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AMPHOLINE–AMPHOLINE INTERACTION AS A CAUSE OF pH GRADIENT DRIFT IN ISOELECTRIC FOCUSING

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SUMMARY

Two mechanisms for the cathodic drift in isoelectric focusing are proposed: (1) Ampholine–Ampholine interaction between acidic and basic species, particularly in the pH range 6–7; the aggregates are slowly split during the focusing process, to an extent proportional to the square of the voltage applied; and (2) the presence of transient species of "poor" carrier ampholytes, particularly at alkaline pH. The resultant of these two "transient states" is to set in motion a "cascade" effect on the "steady-state" species, the net effect of which is a drift to the cathode. The markedly different behaviour of acidic and basic carrier ampholytes is demonstrated via binding to a neutral detergent, Nonidet P40.

INTRODUCTION

From the early days of isoelectric focusing (IEF) in gels, it has been apparent that the steady-state ("equilibrium") reached, with stationary carrier ampholyte distribution and protein patterns, is subject to rapid decay with concomitant transport of samples and Ampholine mainly towards the cathode. This pH gradient instability was called a "plateau phenomenon"[1,2] when linked to the observation of a flattening in the centre of the pH gradient, or "cathodic drift"[3] when linked to the main direction of the flow. A dramatic representation of this phenomenon can be seen in a series of illustrations published by Davies[4], covering an 8-h IEF experiment with haemoglobins. The cathodic drift also occurs when Ampholine is replaced by buffers[5] or when strongly acidic and basic electrolytes are replaced by buffer electrolytes[6]. Even under conditions where pH gradient stabilization is achieved, the residual drift is in the cathodic direction[7,8]. Only recently a unique example of "anodic drift" has been reported[9].

Several explanations have been advanced for the mechanism of pH drift in IEF[10], and experimental evidence in favour of different models has been given by Fawcett[11] and Baumann and Chrambach[12]. A valuable survey of the literature data was put forward by Rilbe[13], who concluded that "most observed forms of pH gradient instability can be explained by electroosmotic water flow and hydration water transport together with secondary phenomena".

During several years of use of the IEF technique, we had constantly observed that pH gradients and protein patterns obtained in the presence of 8 *M* urea were much more stable and sharper than equivalent pH gradients and protein profiles obtained in standard gels without urea or in gels containing iso-viscous additives. We have further investigated this phenomenon and we present here a new model.

EXPERIMENTAL

Materials

Acrylamide, N,N′-methylenebisacrylamide (Bis), ammonium persulphate, N,N,N′,N′-tetramethylethylendiamine and urea were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Ampholine, pH range 3.5–10, and ^{14}C-labelled carrier ampholytes were purchased from LKB (Bromma, Sweden) and sucrose and glycerol (analytical-reagent grade) from Merck (Darmstadt, G.F.R.). Nonidet P40 was obtained from BDH (Poole, Great Britain).

Isoelectric focusing

Gel slabs, 2 mm thick, containing 6% acrylamide (the ratio of acrylamide to Bis being 25:1) and 2% Ampholine, pH 3.5–10, were cast in a standard LKB 2117 Multiphor cell (total volume 62 ml). The anolyte and catholyte were 1 *M* orthophosphoric acid and 1 *M* sodium hydroxide solution, respectively. A constant power of 12 W was delivered with an LKB 2103 power supply for 1 h. The experiment was then continued at a constant voltage (800 V, *i.e.*, 80 V/cm) to the end of the run.

Cathodic drift measurements

The gel was usually cast into two halves[14], one being the control and the other containing either 8 *M* urea, 19% sucrose or 22% glycerol. These three levels of additives are iso-viscous at 20°[15]. The two halves of the gel were physically separated by cutting, after polymerization, a narrow (2-mm) gel strip at their junction. Also the anode and cathode strips were applied separately to the two gel halves. Care was taken to have the same volume for the two gel slabs (30 ml), the same surface area (12 × 10 cm) and the same length and weight of the electrode strips. In addition, identical volumes of anolyte and catholyte were applied to the two halves of the gel. It is well known that in horizontal gel slabs the cathodic drift is manifested as an accumulation of free liquid on the gel surface close to the cathode. Taking advantage of this phenomenon, we have measured the pH drift by carefully wiping away, at given intervals, these pools of liquid near to the cathode with pre-weighed pieces of filter-paper, and by weighing the liquid withdrawn. Care was taken to sweep the same gel area each time, to avoid scratching the gel surface and to avoid withdrawing catholyte from the cathodic filter-paper strip. This operation is easy to perform, gives highly reproducible results and does not physically or mechanically disrupt or distort the focusing gels. Originally, when we attempted to measure the pH drift by removing and weighing, at intervals, the anodic and cathodic filter-paper strips, we found this procedure to be highly erratic and to severely disturb the focusing (*e.g.*, the gel slab was often detached from the glass surface and stretched, resulting in an uneven field strength, distortion of Ampholine patterns and this "mechanically induced" pH drift bore no relationship to the real mechanism of pH gradient instability). In weighing the liquid transported to

the cathode, no correction was made for the possible increase in density of the liquid withdrawn from the additive-containing gels. By this procedure, no "anodic" drift could be measured as at no time was there an accumulation of liquid at the anodic gel surface.

β-Spectrometry

When using ^{14}C-labelled carrier ampholytes, after focusing the slab was cut into 30 slices (each 3 mm wide), which were transferred to plastic vials to each of which 1 ml of distilled water was added. After shaking overnight, 8 ml of Instagel counting solution (Packard) were added and the vials were read on a Beckman 31 33T liquid scintillation counter.

RESULTS

Fig. 1 shows the cathodic drift measured in control gels and in gels containing either 19% sucrose, 22% glycerol or 8 *M* urea. It can be seen that in the first three gels no measurable cathodic drift begins before the first 4 h of focusing. This is in agreement with most of the data available on pH gradient decay (*e.g.*, in the time sequence photographs of Davies[4], the drift is seen to begin after 2–3 h of focusing, but he used a voltage gradient of 120 V/cm, a level which is known to increase the drift markedly[16]).

If we compare the liquid transport in the control and urea-containing gels, we can see a considerable difference between the two systems: whereas in the control gels the drift proceeds at a rate of 60 mg/h, in 8 *M* urea-containing gels it is only 7 mg/h. One might argue that this is merely due to an increased viscous drag in the additive-containing gels. However, when the drift was measured in iso-viscous gels, containing either 19% sucrose or 22% glycerol, the liquid transport was reduced to half that of the control gel (28 mg/h), but was still substantially higher (by a factor of four) than the drift in 8 *M* urea-containing gels. Moreover, in this last instance, the onset of any appreciable drift was much delayed, starting only after 7–8 h of focusing.

Our results on the moderate effect of sucrose and glycerol on cathodic transport of liquid are in agreement with those of Fawcett[11] but contrary to those of Chrambach *et al.*[2]. It is of interest that, in the control gels, although the total amount of liquid transported over a 10-h period (300 mg) is relatively small in comparison with the total gel volume (30 g) (*i.e.*, it is only 1% of the total gel volume, assuming equal densities), its effects on the separation process are disastrous, as it is enough to wash out at the cathodic end most of the proteins with p*I* values above pH 6. It should also be noted that the liquid transported to the cathode comes from the gel itself and not from the anodic or cathodic compartments, as the weight of the electrodic filter-paper strips remained unaltered during IEF (results not shown). Thus, our results indicate a special "urea effect" on the pH gradient decay, an effect which is manifested in two macroscopic features: (a) a much reduced liquid transport to the cathode, even when compared with iso-viscous systems, and (b) a much delayed onset of cathodic drift. We present here a new hypothesis to account for this phenomenon namely that Ampholine–Ampholine interaction is one of the causes of cathodic drift, which is severely quenched by urea via disruption of the complexes. Experiments with ^{14}C-labelled carrier ampholytes have been undertaken to test this hypothesis.

Fig. 2 shows the p*I* distribution profile of focused [^{14}C]Ampholine, in the pH

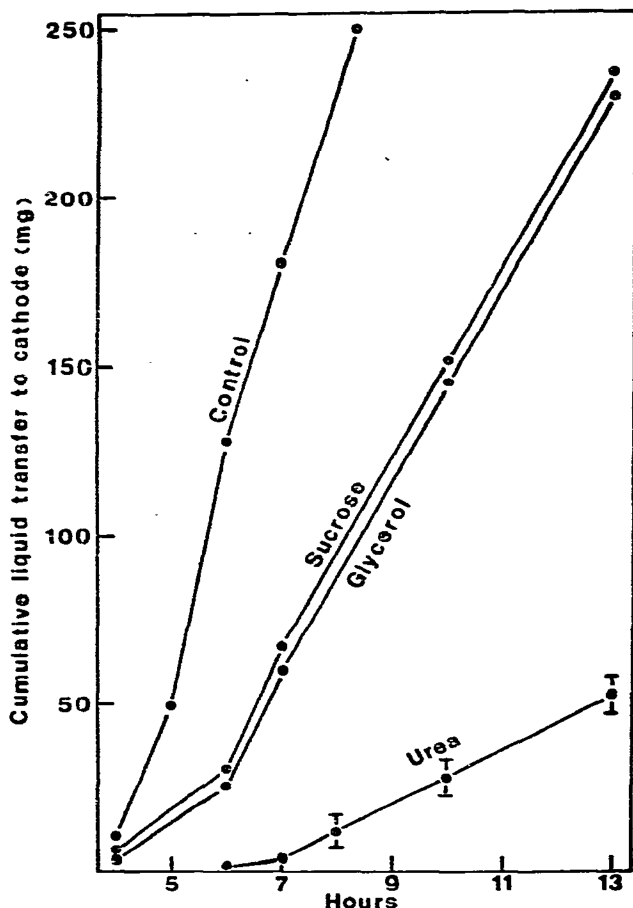

Fig. 1. Cumulative net transfer of liquid at the cathodic gel end. The control gel contains 6% acrylamide and 2% Ampholine, pH range 3.5–10. In addition to this, the other gels are polymerized with 19% sucrose, 22% glycerol or 8 *M* urea. The bars on the urea curve represent the scatter of the results in different experiments.

range 3.5–10. We selected three peaks, one acidic (p*I* 5.2), one neutral (p*I* 6.8) and one basic (p*I* 8.2), which were cut out and re-run in the presence and absence of urea, in order to compare the behaviour of a rather small population of species (for this purpose, 3-mm wide gel strips, representing only one thirtieth of the gel length, were cut parallel to the electrode strips).

In order to ensure that no interacting species, perhaps with widely different p*I* values ,could be isolated, the first run was performed in 8 *M* urea-containing gels. It can easily be demonstrated that, on a 10-cm long gel, containing a pH 3.5–10 gradient, a 3-mm wide gel segment should contain only the Ampholine species that have p*I* values in a pH range of about 0.25 pH unit. However, when these very narrow ranges were re-focused, the picture looked entirely different. As shown in Fig. 3, when Ampholine species occupying 3% of the gel length are re-focused in the absence of urea (with or without glycerol or sucrose), they are spread over a much larger gel length and increasingly wider pH intervals.

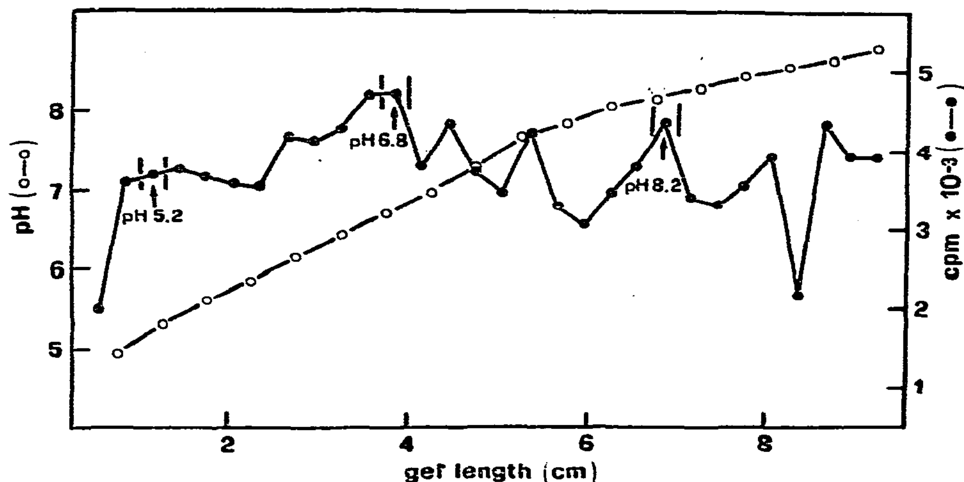

Fig. 2. Distribution of ^{14}C-labelled carrier ampholytes. The gel slab contained 6% acrylamide, 2% Ampholine, pH range 3.5–10, and 1 μCi of labelled Ampholine, pH range 3.5–10. The two vertical lines, arrows and pH values given represent three very narrow populations of Ampholine, contained in a 3-mm wide gel segment, used for the re-focusing experiment shown in Fig. 3.

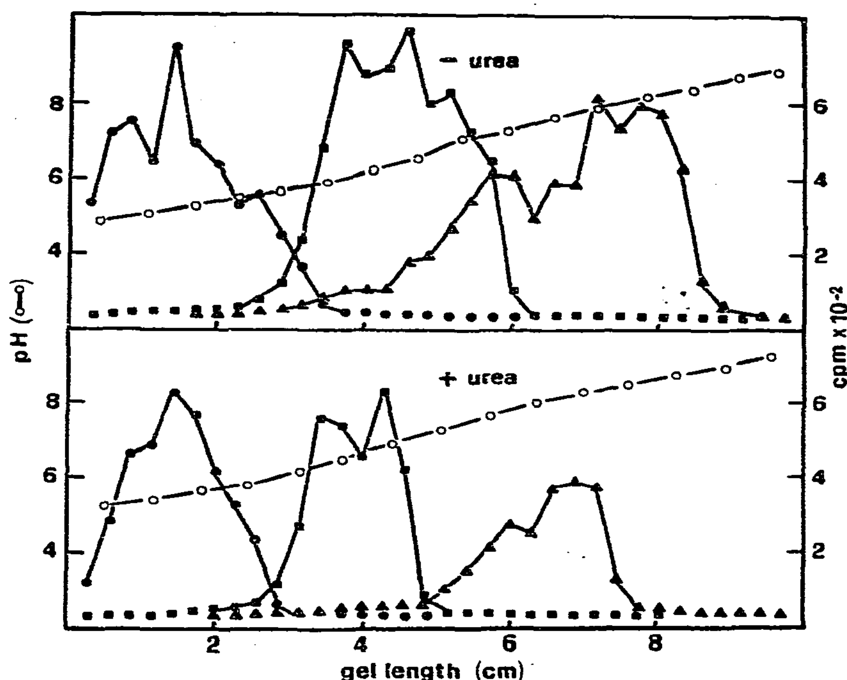

Fig. 3. Results of re-focusing experiment. The pI 5.2 (●), pI 6.8 (■) and pI 8.2 (▲) peaks in Fig. 2 were re-run in a pH 3.5–10 gradient in the absence (above) and presence (below) of 8 M urea. The three peaks were re-run in parallel tracks (side by side), by the laying-on technique by loading at the anodic gel region after 1 h of pre-focusing. After an additional 1 h of focusing, the sample gel strips were removed and focusing was continued for a further 2 h at 80 V/cm. ○, pH gradient.

This phenomenon is not constant, but becomes more and more pronounced on going from acidic to neutral and basic carrier ampholytes. Thus, the p*I* 6.8 peak (spread over 0.25 pH unit and 3% of the gel length) is now spread over 2 pH units (pH 5.5–7.5) and occupies 35% of the gel length. The p*I* 8.2 peak (also spread over 0.25 pH unit and 3% of the gel length) is now distributed over 2.5 pH units (pH 6–8.5) and over 52% of the gel length. This phenomenon is considerably reduced, but not abolished, in 8 *M* urea gels. Thus, the p*I* 6.8 peak now encompasses 1 pH unit (pH 6–7) and 20% of the gel length, while the p*I* 8.2 peak now spans more than 1 pH unit (pH 7.2–8.5) and 25% of the gel length. The significance of these results is discussed below.

DISCUSSION

Our results strongly suggest Ampholine–Ampholine interaction as one of the causes of cathodic drift. The "urea effect" that we have demonstrated is clearly not due to an increased viscosity of the medium. In IEF, urea has recently been demonstrated effectively to disaggregate complexes between Ampholine and acidic dyes[14], heparin[17,18], polyanions[19] and also with one protein, rat incisor phosphoprotein[20]. The possibility of a direct Ampholine–Ampholine interaction has not been suggested before, but is not unreasonable in view of the following considerations.

(1) Structure of carrier ampholytes (CA). Let us consider the structure of a particular class of CAs, the reaction product of pentaethylenehexamine (PEHA) and acrylic acid (AA), as described in the literature[21–23]. It is reasonable to assume that the species of highest p*I* could be the product $(PEHA)_1(AA)_1$, while the species of lowest p*I* could be $(PEHA)_1(AA)_6$, with intermediate stoichiometries at intermediate p*I* values. Incidentally, this is equivalent to stating that species of low p*I* have higher molecular weights than species of high p*I*, a fact that has been demonstrated elegantly by Gelsema *et al.*[24] solely on the basis of conductivity and buffering capacity considerations. Now, at pH 6–7, $(PEHA)_1(AA)_6$ will behave as a polyanion while $(PEHA)_1(AA)_1$ will act as a polycation thus allowing for direct ion–ion binding, stabilized perhaps by secondary interactions (such as weak hydrophobic interactions). Therefore, if the species that focus, especially in the pH range 6–7, are not pure Ampholine species, but aggregates thereof, it is conceivable that, as these aggregates are split by the current, they will have to migrate electrophoretically towards their p*I*, thus setting in motion a "cascade" or "domino" effect, *i.e.*, forcing species encountered in their path to assume a charge and migrate too. This concept was, in fact, already contained in the literature, when it was stated that one of the possible causes of "electrophoretic" migration of "isoelectric" Ampholine could be the "progressive gain or loss of charged ligands" by Ampholine[12].

No-one had extended this idea to the logical conclusion that the most likely ligand of an Ampholine could be another Ampholine molecule. In agreement with our hypothesis of an interaction among acidic and basic carrier ampholytes in the pH range 6–7, Frater[25] has reported that, after a certain duration of focusing, there is an increase in the resistance in the neutral centre of a gel in the pH range 3–10, with a concomitant decrease in bands stainable with Coomassie violet. Simultaneously, resistance at both ends of the gel decreases and the concentration of the Ampholine bands at the ends of the gel increases. Our model would also explain why the drift is

more pronounced at higher field strengths. If the drift is set in motion by Ampholine–Ampholine complexes disaggregating under the influence of the electric field, it should be recalled that as early as 1946 Ogston[26] demonstrated that complexes of charged species will dissociate during an electrophoretic separation to an extent proportional to the square of the voltage gradient.

(2) Behaviour of carrier ampholytes. If the CAs have structures ranging from the two limiting formulae, $(PEHA)_1(AA)_1$ and $(PEHA)_1(AA)_6$, it is clear that their properties must vary greatly in going from one extreme to the other. If we could draw a hydrophobicity scale, for instance, it is clear that the species of high p*I* (especially at their isoelectric point) would be much more hydrophobic than the components of low p*I*. In fact, $(PEHA)_1(AA)_1$, at its p*I*, can have only one positive and one negative charge (*i.e.*, five out of six nitrogen atoms will be deprotonated in the polyamine), whereas $(PEHA)_1(AA)_6$, at its p*I*, will probably have an average of four positive and four negative charges.

This behaviour is demonstrated in the presence of neutral detergents, such as Nonidet P40. We recently stated that carrier ampholytes form complexes with Nonidet P40[27], and Fig. 4 shows these complexes, precipitated in 5% trichloroacetic acid and 30% ethanol. The detergent concentration, after focusing, is no longer uniform throughout the gel, but increases sharply along the Ampholine ridges. Moreover, basic Ampholine species are seen to bind much more detergent than neutral and

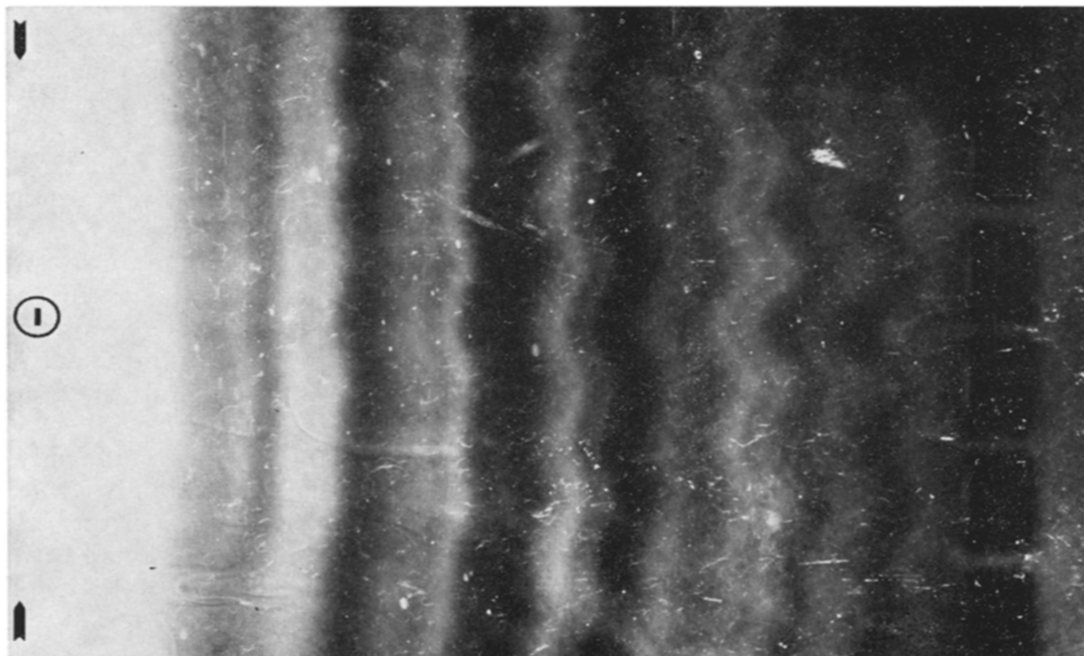

Fig. 4. Binding of carrier ampholytes to Nonidet P40. The gel contained 6% acrylamide, 8 *M* urea and 3% Nonidet P40. After 2 h of focusing, the slab was immersed in 5% trichloroacetic acid and 30% ethanol at room temperature. Opaque bands of the Ampholine–Nonidet P40 complexes reached maximal intensity in 1 h and were photographed in a dark field. The two arrows at the top represent the end of the gel at the cathode, where alkaline Ampholine species form a continuum of white zones of complexes with Nonidet P40.

acidic species. Thus, in addition to ionic interactions in the pH range 5–7, in the basic pH region there might be carrier ampholytes interacting also via weak hydrophobic bonds. If this is so, this might explain the prevalently unidirectional (cathodic) drift.

(3) "Poor" versus "good" carrier ampholytes. Our hypothesis fails to explain why, upon re-focusing, an Ampholine group with a p*I* range of 0.25 spreads over 1 or more pH units even in the presence of 8 *M* urea. It might be argued that even 8 *M* urea fails to disaggregate these complexes completely, but it is difficult to believe in such strong Ampholine interactions. Therefore, we have to resort to a second hypothesis, presented in 1976 by Rilbe (quoted in ref. 28). According to Rilbe, there are "poor" (focusing broadly) and "good" (focusing sharply) carrier ampholytes. Also, "poor" carrier ampholytes are important to the economy of IEF, as they are needed to fill up partially conductivity gaps in between "good" carrier ampholytes. However, on prolonged focusing, also these "poor" CAs will keep condensing at their respective p*I* values, thus building up zones of "transient species", which will move against a background of "steady-state species". In fact, the coexistence of "steady state" and "transient state" in IEF has recently been demonstrated[29].

By a similar reasoning, we could imagine that these transient peaks might set in motion a "cascade" or "domino" effect on the "steady-state" peaks. This hypothesis would explain the distribution profile of Fig. 3. The broad re-distribution in the absence of urea could be due to "good" and "poor", as well as interacting, carrier ampholytes. The narrower re-distribution in the presence of 8 *M* urea would only represent the actual profile of "good" and "poor" CAs, originally present in the 3-mm gel slices, in the absence of any Ampholine–Ampholine complex. If this is so, then we should also conclude that there are more "poor" CAs in the alkaline pH region than in the acidic zone, as the peaks in Fig. 3 keep progressively broadening toward the alkaline end, and as the drift is mostly cathodic.

Is it reasonable to assume that "poor" CAs are mostly found above pH 7? We believe that it is. According to Svensson[30], "good" CAs must exhibit a very small p*I*—p*K* difference (the limiting value being 0.6; "poor" carrier ampholytes will have p*I*—p*K* values of 2 or greater). Now, above pH 7, the only p*K* values that a CA can exploit to fulfill this requirement are three out of six p*K* values of the amino groups in PEHA. However, below pH 7, a CA has many more chances of fulfilling the Svensson inequality because its p*I* can be close to either the p*K* of an amino group in the polyamine or the p*K* of the carboxyl group of acrylic acid (or other compounds, such as itaconic, sulphonic or phosphonic acids, often used in mixture with acrylic acid).

We conclude by stating that our two hypotheses are not, by any means, the only explanations of cathodic drift. Other phenomena must also occur and certainly a role must also be played by electroendoosmosis, generated by fixed charges in the gel matrix [13]. We also agree that the experimental evidence which we have presented here for the two hypotheses is only indirect. However, a direct demonstration of Ampholine–Ampholine interaction might be a difficult, if not impossible, task.

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