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pH MEASUREMENTS AFTER ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENTS

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SUMMARY

It is impossible to measure pH values in immobilized pH gradients either with a surface electrode or by cutting gel slices and eluting in 10 m*M* potassium chloride solution. The use of reversible gels, cross-linked with bisacrylylcistamine, improves the measurements in the acidic region, but gives false values in the alkaline region, owing to the buffering power of added 2-mercaptoethanol or dithiothreitol. By using mixed-type gels, containing Immobilines and 1% carrier ampholytes, accurate and reliable pH measurements can be obtained. The discrepancy between the theoretical slope of the immobilized pH gradient and the actual pH values obtained by reading the pH of eluted Ampholine focusing in the same gel fragment is less than 0.1 pH unit over a 1 pH unit span. The effects of temperature and of carbon dioxide absorption on pH readings are demonstrated and evaluated.

INTRODUCTION

Much attention has been devoted in the past to pH measurements after isoelectric focusing (IEF) in carrier ampholyte buffers (CAs), as the p*I* determined by IEF also represents the intrinsic isoionic point of a pure protein[1]. It was also soon appreciated that pH assessments should be made at the same temperature of the IEF fractionation, as the d(pH)/d*T* and d(p*I*)/d*T* curves for CAs and proteins diverge, especially as a function of the histidine and lysine residue content of the latter and at alkaline pH[2,3]. In a series of papers, Gelsema and co-workers[4–6] also made a thorough study of all the factors affecting pH measurements and of the possible correction factors to be used, especially in the presence of additives, such as sucrose, glycerol and ethylene glycol. Actually, the additive altering most extensively the solution pH is urea (8 *M* solutions), for which Ui[7] has suggested an overall correction factor of 0.42 pH unit, Josephson *et al.*[8] of 0.9 pH units and Gianazza *et al.*[9] a variable correction term, ranging from 0.4 to 0.6 pH unit from acidic to alkaline pH values. The interference with pH values above pH 8 from atmospheric carbon dioxide absorption was also evaluated[10]. The large volume of data published (up to 1984, more than 6000 articles dealing with protein IEF had appeared) has allowed the compilation of tables listing p*I* values for *ca.* 1000 different proteins and isoforms thereof[11,12].

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With the advent of immobilized pH gradients (IPGs)[13], the art of separating proteins in a pH gradient has evolved into an exact science. In a series of papers[14–17] we have described a computer program for the generation and optimization of any narrow or extended pH interval with Immobiline chemicals. The program, first adopted for a multi-chamber mixing device[14], was then applied to two-chamber mixers[15] and recipes were given for any possible pH interval, spanning from a minimum of 1.5 to a maximum of 7 pH units, with[16] or without[17] the aid of strong titrants. Recently, we have also given formulations for non-linear, extended pH gradients to be used in the first dimension of two-dimensional maps of complex samples, such as cell lysates and biological fluids[18]. However, even with IPGs the assessment of pH gradients has not improved; in fact, the situation has worsened. Because the buffering ions and titrants are grafted to the polyacrylamide matrix, pH determinations with surface electrodes are totally meaningless[19]. Even more traditional methods, such as cutting gel segments along the separation axis and eluting them in 10 m*M* potassium chloride solution for pH readings, as routinely performed in CA-IEF[20], lead to completely erroneous results, as no free ions are eluted from the matrix to buffer the supernatant.

By exploiting an original idea developed in our laboratory[21], we have recently described a novel approach to pH gradient fractionation of proteins, namely a mixed Ampholine–Immobiline gel[22]. In these gels, the primary, IPG gradient stabilizes the secondary, CA gradient. The latter, while increasing the background conductivity for faster focusing of proteins, should in principle allow easy measurements of the pH gradient profile along the separation axis. This paper deals with this novel method of pH determination in IPG matrices.

EXPERIMENTAL

Immobiline buffers and Ampholine carrier ampholytes were purchased from LKB (Bromma, Sweden), acrylamide monomers and polymerization catalysts from Bio-Rad Labs. (Richmond, CA, U.S.A.), Gel Bond PAG from Marine Colloids (Rockland, ME, U.S.A.) and Pharmalyte carrier ampholytes from Pharmacia (Uppsala, Sweden).

The IEF experiments in IPGs were carried out by using the LKB Ultrophor apparatus together with an LKB 2197 constant power supply and, for cooling, an LKB 2209 Multitemp. For gel casting the LKB 2117-901 gradient gel kit with the microgradient mixer, and for pH measurements a pH M64 research pH meter from Radiometer (Copenhagen, Denmark), were used. IPG were cast according to published methodologies[23]. The gel dimensions were 11.5 × 11 × 0.07 cm. The chambers of the microgradient mixer were each filled with 5.5 ml of a solution containing 3.5% T, 4% C and Immobilines in concentrations calculated to give pH gradients of 6.8–7.8, 4.5–5.5, 7.0–7.5 and 7.0–8.0. For the pH 6.8–7.8 gradient, the acidic chamber contained the following Immobilines (each a stock 0.2 *M* solution): 251 μl of p*K* 7.0 and 170 μl of p*K* 3.6, the corresponding amounts for the basic chamber being 344 μl and 46 μl, respectively. For the pH 4.5–5.5 gradient, the acidic chamber contained 152 μl of p*K* 3.6, 88 μl of p*K* 4.6 and 183 μl of p*K* 6.2, the corresponding amounts for the basic chamber being 76 μl of p*K* 3.6, 148 μl of p*K* 4.6, 136 μl of p*K* 6.2, 45 μl of p*K* 7.0 and 55 μl of p*K* 9.3. For the pH 7.0–7.5 gradient, the acidic chamber

contained 276 μl of p*K* 7.0 and 145 μl of p*K* 3.6, the corresponding amounts in the basic chamber being 318 μl and 83 μl, respectively. For the pH 7.0–8.0 gradient, the acidic chamber contained 495 μl of p*K* 3.6, 100 μl of p*K* 7.0, 136 μl of p*K* 8.5 and 310 μl of p*K* 9.3, the corresponding amounts for the basic chamber being 335 μl, 90 μl, 101 μl and 253 μl, respectively.

The catalysts (4 μl of 40% ammonium persulphate and 5 μl of N,N,N′,N′-tetramethylethylenediamine per chamber) were added directly to the gradient mixer immediately before filling the gel into the cassette. After focusing at 2000 V and 4 W maximum power for different time periods (see Results) at 10°C, the gels were cut in 1 × 0.5 × 0.07 cm strips (a volume of *ca.* 35–40 μl) and eluted with 300 μl of 10 m*M* potassium chloride solution for 1 h. pH values were then measured at room temperature or at 10°C, in air of under a nitrogen atmosphere[20].

RESULTS

Fig. 1 shows the results obtained for pH gradient measurements in an IPG gel close to neutrality (pH 7–8) when cutting gel strips and assessing the pH of eluates from them. The solid line represents the theoretical pH 7–8 gradient assuming linear mixing between the two extremes. The points closely following this curve are actual pH measurements performed in fractions of the IPG matrix eluted from the gradient mixer directly into a fraction collector in the absence of catalysts (*i.e.*, by preventing

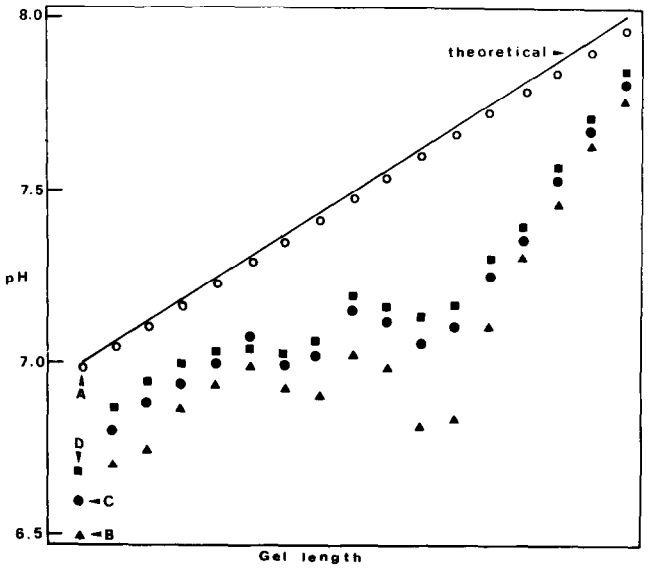

Fig. 1. pH gradient determinations in IPG gels in the absence of carrier ampholytes. A pH 7–8 IPG gel was cast and run for 6 h at 2000 V and 10°C. Seventeen gel slices were cut from anode to cathode, 300 μl of 10 m*M* potassium chloride solution were added and the mixture was allowed to equilibrate under nitrogen for different times. Solid line, theoretical IPG slope. A, Gradient measured in unpolymerized liquid fractions collected from the gradient mixer into a fraction collector; B, C and D, gradients measured in slice eluates after 2 h (▲), 4 h (●) and 18 h (■), respectively (unpublished experiments with G. Artoni and E. Gianazza).

gel polymerization). It can be seen that, in solution, the expected and experimental gradients almost coincide. However, once the gel has been polymerized, run for 6 h at 2000 V to remove unbound material and the pH assessed in gel segments, the situation is entirely different. As shown by the lower points, the discrepancy between theoretical and experimental is very large, of the order of 0.3–0.8 pH unit (over barely 1 pH unit interval, *i.e.*, as much as an 80% error). Even when allowing the supernatant to equilibrate with the gel slices for long times (up to 18 h), the error is reduced, but still unacceptably large (a difference of as much as 0.4 pH unit). These findings led us to abandon direct pH measurements in plain IPG matrices and to try such determinations in mixed Ampholine–Immobiline gels.

In Fig. 2 we have addressed the question of the minimum level of CAs to be added to an IPG matrix for a correct assessment of a pH gradient in gel slice eluates. Three different gels were run, equilibrated in 0.3%, 1% and 2% carrier ampholytes in the pH range 6–8 (the narrowest commercially available). It can be seen that 0.3% of CAs in the gel give too large a discrepancy from the expected to the experimentally found pH values, with readings diverging by as much as 0.2–0.3 pH unit. The 1% and 2% CAs give curves more closely approaching the theoretical pH slopes, especially on the anodic side of the gel. This might simply be due to a dilution factor; in general, a slice corresponding to 35 μl of gel volume is diluted to 300 μl with 10 m*M* potassium chloride solution for pH readings; the 0.3% CA gel would have too little buffering power in such a diluted solution. Hence a level of 1% of CAs in an IPG gel was routinely adopted for pH determinations.

We next investigated the stability of such gradients with time and the minimum

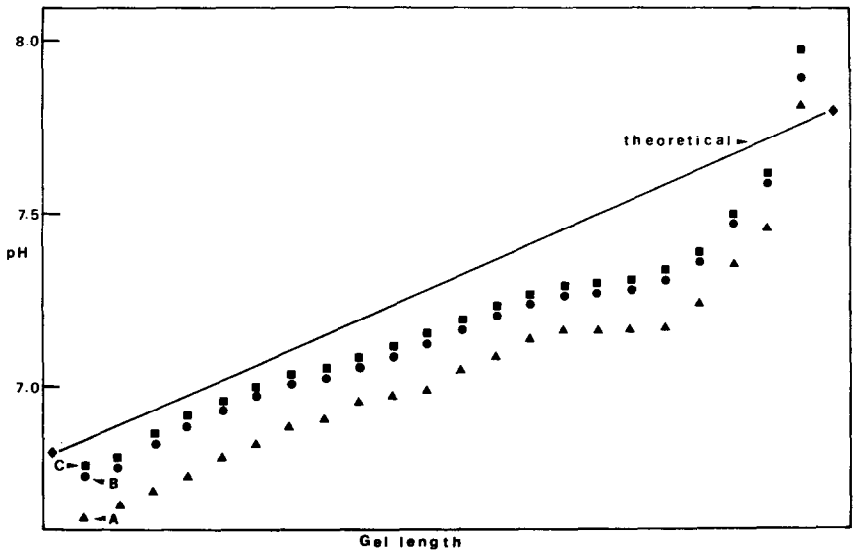

Fig. 2. Minimum requirement for Ampholine in an IPG gel. A pH 6.8–7.8 IPG gel, run for 6 h at 2000 V and 10°C, was segmented into 22 slices and 300 μl of 10 m*M* potassium chloride solution were added to each. Solid line, theoretical pH slope, A, B and C, pH measurements in gel slices from three different IPG gels containing 0.3% (▲), 1% (●) and 2% (■) pH 6–8 Ampholines, respectively. pH determinations at 23°C in air.

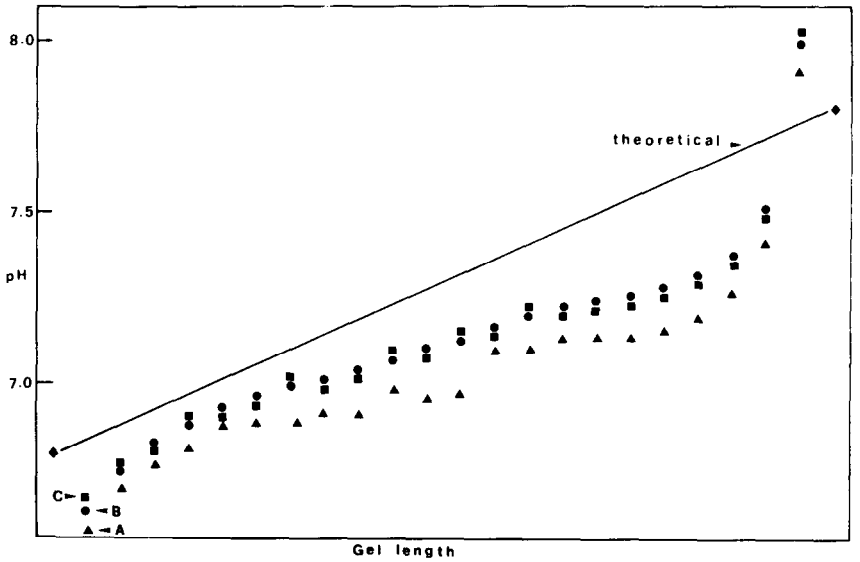

Fig. 3. Time requirement for pH establishment in mixed Ampholine–Immobiline gels. Conditions as in Fig. 2, except that the pH 6.8–7.8 IPG gels contained 1% pH 6–8 Ampholine. pH determinations in gel eluates after (A) 3 h (▲), (B) 6 h (●) and (C) 22 h (■) of focusing at 2000 V and 10°C in the IPG gels. pH measurements at 23°C in air.

focusing time needed for proper pH evaluation. The results are shown in Fig. 3; it can be seen that at 3 h the pattern is not fully established and erratic pH measurements ensue. At 6 h the Ampholine gradient is fully established and essentially no variation is seen on prolonged focusing for up to 22 h. This is especially important in view of the well known pH gradient instability of a conventional CA gel (cathodic drift)[23]; it suggests that indeed the primary IPG gradient also "immobilizes" the secondary, CA-generated pH gradient in the gel. What is surprising is that the carrier ampholytes are not quite at the steady state after 3 h in IEF, whereas in general they are after barely 1 h in conventional IEF[24]; it cannot be excluded that the slower focusing could be due to transient interaction of CAs with the underlying Immobiline buffers.

As can be seen from Figs. 2 and 3, there is a systematic discrepancy between the theoretical IPG pH slope and the measured slope, the latter being almost parallel and at consistently lower (0.1–0.2 pH unit) pH values. It should be remembered, however, that IPGs are measured and run always at 10°C, whereas our experimental readings were taken at room temperature (23°C). When the same pH assessments were repeated in an IPG pH 6.8–7.8 gradient containing 1% CA in the pH range 6–8 at 10°C (with the electrode also equilibrated at 10°C), the results in Fig. 4 were obtained; at lower temperatures, the theoretical and experimental curves almost coincide and only diverge towards the alkaline extreme. The pH shift at lower temperatures is an increase of the order of 0.15 pH unit over most of the pH range. In acidic pH ranges, *e.g.*, in a pH 4.5–5.5 span (see Fig. 5), the temperature effect can still be appreciated, but it is very minute, barely 0.05 pH unit over most of the gradient. This is in general agreement with the well known temperature coefficient (dpK/dT) of

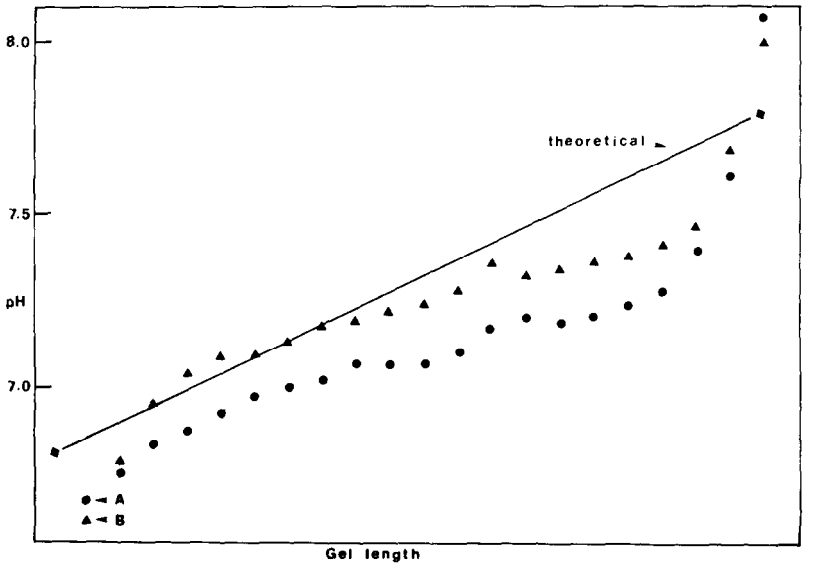

Fig. 4. Effect of temperature on pH measurements in alkaline gradients. Conditions as in Fig. 2, except that the pH determinations were made in slice eluates at (A) 23°C (●) and (B) 10°C (▲) in air. Solid line, theoretical pH slope.

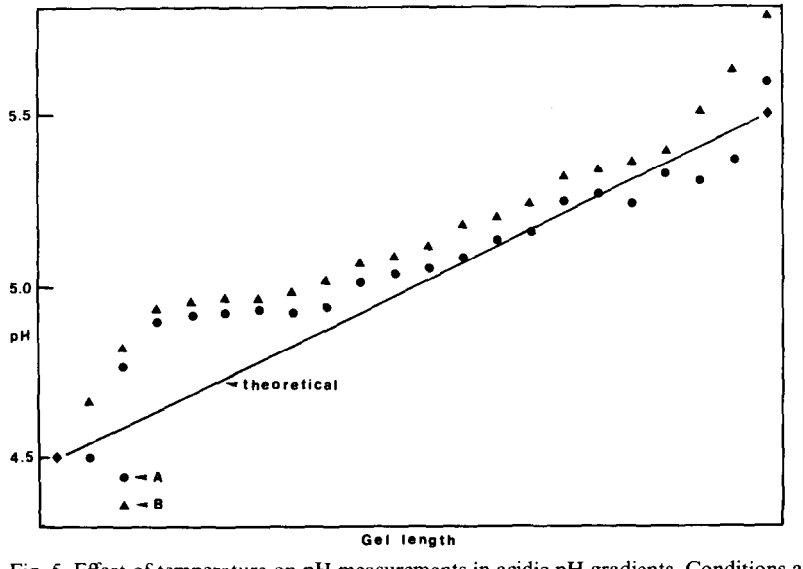

Fig. 5. Effect of temperature on pH measurements in acidic pH gradients. Conditions as in Fig. 2, except that the IPG gel contained a pH 4.5–5.5 gradient and that pH determinations were made in slice eluates at (A) 23°C (●) and (B) 10°C (▲) in air. Solid line, theoretical pH slope.

Immobiline chemicals[13], which is fairly large for the alkaline species and much reduced for the acidic buffers.

However, even when correcting for the temperature effect, the pH 6.8–7.8 gradient still exhibits a marked deviation from the theoretical slope towards the alkaline extreme. As the pH gradient tends to flatten in this region, it cannot be excluded that this phenomenon could be due to absorption of carbon dioxide by the gel slices as they are extracted in 10 m*M* potassium chloride solution. To test for this, the experiments were repeated under an inert atmosphere, by flushing each test-tube with nitrogen and immediately sealing it with a stopper. Indeed, as shown in Fig. 6, there is an increment of pH readings of about 0.1 pH unit, thus bringing the theoretical and experimental profiles into closer agreement.

It has been reported[25,26] that different commercial carrier ampholytes could produce different results for the linearity of the pH gradient and the distribution of protein zones along it. In particular, Pharmalytes have been recommended for two-dimensional maps as they appear to have a better conductivity around neutral pH, presumably owing to the presence of a more diversified number of carried ampholytes in this region, with better buffering power and conductivity profiles[27]. We therefore repeated the above experiments by replacing 1% Ampholine with 1% Pharmalytes in the IPG gel. The pH gradients at different time intervals are plotted in Fig. 7; the pH readings appear to be better with less scatter of the experimental points, but the difference between these profiles and those reported in Figs. 2 and 4 is too small to allow us to make a clear distinction between different types of CA species.

Finally, we have tried to assess the lower limit of the pH range of an IPG gel that can be safely measured by the addition of regular, 2 pH unit wide carrier ampholyte intervals. As can be seen from the above data, a 2 pH unit CA gradient can be super imposed on a 1 pH unit IPG gradient and be converted essentially into a

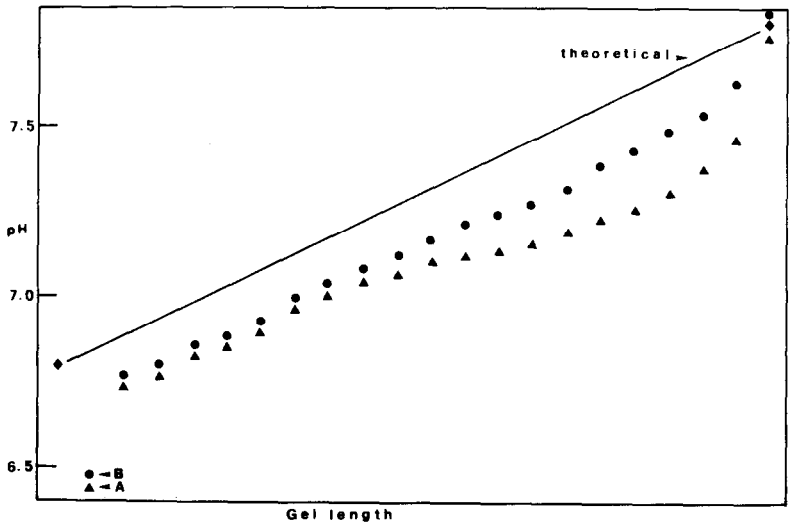

Fig. 6. Effect of carbon dioxide on alkaline pH readings. Conditions as in Fig. 2, except that the pH in the slice eluates (IPG pH 6.8–7.8 gel) was determined either (A) in air (▲) or (B) under a nitrogen atmosphere (●) at 23°C. Solid line, theoretical pH slope.

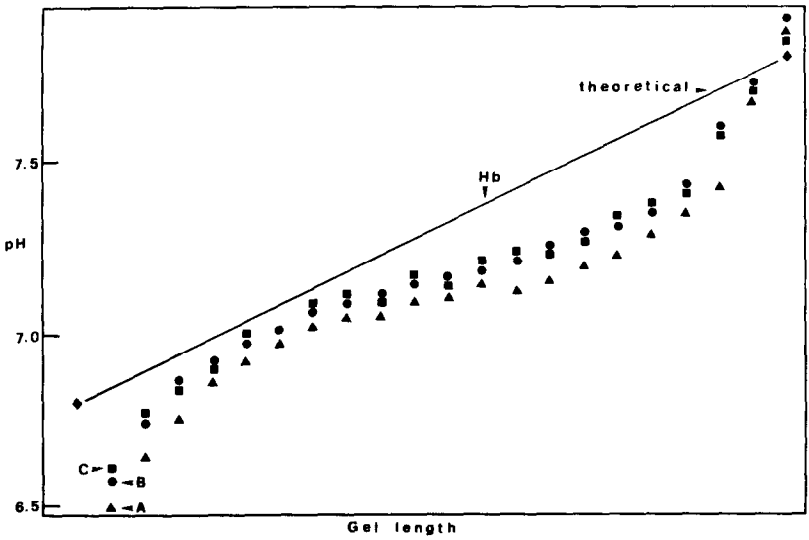

Fig. 7. Effect of changing the carrier ampholytes. The IPG pH 6.8–7.8 gel was equilibrated in 1% Pharmalyte instead of 1% Ampholine. The pH in the slice eluates was read at 23°C after (A) 3 h (▲), (B) 6 h (●) and (C) 24 h (■) of focusing in air. The pH gradient appears to be smoother than in presence of 1% Ampholine. The position of focused HbA is marked by an arrow. Solid line, theoretical pH slope.

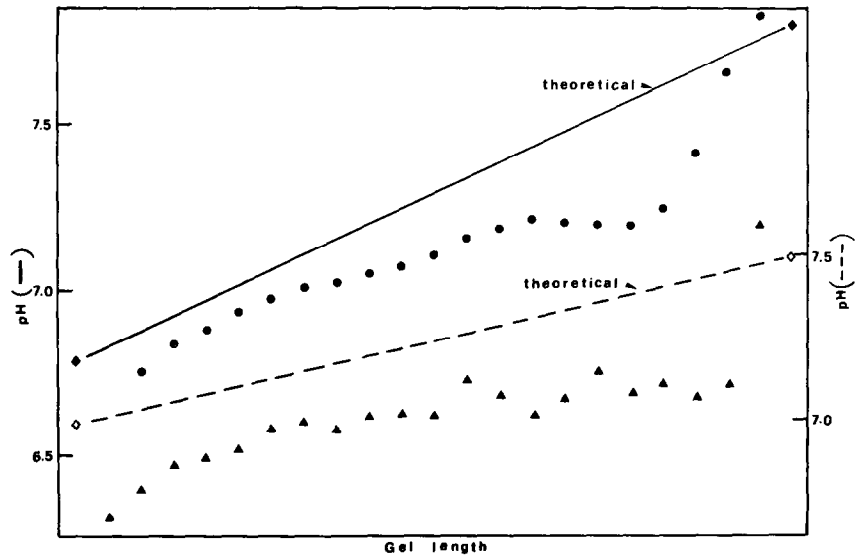

Fig. 8. Effect of narrowing the Immobiline pH gradient span. A 1 pH unit (pH 6.8–7.8) (●) and a 0.5 pH unit (pH 7.0–7.5) (▲) IPG gel, equilibrated with 1% pH 6–8 Ampholine were run for 6 h at 2000 V and 10°C. The pH of the eluates was assessed in slices eluted with 300 μl of 10 mM potassium chloride solution at 23°C in air. It can be seen that the primary IPG gradient can convert a 2 pH unit Ampholine gradient into either a 1 pH unit or a 0.5 pH unit, according to the pH range of the underlying immobilized pH gradient.

narrow, 1 pH unit interval by the superior buffering power of the primary, immobilized pH gradient. As shown in Fig. 8, even in a narrower pH range (in this instance 0.5 pH unit, 7.0–7.5), most of the gradient over the gel length is confined to this interval, even though the secondary pH gradient encompasses 2 pH units. Presumably the excess Ampholines, outside the primary pH gradient, are confined to the filter-paper strips that act as catholyte and anolyte reservoirs at the gel extremities, so that they will alter locally the pH gradient only in the extreme 10% of the gel length at the anode and 10% at the cathode. It should be noted that in all experiments the electrodic wicks were not soaked in a free acid and a free base, as is customary in IEF and in IPGs, but were impregnated directly in carrier ampholytes.

DISCUSSION

Some interesting conclusions can be drawn from the above experiments as follows.

pH gradient width

When a CA gradient is superimposed on an IPG gradient, the latter will reduce the width of the former to its own pH extremes. This will only be valid, however, if the amount of CAs is not much greater than 1% as a final gel concentration. A standard Immobiline gel will contain *ca.* 10 m*M* buffering ion plus varying amounts of titrant (at pH = p*K* of the buffering species, the titrant concentration will be 5 m*M*); this would correspond to a 2% Ampholine concentration in conventional CA-IEF. Thus, in presence of 1% CA, the Immobiline gel will exhibit twice the buffering power of the added carrier ampholytes, so that it will be effective at dictating the span of the pH gradient even when wider Ampholine gradients are added. However, if the concentration of the added Ampholines is 2% or greater, the two systems will rapidly come into conflict and should only be mixed when they encompass the same pH range[22]. The alternative would be to change the electrode filter-paper strips at regular intervals, so that the CA species outside the primary IPG interval would be eluted from the Immobiline matrix. We have tried this approach successfully when too much salt present in the system had to be removed from the gel phase. In this instance, the new filter-paper strips should simply be soaked in distilled water, so that the electric current will saturate them with the ions to be removed electrophoretically from the IPG matrix[28].

Accuracy of pH measurements

For a proper assessment of pH values, a 1% Ampholine concentration in the IPG gel is needed. In alkaline ranges, the concomitant effect of temperature and carbon dioxide absorption markedly lowers the pH readings, by as much as 0.2–0.3 pH unit in a 1 pH unit range. Thus, as recommended in the past for conventional IEF, pH readings should be taken at low temperatures (for IPGs at 10°C) and under an inert gas (nitrogen) atmosphere. With these precautions, the discrepancy between predicted and experimentally found pH values never exceeds 0.1 pH unit. For instance, when focusing haemoglobin A (HbA) in IPGs[29], we have always interpolated a p*I* value (at 10°C) of 7.33 ± 0.05. In mixed Ampholine–Immobiline gels, the p*I* of HbA at 23°C in air was as low as 7.05 but, on reading at 10°C under nitrogen the

value was 7.25 ± 0.1, in acceptable agreement with the expected value. Of course, pH readings close to the anode and cathode extremes are meaningless, as here the pH decays or increases exponentially, owing to diffusion of lower and higher p*I* carrier ampholytes present in the wider CA range. When working in even narrower pH ranges (*e.g.*, 0.5 pH unit), we have shown the feasibility of still measuring a pH gradient (see Fig. 8) but definitely with a greater scatter of experimental points. This is probably due to problems related to the electrode, rather than to the mixed CA-IPG technique adopted. In reality, over a 0.5 pH unit span, as we cut more than 20 gel slices, the pH increment in adjacent slices is expected to be of the order of 0.025 pH unit. Notwithstanding the availability of modern pH meters reading to three decimal places, in reality the error in pH readings is already greater than 0.05 pH unit, so that the reading of such small pH increments becomes problematic. The alternative would be to read such minute pH increments with a differential pH meter, which has a sensitivity down to five decimal places[30] (work in progress). Otherwise, in ranges of only 0.5 pH unit or narrower, one could try to prepare a carbamylated charge train[31], with a series of spots at well defined charge intervals and p*I* values, focusing within the limits of the selected IPG gradient. Finally, in ultranarrow pH gradients, *e.g.*, 0.1–0.2 pH unit, there will be no alternative but simply to rely on the calculations made with the Henderson–Hasselbalch equation for creating such a narrow range and interpolate the p*I* value simply from the focusing position in the gel.

Other approaches

pH assessments in a plain IPG gel with a surface electrode should not be attempted, as there will be some electromotive force and the pH meter will flash some values; the physico-chemical meaning of such readings, however, remains a mystery to us. Even in conventional CA-IEF, in fact, readings with a surface electrode are not completely reliable. However, such measurements have been reported for IPGs[32]; as the data were confined to acidic pH ranges, it is possible that the higher conductivity of these pH gradients will allow more reliable readings. In fact, in the past we have tried to make pH measurements in IPG gels via conductivity determinations by using the well known equation

$$\lambda = c\, m\, F \tag{1}$$

where λ (μS cm^{-1}) is the conductivity, c is the molarity of an ion having a given mobility m and F is the Faraday (96 500 C mole^{-1}). By assuming that c is the proton molarity at the pH in the gel at which conductivity measurements (in μS cm^{-1}) have been taken, and by knowing its mobility in free solution ($314 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}), substitution of the known values of λ, m and F in eqn. 1 would allow the determination of c (in this instance [H$^+$]) and from this value a measure of pH (it is assumed here that the proton mobility does not change in a gel phase). This relationship did seem to hold, in fact, up to pH 5, above which the theoretical and experimental curves began to diverge[33]. In another approach, we tried to prepare reversible gels cross-linked with bisacrylylcystamine[34]. The excised gel slices would then be solubilized with excess of 2-mercaptoethanol or dithiothreitol and the pH read in solution. Again, this approach did seem to work but only up to *ca.* pH 7; at higher pH the excess of thiol reagent used in solubilizing the gel began to buffer and the theo-

retical and experimental curves diverged as the pH increased[35]. It therefore appears that at present the only reliable method for pH assessments in IPGs is still the use of mixed Ampholine–Immobiline gels. A preliminary report on such an idea has also been presented by Fawcett and Chrambach[36].

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