ITACONIC ACID CARRIER AMPHOLYTES FOR ISOELECTRIC FOCUSING

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SUMMARY

Commercial carrier ampholytes, obtained by coupling polyethylene poly-amines to acrylic acid, exhibit a conductivity minimum in the pH range 5.5–6.5 owing to the lack of appropriate pK values of the polyamine in this pH region. By replacing acrylic with itaconic acid, it has been possible to effect substantial improvements in the pH range 5.5–6.5 as itaconic acid has a pK_2 value of 5.45. Upon coupling, the pK of the γ-carboxyl group remains virtually unaltered. With itaconic acid carrier am-pholytes it has been possible to improve the conductivity in the pH range 5.5–6.5 by as much as 400% compared with conventional carrier ampholytes. It is suggested that the commercial products should be supplemented with itaconic acid carrier am-pholytes in order to obtain a more uniform conductivity and buffering capacity in the pH range 3–10.

INTRODUCTION

Svensson[1,2] conceived isoelectric focusing (IEF) as the formation of a natural pH gradient due to the attainment of the isoelectric state by amphoteric compounds with a wide variety of isoelectric points (pI). This concept was soon realized in prac-tice with the synthesis by Vesterberg[3] of carrier ampholytes with good conductivity and buffering capacity in the pH range 3–10. Vesterberg[3] proposed a very simple and elegant procedure, by which aliphatic polyamines reacted with acrylic acid, thus form-ing a highly heterogeneous mixture of polyamino polycarboxylic acids. This synthesis was also described in more detail by Vinogradov *et al.*[4] and Righetti *et al.*[5]. It soon became apparent, however, that the commercial product (Ampholine) did not exhibit an even conductivity in the pH range 3–10. Thus Davies[6], comparing Ampholine in the pH ranges 3–6 and 5–8, found that in the latter range a minimum of conductivity was centred around pH 6 (range 5.5–6.5). The resistance values of Ampholine focused around pH 6 were 5–6 times higher than the average resistance of Ampholine of pH 3–6. The existence of a minimum around pH 6 has also been reported by Hag-lund[7] and Righetti *et al.*[5]. Recently, Fawcett[8] has reported the same conductivity

minimum (centred around pH 6.2) not only in Ampholine, but also in Servalyt. Frater's data[9] essentially confirm these findings, although he reports a minimum around pH 4.7 (range 4.5–5.5).

Attempts to synthesize carrier ampholytes with sulphonic and/or phosphonic groups instead of carboxyl groups have worsened the situation: these compounds[10] present a wide gap in the pH range 3.5–6.0.

A minimum of conductivity leads to a minimum of buffering capacity, which in turn means that, whenever these pH ranges are used, the highest potential drop will be located in the pH range 5.5–6.5, while other parts of the gradient will be underfocused. The high field strength in the pH range 5.5–6.5 will often generate considerable Joule heat, with deleterious effects, especially when using high-voltage techniques[11,12]. No attempts have been described so far to correct this deviation from the ideal properties of carrier ampholytes, as envisaged by Svensson[1,2].

In this paper we describe how, by coupling polyamines with itaconic acid, we have been able to effect substantial improvements in the pH range 5.5–6.5.

EXPERIMENTAL

Materials

Tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA) were obtained from Hoechst Italia (Milan, Italy) and acrylic acid, itaconic acid, acrylamide and N,N'-methylenebisacrylamide (BIS) were purchased from Merck-Schuchardt (Munich, G.F.R.). BIS was recrystallized from acetone and the acrylamide from chloroform, as described by Loening[13]. Ampholine, pH 3.5–10, was obtained from LKB (Bromma, Sweden) and Servalyt pH 2–11 was a gift from Dr. N. Grubhofer (Serva, Heidelberg, G.F.R.).

Coupling reaction

The distillation of acrylic acid, TEPA and PEHA, and their coupling process, have been described previously[5]. The scheme of the reaction chamber has been reported by Gianazza and Righetti[14]. As itaconic acid has a low solubility in water (83 g/l at 25°), it was added finely ground to the polyamine solution in the reaction chamber. In a typical experiment, 74 g of PEHA in 148 ml of water were placed in a flask under nitrogen with continuous stirring and 63 g of itaconic acid (powdered) were added over a 1-h period. This gives an N:COOH ratio of 2:1. The flask, tightly stoppered, was then transferred into a vaseline oil-bath at 90° and the reaction allowed to proceed for 4 days with stirring. Under these conditions, titration[3] with potassium permanganate solution revealed that more than 99 % of the itaconic acid had reacted.

Isoelectric focusing

IEF was performed in a thin-layer gel, using an LKB Multiphor 2117 chamber and an ISCO Model 492 constant-wattage power supply. The gels contained 2 % Ampholine or 2 % Servalyt or 2 % synthetic carrier ampholytes and 5 % acrylamide (the acrylamides:BIS ratio being 25:1). IEF was run for 2 h at 13–15 W (final voltage 1000 V) at 4°[12].

Conductivity measurements

Immediately after IEF, the gel was carefully cut into 6 × 0.5 × 0.2 cm slices and each fraction was eluted for 4 h in 8 ml of distilled water. Conductivity measurements were made with a Philips PW 9501 conductimeter in a Philips PW 9512/00 cell at 25°, then pH measurements were made at 25° in each fraction with a Radiometer pH meter, using a combination electrode.

RESULTS

Fig. 1 shows conductivity and pH profiles in TEPA–acrylic acid in comparison with TEPA–itaconic acid carrier ampholytes. TEPA–acrylic acid ampholytes exhibit a conductivity minimum around pH 5.5, while TEPA–itaconic acid ampholytes display a consistently higher and more uniform conductivity in the pH range 3–10. In the region of the minimum, the itaconic acid compounds have a conductivity that is five times higher than the conductivity of acrylic acid ampholytes.
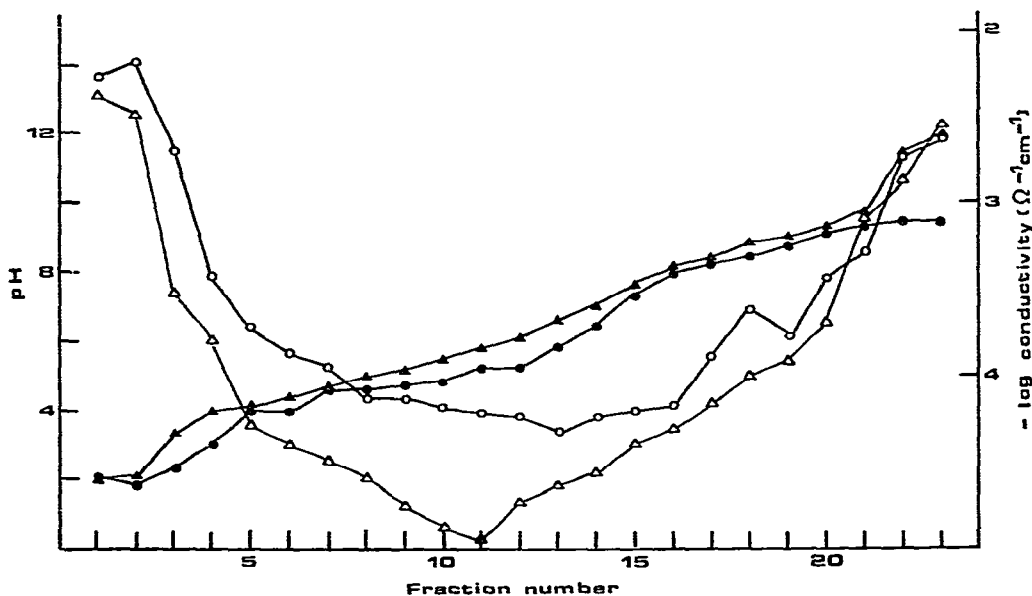

Fig. 1. pH gradients and conductivity profiles of TEPA–acrylic acid (▲, pH; △, conductivity) and TEPA–itaconic acid carrier ampholytes (●, pH; ○, conductivity). IEF, conductivity and pH measurements as described under Experimental. Gel segments 6 cm long and 2 mm thick were cut at 0.5-cm intervals along the focusing path. Each point is the average of three measurements.

Fig. 2 shows a comparison of the same parameters for PEHA–acrylic and PEHA–itaconic acid ampholytes. PEHA–acrylic acid ampholytes exhibit a conductivity minimum around pH 6, while again PEHA–itaconic acid ampholytes exhibit a constantly higher conductivity, which, in the region of the minimum and in its surroundings, is two to three times higher than the conductivity of PEHA–acrylic acid ampholytes.
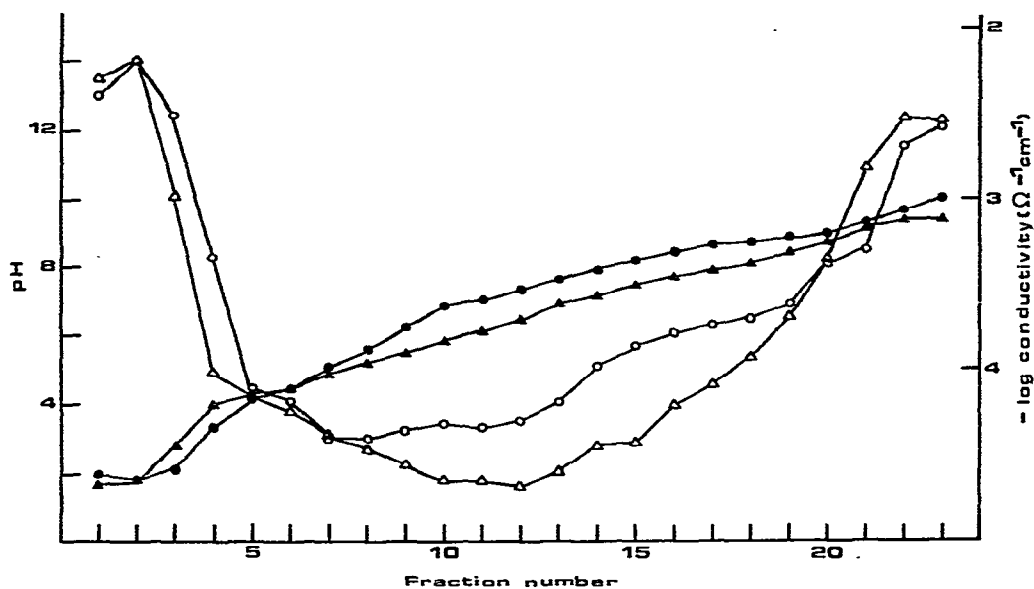
Fig. 2. pH gradients and conductivity profiles of PEHA–acrylic acid (▲, pH; △, conductivity) and PEHA–itaconic acid carrier ampholytes (●, pH; ○, conductivity). All other conditions as in Fig. 1.
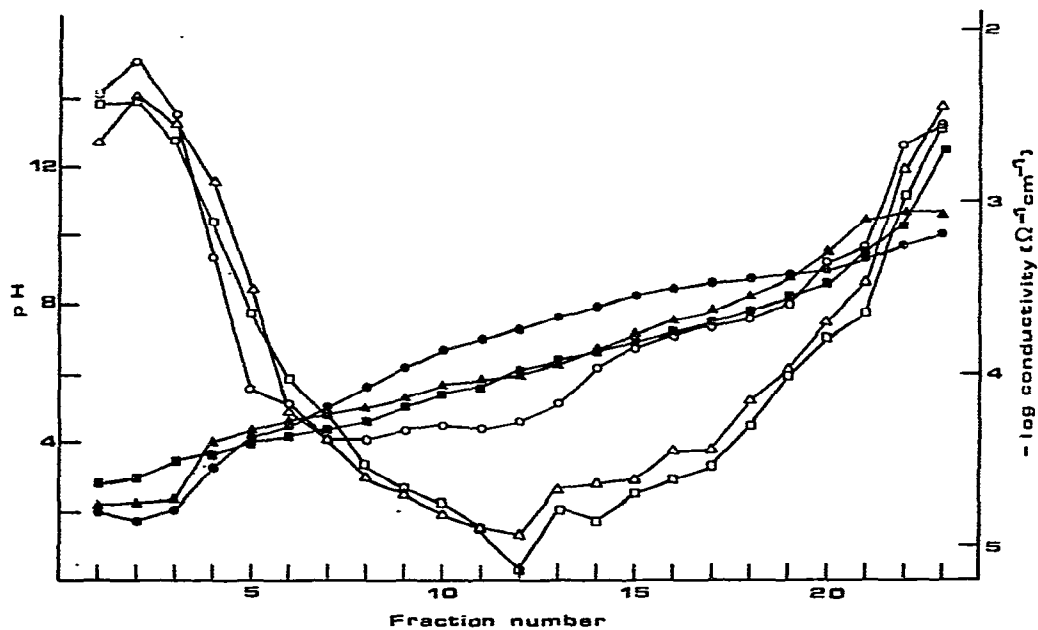

Fig. 3. pH gradients and conductivity profiles of PEHA–itaconic acid carrier ampholytes (●, pH; ○, conductivity), Ampholine (▲, pH; △, conductivity) and Servalyt (■, pH; □, conductivity). All other conditions as in Fig. 1.

Fig. 3 reports the same parameters measured in Ampholine, Servalyt and PEHA–itaconic acid ampholytes. Ampholine and Servalyt display very similar conductivities and pH gradients over the whole pH range 2.5–11. Perhaps there is a trend for Servalyt to exhibit a higher conductivity at acidic pHs (possibly due to sulphonic and/or phosphonic groups) with a lower conductivity at alkaline pHs. Both commercial products, however, display the same conductivity minimum centred at pH 6. In comparison, in the pH region 5.5–8.5, the PEHA–itaconic acid ampholytes display conductivities that are 400–500% better than the conductivities of both Ampholine and Servalyt.

DISCUSSION

We believe that the conductivity minimum in the pH range 5.5–6.5 is not due to the lack of amphoteric species focusing in this region, but to the fact that the compounds focusing in the pH interval 5.5–6.5 are poor carrier ampholytes, i.e., they exhibit too high a value of $pI - pK_1$. If we consider TEPA, for instance, it has a pK_3 of 7.9 and a pK_4 of 4.3. When coupled with acrylic acid (pK 4.25), these two values are lowered to 7.7 and 4.1 (ref. 3); coupled acrylic acid will lower its pK to ca. 3.7 (ref. 3). It is clear that, in TEPA ampholytes, species which focus at around pH 6 will be very poor carrier ampholytes, as they will exhibit $pI - pK$ values close to 2 (an excellent ampholyte would have a $pI - pK$ value close to 0.6). It is possible that even more complex polyamines, such as PEHA, do not have pK values of their amino groups close to pH 6. The situation is not really improved when the polyamine is obtained by polymerizing ethyleneimine with itself or with other amines, such as in the case of Servalyt[15]. The next logical step was therefore to see if we could introduce a functional group with a pK close to 6 by changing acrylic acid with another, more appropriate, compound and we considered fumaric, maleic and itaconic acid. Fumaric acid (pK_1 3.03; pK_2 4.54) and maleic acid (pK_1 1.93; pK_2 6.58) were discarded because they are unreactive and have unfavorable pKs. Only maleic acid, in fact, has a promising pK (pK_2 6.58). In reality, once reacted, it will behave as an N-substituted aspartic acid, and thus exhibit a pK_1 of 2.09 and a pK_2 of 3.86 (or values very close to these, due to the N-substitution).

Itaconic acid (pK_1 3.85; pK_2 5.45) seemed to be the ideal compound. It reacts fairly well and, once reacted, only the carboxyl group in the β-position to the amino group will lower its pK from 3.85 to ca. 3.35, while the γ-carboxyl group will be virtually unaffected or, at the most, will lower its pK by 0.1 pH unit (as determined by comparison with γ-aminobutyric acid).

Itaconic acid carrier ampholytes have not been reported previously nor used in commercial products. It is stated, in fact, that "Ampholine carrier ampholytes are obtained by coupling acrylic acid to a mixture of polyalkylene polyamines"[6]. Servalyt products are produced by reacting polyamines with sulphonic and phosphonic derivatives and with acrylic acid, in order to cover the pH gap 3.5–6 (ref. 15). The use of itaconic acid, however, is covered in Vesterberg's patent[16] but, in the light of the information available, and from our results, it seems logical to conclude that it has really never been used and was mentioned only to protect the patent.

Given the finding that itaconic acid carrier ampholytes improve the conductivity in the pH range 5.5–6.5 by as much as 400%, we strongly recommend them as additives

to the commercial products. For instance, acrylic acid and itaconic acid carrier ampholytes could be mixed in appropriate amounts to obtain a better conductivity and buffering capacity in the pH range 5.5–6.5. The mixture would also probably show many more amphoteric species. Alternatively, mixed-type ampholytes could be synthesized in a sequence, by starting the reaction with itaconic acid (slower reacting species) and then by adding to the reaction mixture, 2 days later, appropriate amounts of acrylic acid. It should also be possible, by increasing the temperature and/or using catalysts, to reduce the reaction time of itaconic acid from 4 days to 1 or 2 days, as is the case now with acrylic acid.

ACKNOWLEDGEMENTS

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