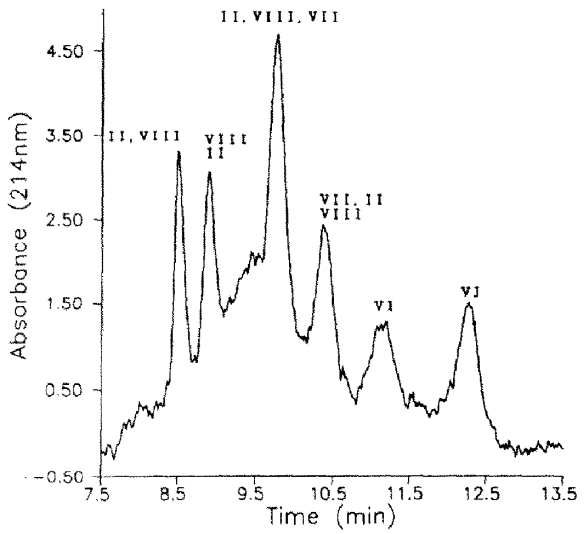

Fig. 4. CZE of histones obtained with a Waters Quanta 4000E Ion Analyzer. Capillary, 37 cm × 100 μm I.D., coated with a layer of poly(AAEE); running buffer, 50 m*M* Tris–acetate (pH 8.0) in 7 *M* urea; sample, mixture of all histone fractions (5 mg/ml) dissolved in 110 m*M* phosphate buffer (pH 2.0) and 0.03% HMP-cellulose; injection, 8 s at 15 kV; run, 15 kV, 95 μA, 25°C; detection at 214 nm.

As we became aware, during this work, of the data of Lindner et al. [8] on the CZE of histones, we tried to compare histone patterns under the two different electrokinetic protocols. Fig. 4 shows the CZE profile of a mixture of all histone samples, run at pH 8.0 (7 *M* urea) in a coated capillary. Six major fractions are resolved, identified by running separately the four different histone standards. Note that here the transit times should follow the p*I* spectrum, the early-eluting peaks representing the p*I* 12 species and the slow-migrating bands the p*I* 11 fractions.

4. Discussion

It might be asked how well our histone p*I* data are correlated with the literature in the field. Owing to recent progress in nucleic acid research, an exponentially growing number of protein sequences are being published, derived from gene sequences. We have taken, at random, a number of histone sequences published in the period 1987–89 and calculated the resulting p*I* value with the help of our IPG program [18] (modified not to account for the ionic product of water and without any correction for activity coefficients of ions). For example, for mouse (*Mus musculus*) Liu et al. [23] have reported a 125 amino acid (AA) sequence for histone H2B, resulting in an M_r of 13 765 and a calculated p*I* of 11.45. In the same species, for histone H1.0, a 193 AA sequence has been published, resulting in an M_r of 20 715 and a p*I* of 11.95 [24]. In a third report, mouse histone H1.1 was given an AA sequence of 211, resulting in an M_r of 21 135 and a p*I* of 11.84 [25]. In muscovy duck, histone H2A was assigned an AA sequence of 128, resulting in an M_r of 13 825 and a p*I* of 11.98 [26], while histone H2B had a sequence of 125 AA, resulting in an M_r of 13 765 and a p*I* of 11.37 [26]. In *Tetrahymena pyriformis*, Histone H1 had a sequence of 165 AA, an M_r of 17 943 and a p*I* of 11.33 [27]. In *Caenorhabditis elegans*,

histone H1.1 was assigned a sequence of 207 AA, an M_r of 21 263 and a p*I* of 11.80 [28]. In *Homo sapiens*, histone H2A.X was given a sequence of 142 AA, with a corresponding M_r of 15 013 and a p*I* of 11.97 [29]. The present data on p*I* values of all classes of histones fall nicely in all these literature ranges. However, they do not agree with the data of Valkonen and Piha [30], who reported, in conventional IEF, p*I* values ranging from 9 (histone H_1) to 10.5 (histone H_4).

It might be asked what effects the various additives have on the true p*I* values of histones. We have on purpose avoided giving precise p*I*s, as pH measurements in such extremely alkaline gradients are besieged by severe problems. We have reported, however, that 8 *M* urea increases the p*K* values of both acidic and alkaline Immobilines by as much as 1 pH unit [12]. It is nevertheless to be expected that also the p*K* values of acidic and basic groups of proteins will shift to a similar extent so that, on a relative scale, the p*I* positions will be very much the same as predicted by the theoretical curve in Fig. 1. The other additives should not have, per se, much effect on the IPG interval reported here. We have already described that detergents do not modify the p*K* values of Immobilines [12]; in addition, the Ampholines used focus only in the lower IPG interval (pH 10–11), as also visible from their refractive lines, once focused. Additionally (and also in order to answer to one referee's comment), it would have been useful to compare gel slab focusing with capillary IEF and not with capillary zone electrophoresis. The problem is that there does not seem to be, at present, an easy way to perform IPGs in capillaries, owing to difficulties in pumping in such minute gradient volumes, amplified also by major difficulties in avoiding interference from silanols at such extremely alkaline pH values.

Focusing with IPGs at pH extremes (both acidic and alkaline) requires respect of basic laws in physics. In early attempts made in 1985 by one of us (P.G.R., on a summer sabbatical in Tucson, AZ, in M. Bier's laboratory), the mildly forbidden pH 3–4 region was explored, in an attempt to focus dansylated amino acids [31]. We had severe experimental problems, as we overlooked the fact that, at pH 3, the gel has to bear a 1 m*M* net negative charge, which results in electroendosmotic (EEO) flow of positive charges (with their hydration water) to the cathode and on complete anode dehydration (with severe risks of sparks and gel burning). In those days, EEO was not in the limelight as it is today, owing to the advent of the CZE technique. As a result of these experiments, we had to include, in our IPG computer modelling, the contribution of the dissociation products of water to conductivity and buffering capacity (which can be neglected in the pH 4–10 range) [32]. If things were so difficult at pH 3–4, then they should be impossible in the pH 10–12 range where, at the cathodic gel extreme, the gel should bear a 10 m*M* net positive charge and the EEO flux should be ten times higher. The fact is that the combination of 8 *M* urea and a sorbitol gradient acts as a very efficient conductivity quencher, markedly reducing the anode-directed water transport. Quenching of conductivity is also necessary in order to sharpen the highest p*I* components. It is in fact seen in Fig. 2A that the higher p*I* species are diffuse, as opposed to the sharper lower p*I* components. The pattern in Fig. 2A resembles an elution profile in chromatography, where late-eluting peaks are more diffuse. The mechanism here is different, however: the voltage gradient at the pH 12 extreme is 100 times less than at the pH 10 gel end, as it follows the 100-fold difference in conductivity between the two gel extremes. We tried to even out this huge difference by the combined action of two additives: the small amount of carrier ampholytes, which focus only in the pH 10–11 interval, increasing the conductivity in this region, and the sorbitol gradient, which quenches the conductivity in the pH 11–12 interval. This seems to be a successful strategy, as shown in Fig. 3, a truly amazing achievement for any focusing system.

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