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MECHANISM OF WATER EXUDATION FROM MIXED-BED AMPHOLINE–IMMOBILINE GELS FOR ISOELECTRIC FOCUSING

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SUMMARY

In mixed-bed Ampholine–Immobiline gels, water is exuded from the polyacrylamide matrix with severe risks of short-circuits, sparks and burning on the gel surface. Such water loss is proportional to the amount of carrier ampholytes added to the immobilized pH gradient. It is hypothesized that, on diffusing away from the p*I* zone, the charged species in equilibrium with the isoelectric form (cations and anions) become more hydrated and sequester water from the surroundings. As the voltage drop forces these charged species back to the p*I* zone, they return to a state of zero net charge and release the excess water. This results in accumulation of water at the Ampholine p*I* zone and dehydration on either side of the peak. Chaotropes, which disrupt the water structure (*e.g.*, 8 *M* urea), or polyols (*e.g.*, sucrose, in 30–50% concentration), which compete for available water, are effective in disrupting this water pumping phenomenon and minimizing gel exudation.

INTRODUCTION

In 1982, immobilized pH gradients[1] (IPG) were introduced to overcome the problems with conventional isoelectric focusing[2] (IEF) in amphoteric buffers, namely (a) instability of the pH gradient with time (cathodic drift); (b) lack of even conductivity and buffering capacity[3]; (c) extremely low and unknown ionic strength[4]; and (d) limited load capacity, mostly due to isoelectric precipitation caused by the low ionic strength environment[5]. Since then, IPGs have performed extremely well and have effectively proved their capability to solve the above problems[6]. However, we have recently become aware of another problem, namely that in IPGs, especially in the intervals across neutrality and with hydrophobic or poorly soluble proteins, the protein sample often precipitates at the application point and/or smears on the gel surface rather than collecting into sharply focused bands. This unexpected problem required a new solution; we therefore resorted to a combination of two types of

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techniques, a primary, immobilized pH gradient supporting a secondary, carrier ampholyte-generated pH gradient. This accomplished two functions: (a) in focusing soluble proteins across neutrality, it increased the background conductivity to an extent that allowed rapid protein migration; (b) in focusing membrane proteins in detergent solutions, it provoked the formation of mixed detergent–Ampholine micelles, with a higher solubilizing power for membrane components[7,8]. The mixed bed technique appears most versatile and is rapidly becoming popular.

However, even with the latter, efficient technique, there appeared to be one additional problem: when the gel was focused at high voltages (>2000 V), as is typical of IEF in IPGs, it started to exude water, followed by sparks and often burning. In this paper we propose a mechanism for this water transport (not of electroendosmotic origin) and suggest simple solutions for it.

EXPERIMENTAL

All chemicals (Immobilines, carrier ampholytes, acrylamide, bisacrylamide, TEMED, persulphate and urea) and equipment (Ultrophor electrophoresis chamber, Multitemp thermostat and Macrodrive 5 power supply) were obtained from LKB (Bromma, Sweden). For casting of mixed-bed Immobiline–Ampholine gels, see refs. 7 and 8. For photographing the ridges of focused carrier ampholytes (refractive index gradients) we used shallow side illumination against a black background[9]. For measuring water exudation, two techniques were adopted: (a) the liquid was carefully blotted from the gel surface with a pre-weighed Kim-wipe tissue; (b) the gel was weighed before and after blotting and the difference in weight was taken as exuded liquid. These methods gave identical results.

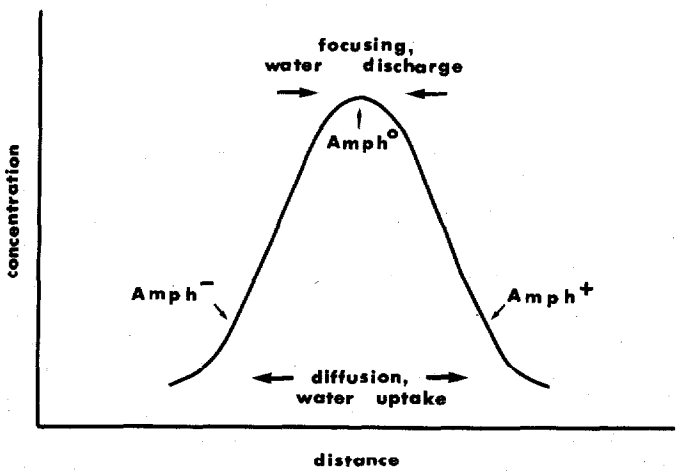

Fig. 1. Hypothetical model of the concentration distribution of a focused carrier ampholyte. The peak is subjected to two opposite forces: a focusing process, driven by the electrical field, and a diffusion process, driven by the absolute concentration in the zone. The diffusion event is depicted as a hydration step, as the anions ($Amph^-$) and cations ($Amph^+$) in equilibrium with the isoelectric species ($Amph^0$) acquire more hydration water. The focusing event is represented as a water deposition step at the p*I* zone.

RESULTS

Fig. 1 depicts a steady-state distribution profile of a focused, amphoteric buffer (carrier ampholyte). According to Svensson[10], this profile should approach a gaussian distribution and should represent the balance of two opposite forces: diffusional, which tends to remove the species from the p*I* zone, and electrical, which tends to transport the ampholyte back to its p*I* position. Thus, throughout a conventional IEF experiment, each amphoteric buffer component is subjected to a "to-and-fro" movement (which also ensures a substantial background conductivity). In the p*I* zone the carrier ampholytes (which are a mixture of oligoamino and oligocarboxylic compounds) have zero net charge; this also represents a state of minimum charge. As they diffuse away from the p*I* zone, they acquire additional charges (negative in the anodic direction, positive towards the cathode) and thus they increase their hydration shell. Once they have acquired sufficient net charge, the electric field will drive them back to the p*I* zone, where they will again be isoelectric and discharge the excess water. As depicted in Fig. 1, it is our contention that this to-and-fro oscillation of each ampholyte about the p*I* is concomitant with a process of water uptake (away from the p*I*) and water discharge (in the p*I* zone). Each ampholyte thus represents a mini-water pump with a flow quasi-symmetric towards its p*I* direction.

In order to prove this hypothesis, we performed the experiment illustrated in Fig. 2: water exudation was measured as a function of Ampholine load in an IPG gel. Clearly there is a positive correlation between the water transport and the amount of carrier ampholyte in the gel, suggesting that the latter act as units for water pumping. Conversely, addition of chaotropic agents or water-sequestering molecules should suppress or strongly reduce this effect. A typical chaotrope is urea, which

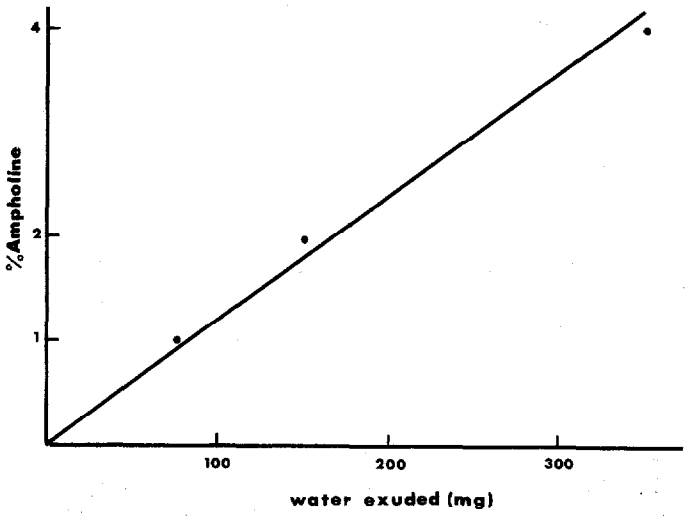

Fig. 2. Water exudation as a function of level of carrier ampholyte in the Immobiline gel. A 5%T, 4%C IPG gel, in the p*I* range 4–8, was polymerized, washed, dried, divided into three identical strips (8 cm wide, 11 cm long, 1 mm thick) and reswollen in 1%, 2% or 4% Ampholine in the pH range 4–8. After focusing for 6 h at 2000 V (at equilibrium) at 10°C, the total water exuded by each gel was carefully blotted and weighed.

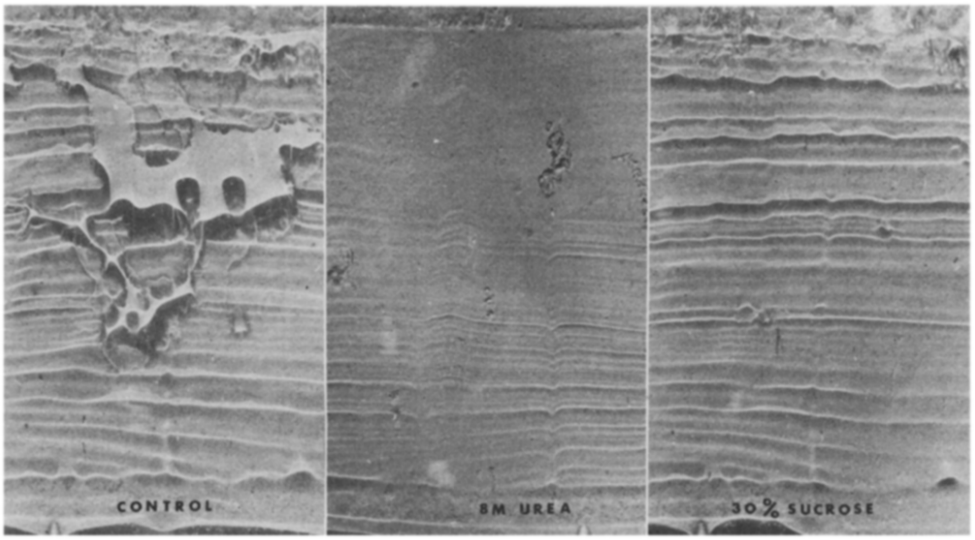

Fig. 3. Quenching of water exudation in different additives. The same gel as in Fig. 2, divided into three strips, was reswollen either in 2% Ampholine alone (left), in 2% Ampholine + 8 *M* urea (centre) or in 2% Ampholine + 30% sucrose (right). After focusing for 6 h at 2000 V, the Ampholine ridges were photographed with shallow side light against a black background.

disrupts the hydrogen bonds of water clathrates, while polyols (*e.g.*, soluble dextrans) or simpler molecules, such as sucrose, should effectively compete with carrier ampholytes for hydration water. As can be seen in Fig. 3, these expectations are fulfilled: whereas in the control gel water exudation is clearly visible along the Ampholine ridges in the gel surface, this phenomenon is abolished in 8 *M* urea and strongly diminished in a 30% sucrose gel. In the latter two instances, in fact, the focused Ampholine zones are less prominent than in the control gel, suggesting that the amphoteric buffers are less hydrated than in the absence of chaotropes or water-sequestering agents.

DISCUSSION

According to Svensson[10], the focusing of a protein in IEF can be described by the following differential equation, which represents the equilibrium condition between simultaneous electrophoretic and diffusional mass transport:

$$C\,u\,E = (\mathrm{d}C/\mathrm{d}x)\,D$$

where C is the protein concentration at the level x in the separation column, u is its mobility ($cm^2/V \cdot s$) at that point, E (V/cm) is the field strength and D (cm^2/s) is its diffusion coefficient. This equation applies also to the carrier ampholytes (the amphoteric buffers creating and stabilizing the pH gradient in the gel) with the proviso that their diffusion coefficients will be much greater than for proteins (600 daltons *vs.* an average 60 000 daltons for the latter)[11]. Thus their oscillation about the p*I* position and their ability to accumulate water will be much greater than in the case

of proteins. This water transport, though, will not be completely symmetrical: as anions (*e.g.*, carboxyl groups) are considerably more hydrated than cations (*e.g.*, amino groups), it is to be expected that these two ionic species, in equilibrium with the truly zwitterionic or isoelectric form, will transport water in an asymmetric fashion, the anionic shoulder of the Ampholine gaussian being at any given time more "hydrated" than its cationic counterpart.

It should be appreciated that the phenomenon described here is completely different from electroendosmosis; in the latter, water transport (which can be in either direction, anodic or cathodic) is generated by fixed charges in the anticonvective matrix (*e.g.*, agarose, cellulose, polyacrylamide, dextrans) or in the container (electrophoretic chamber). The fact that electroendosmosis results, in most instances, in water transport towards the cathode is purely accidental and is due to the condition that most capillary systems used for support contain fixed negative charges (carboxyls and sulphate for agarose and polyacrylamide, carboxyls alone for cellulose and dextrans), which force an equivalent number of cations to migrate to the negative pole and there, by deprotonation, discharge their water. We have in fact demonstrated that, by incorporating positive charges in a polyacrylamide matrix, the electro-osmotic flux is reversed to the anode[12]. In the present case the water transport is not towards, but away from the electrodes; the system can be envisaged as a series of mini-water pumps linked together in a continuous fashion from the anode to the cathode, each one building up water at its p*I* position. Hence the fact that their hydration shell is substantially reduced with chaotropes or with water-sequestering agents[13] has the beneficial effect of quenching this problem of water exudation from mixed-bed Ampholine–Immobiline gels (the phenomenon can never be totally suppressed, but can be reduced to such an extent as to allow proper focusing conditions and the application of sufficiently high voltages, *e.g.*, 2–2500 V). It cannot be denied that part of this phenomenon could also be due to the increased viscosity of 8 *M* urea or 30% sucrose solutions, which could dampen the oscillation of carrier ampholytes and thus reduce their water transport; however, owing to their low molecular mass, the viscosity effect should not be as prominent as it is with macromolecules.

At this point it is natural to ask why water exudation is not seen in conventional IEF gels, as our proposed model in Fig. 1 should apply here also. In reality, in plain IEF in amphoteric buffers, in the absence of carrier ampholytes, the situation is different: the stack of focused carrier ampholytes is never completely arrested but continues to drift slowly towards the cathode as a result of the strong, underlying electro-osmotic flow and of the fact that the cations in equilibrium with the isoelectric species have a higher electrophoretic mobility than the anions. Hence the displacement of the train of zones towards the cathode obliterates the micro water transport in each Ampholine zone and results in an unidirectional (and abundant) accumulation of water at the cathode. Conversely, in IPGs, the primary pH gradient, which is covalently bound to the matrix, prevents the decay of the secondary, carrier ampholyte-generated gradient, thus amplifying the water transport of each ampholyte at its p*I* position.

CONCLUSION

As mixed-bed Ampholine–Immobiline gels will become increasingly popular,

some guidelines for their proper use may be useful: (a) when the gels are run in 8 *M* urea, they can be left on overnight even at 200 V/cm without any danger; (b) when they are run in 30% sucrose, it is best not to exceed 120 V/cm in an overnight run; (c) if for any reason it is impossible to add urea or sucrose, the gel should be run overnight at voltages not exceeding 70 V/cm and then the bands can be sharpened at 200 V/cm for a short time and under constant observation to prevent burning[14]. In a gel that does not contain additives, reducing the amount of Ampholines to a low level (*e.g.*, 0.5%) minimizes water exudation (in gels with additives, we have loaded as much as 4% of ampholytes).

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