*Journal of Chromatography*, 166 (1978) 55–64


CHROM. 11,157

# COEXISTENCE OF STEADY STATE AND TRANSIENT STATE IN ISOELECTRIC FOCUSING

PAOLO AROSIO, ELISABETTA GIANAZZA and PIER GIORGIO RIGHETTI
*Department of Biochemistry, University of Milano, Via Celoria 2, Milan 20133 (Italy)*
(Received May 20th, 1978)

## SUMMARY

The possibility of transforming a wide (pH 3.5–10) into a narrow pH gradient in isoelectric focusing (IEF) has been examined by the use of different amino acid terminators at the electrodes, *viz.* 50 m*M* Asp, 50 m*M* Trp, 45 m*M* Phe, 0.5 *M* Gly or 0.5 *M* Lys. The pH gradient formation and decay seem to be insensitive to the type of amino acid used or to its molarity. While Ampholine and protein samples reach equilibrium positions, large amounts of amino acids move as two wave fronts, from the anode and cathode, unsuccessfully trying to reach their p*I* positions. Thus, the steady state and transient state coexist in IEF and are quite insensitive to each other. The formation and stabilization of the pH gradient in IEF do not require a preformed "pH-cage", nor can IEF be regarded as isotachophoresis, since the same ion acts simultaneously as the "leading" and "terminating" ion in our system. When the same buffer is used simultaneously at the anode and cathode, the gel loses its polarity so that the system becomes insensitive to the choice of electrode position, or to polarity reversal at any time during the IEF experiment.

## INTRODUCTION

Rilbe[1–3] conceived isoelectric focusing (IEF) as an equilibrium method in which amphoteric compounds are segregated according to their p*I* values in a pH gradient, formed by electrophoresis of amphoteric buffer substances known as carrier ampholytes. This concept implies the formation of an even and stable pH gradient, characterized by a good buffering capacity and good conductivity in each region of the gradient itself. In such a system, as was also demonstrated by Almgren[4], the distribution of a uni–uni-valent ampholyte about its p*I* would be gaussian or nearly so. This concept has been challenged by Brown *et al.*[5,6] who have reported that most ampholytes do not focus as gaussians, but as asymmetric peaks spread over more than 10–15% of the separation column and up to 40% of the column length at higher loads. It is also known that most of the present-day IEF systems are plagued by a marked instability of the pH gradient, which results in a progressive "cathodic drift"[7] or "plateau phenomenon"[8]. In a recent series of articles[9–15], Chrambach's group has demonstrated that the same focusing results can be obtained by using mixtures of

non-amphoteric, as well as amphoteric, buffers, including mixtures of amino acids. They have used this technique, called "buffer focusing" (BEF), for the study of pH gradient formation and decay in IEF and for demonstrating a possible parallelism between IEF and isotachophoresis (ITP). Their results can be summarized as follows:

(a) The finding that non-amphoteric buffers, placed in an electric field between the acid anolyte and the basic catholyte, can produce stable pH gradients suggests that focusing is non-isoelectric.

(b) This hypothesis, in turn, suggests that focusing is steady-state stacking (ITP) (in the order of the p$K$ values for non-amphoteric compounds and in the order of the p$I$ values for amphoteric compounds) under conditions such that the buffers used as catholytes and anolytes in ITP are replaced by base and acid, thereby providing a "pH-cage" which prevents the stack from migrating out of the separation column.

The spin-offs of these studies are two-fold.

(1) It is possible to stabilize pH gradients in IEF against decay (cathodic drift) by equalization of the anolyte pH with the p$I$ of the most acidic amphoteric component in a given Ampholine mixture. In the case of Ampholine pH 6–8, this is achieved by using Thr at the anode and His at the cathode.

(2) An ITP system having multiple trailing buffer constituents (a cascade stack) can be converted into a cascade IEF when the leading and trailing constituents in ITP are replaced with strong acids and bases. Conversely, an IEF system can be transformed into a cascade stack when the electrolytes are changed into the appropriate leading and terminating buffers of the corresponding ITP system.

In the present report, we have investigated further the behaviour of IEF systems in the presence of different anolytes and catholytes. Our results, quite unexpectedly, do not fit into the general pattern previously reported[9–15].

## MATERIALS AND METHODS

Acrylamide, N,N'-methylenebisacrylamide (Bis), ammonium peroxodisulphate and tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.), Coomassie Brilliant Blue G-250 from Serva (Heidelberg, G.F.R.) and urea (ultrapure) from Mann Labs. (New York, N.Y., U.S.A.). The amino acids Asp, Phe, Trp, Gly and Lys (analytical grade), as well as equine myoglobin and bovine serum albumin (BSA), were from Sigma (St. Louis, Mo., U.S.A.), while [$^{14}$C]Gly was purchased from New England Nuclear (Boston, Mass., U.S.A.). Horse spleen ferritin was prepared in our lab, while the sample of casein digested with *Lactobacillus bulgaricus* was a gift from Dr. S. Carini (University of Milano).

### *Isoelectric focusing*

IEF was performed in gel slabs in an LKB Multiphor 2117 chamber, and in tubes (3 mm I.D.)[7] using an LKB constant-wattage power supply[16]. The slab (0.7 mm thick) contained 5% acrylamide and 2% Ampholine pH 3.5–10. In some experiments, 8 $M$ urea was added to the gel phase. The anolyte and catholyte used were 2% Ampholine pH 3.5–10 in control gels, or 50 m$M$ Asp, 50 m$M$ Trp, 45 m$M$ Phe, 0.5 $M$ Lys and 0.5 $M$ Gly. For Asp, Trp and Phe, these molarities were dictated by the solubility limits, while the two high-molarity terminators were selected for

studying the effects of increasing electrolyte concentrations on the pH gradient. It should be noted that, in all the experiments, the same amino acid, at the concentration given, was used both as the anolyte and the catholyte. Therefore, at the start of the experiment the pH of the anode and cathode strips was identical, and was the isoelectric (or isoionic) pH of the free amino acid dissolved in distilled water at the molarities given.

The IEF gel slabs were run for a minimum of 6 h up to 48 h with voltage gradients of 40 V/cm up to 80 V/cm, as described in the Results section. The pH was monitored by cutting and eluting 5-mm gel slices in 300 $\mu$l of 10 m*M* KCl (ref. 17). The distribution of the amino acids in the electrode strips or in the gel slab was monitored either by radioactivity counting ([$^{14}$C]Gly), by scanning at 280 nm or by use of the amino acid auto-analyzer on eluates from the gel or from the electrode strips. The gels were stained by the method of Blakesley and Boezi[18] as described by Righetti and Chillemi[19].

## RESULTS

Fig. 1 shows the types of pH gradients obtained when an Ampholine pH 3.5–10 gel slab is run using either the usual 1 *M* NaOH catholyte and 1 *M* $H_3PO_4$ anolyte (or another control containing 2% Ampholine pH 3.5–10 at both the anode and cathode) or other types of buffers, such as 50 m*M* Asp, 50 m*M* Trp, 0.5 *M* Gly and 0.5 *M* Lys (each amino acid was used simultaneously at the anode and cathode, at its isoelectric pH: pH 2.9 for Asp, pH 6.2 for Gly, pH 5.9 for Trp and pH 9.8 for

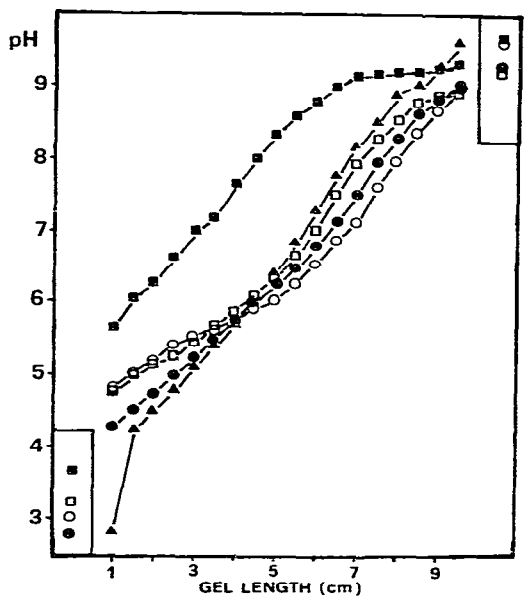


Fig. 1. pH gradients with different electrode buffers. ▲, Control, 1 *M* NaOH at the cathode and 1 *M* $H_3PO_4$ at the anode; ●, 0.05 *M* aspartic acid; ○, 0.05 *M* tryptophan; □, 0.5 *M* glycine; ■, 0.5 *M* lysine. The points in the boxes represent the pH values of the electrode strips at the end of the run.

Lys). The control (with NaOH and $H_3PO_4$) gives an approximately straight line with a relatively marked discontinuity in pH at the extremes of gel length, possibly due to diffusion of acid and base, and the pH gradients obtained using Gly, Asp or Trp are similar. The curves for the amino acids exhibit a slight flattening around pH 5.7, which is essentially independent of the type of electrolyte used. At the two extremes of gel length there are no marked jumps in pH between the actual pH gradient in the gel and the electrolyte pH. Interestingly, the Lys–gel slab shows a similar pH gradient, except that it is shifted toward somewhat higher pH values. While the pH at the cathodic end of the gel is not greatly influenced by the pH of the various buffers used (it ranges from pH 9 to 9.6), the pH at the anodic end is more sensitive to the type of terminator used. The actual pH gradients vary as follows: control, pH 2.8–9.6; Asp, pH 4.3–9.0; Gly, pH 4.8–9.0; Trp, pH 4.8–9.0 and Lys, pH 5.6–9.4.

The question arose as to whether equilibrium focusing patterns could be achieved by macromolecules under these "unorthodox" IEF conditions. Fig. 2 shows the IEF profiles of a mixture of horse spleen ferritin, BSA and myoglobin in one track, and of a casein digest in the other, using the different terminators of Fig. 1. All the sample species, from the low-molecular-weight peptides up to high-molecular-weight macromolecules (440,000 daltons for ferritin), reach their equilibrium positions, as demonstrated by the constancy of the p*I* values with time in the different systems used. The resolution obtained with amino acid electrolytes is quite similar to that with the control, except for very acidic samples, due to the compression of the pH gradient in this region with the amino acid terminators. Moreover, upon prolonged electrolysis, up to 48 h, the cathodic drift was often more pronounced in the control (even when using 2% Ampholine as electrolyte) than in the gels with amino acid electrolytes.

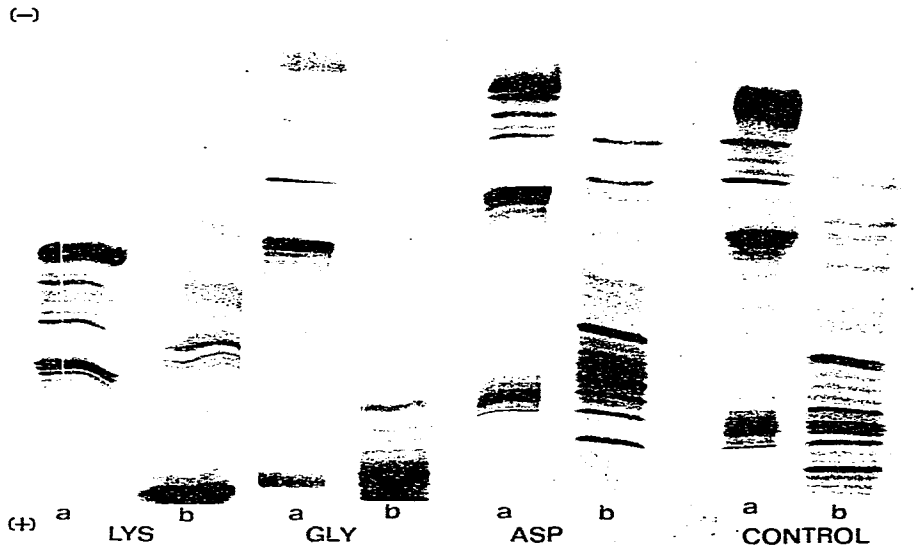


Fig. 2. IEF patterns of a mixture of ferritin, myoglobin and BSA (a) and of a casein digest (b) using different terminator buffers: LYS = 0.5 *M* lysine; GLY = 0.5 *M* glycine; ASP = 0.05 *M* aspartic acid; CONTROL = 1 *M* NaOH and 1 *M* $H_3PO_4$.

This behaviour of pH gradient formation suggests a modification of pH also in the electrode reservoirs containing the amino acids. We thus conducted a "tube" experiment using the anolyte and catholyte as free liquids and monitoring the pH changes with time using two different terminators, 45 m*M* Trp and 50 m*M* Phe. As shown in Fig. 3, in each case, starting at the same pH in the two chambers (pH 6.4 for Phe and pH 5.9 for Trp), the pH progressively increases at the cathode and decreases at the anode. The rate of change of pH is faster at the cathode than at the anode, even though in the latter case the relative jump in pH from the starting to the end point is larger. The two amino acids (as well as other monoaminomonocarboxylic acids tested) exhibit similar behaviour. The only possible explanation for these pH changes is that the amino acids at the electrodes are titrated by Ampholine from the gel. In fact, in gel-slab IEF, when the electrolytes absorbed by the filter-paper strips were eluted and analyzed with the amino acid auto-analyzer, the amino acids used as electrolytes were found to contain acidic Ampholine (at the anode) and basic Ampholine (at the cathode) (not shown). These results are in contrast to the findings of Nguyen and Chrambach[15].

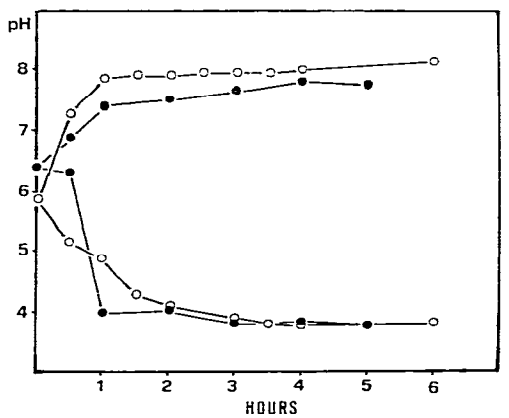


Fig. 3. pH modification at the electrode tanks during IEF in gel cylinders. The electrolyte chambers contained 150 ml solution. ○, 0.05 *M* tryptophan; ●, 0.045 *M* phenylalanine.

Since the various pH ranges obtained with the different terminators are similar, it is of interest to determine whether the amino acids used as electrolytes remain stationary or are swept away in the IEF system and reach their p*I* positions. We thus repeated the previous experiment in a gel slab, placing 50 m*M* Phe or 45 m*M* Trp at the electrode filter-paper strips. As shown in Fig. 4, even after focusing for 20 h, most of the amino acids are still at the electrodes (at the anodic side) or have barely moved into the gel (at the cathodic side). The anionic form of the amino acid tends to move faster than its cationic counterpart, a phenomenon recently demonstrated also with other amino acids[20]. Similar results are obtained when following the rate of movement of [$^{14}$C]Gly from the cathodic and anodic ends, respectively (Fig. 5). At any given time, the peak travelling from the cathode moves faster and contains more radioactivity than the corresponding peak moving away from the anode. In all cases, even after focusing for 20 h, the amino acids are just about everywhere

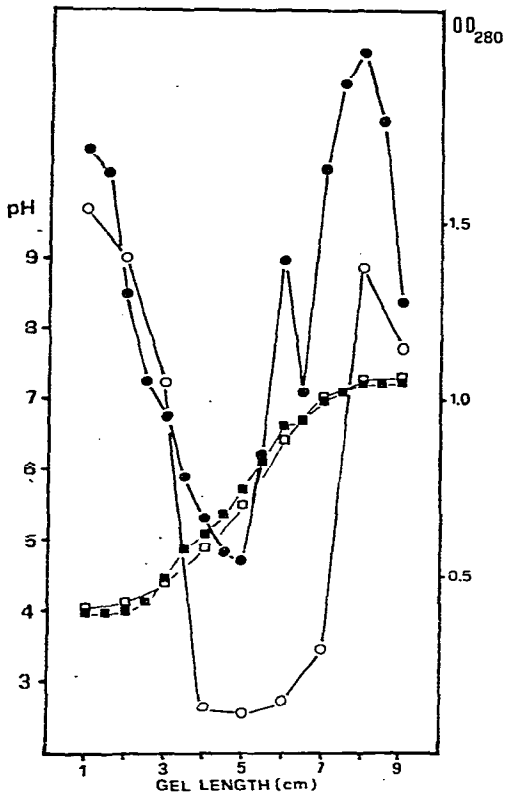


Fig. 4. Amino acid distributions (circles) and pH gradients (squares) using tryptophan (○, □) and phenylalanine (●, ■) as electrolytes. Gel-slab experiments with 1 ml electrolyte soaked in filter-paper strips focused at 200 V for 24 h.

except at their p*I* positions. Thus, in the case of Phe and Trp, the anodic peak is centered at pH *ca.* 4, while the cathodic peak is at pH *ca.* 7.3. In the case of Gly, the anodic front has moved to pH *ca.* 5, while the cathodic wave has moved down to pH *ca.* 7.5. It might be argued that, at least in the case of Trp and Phe, aggregation phenomena, precipitation and/or adsorption to the filter-paper strip may occur. We thus repeated the experiments in the presence of 8 *M* urea, either in the filter-paper strips, or in the gel, or in both, but with identical results with the non-urea systems (not shown).

The differential movement of amino acids from the anode and cathode can also be seen in Fig. 6, which shows the loss of radioactivity from the anodic and cathodic filter-paper strips soaked in 0.25 *M* Gly. After 20 h the anode still retains 80% of the amino acids while at the cathode only 20% of the Gly remains. Yet, even though these considerable amounts of amino acids are far from their p*I* values, and tend to move as two wavefronts from the anode and cathode, the pH gradient in the gel forms, the proteins reach their p*I* positions and the entire system seems to be quite insensitive to the bulk of these unfocused components.

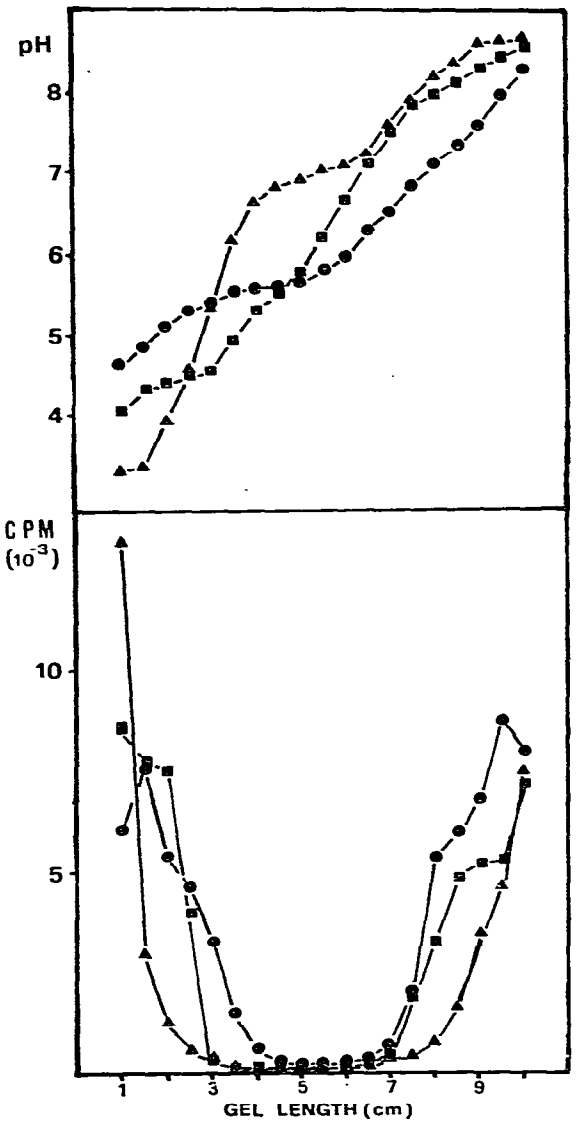


Fig. 5. [$^{14}$C]Glycine distribution and pH gradients at 1.5 (▲), 4 (■) and 20 h (●). 0.25 *M* Glycine at the electrodes, no glycine in the gel and 2% Ampholine pH 3.5–10 in the gel slab.

## DISCUSSION

Our results demonstrate the following aspects of IEF.

(1) The mechanism of pH gradient formation and stabilization (or immobilization) within the separation column does not depend on a preformed "pH cage" acting as a barrier preventing the stack from diffusing out of the column, as previously hypothesized[9]. The pH gradient forms and is just as stable even when the same substance, at the same pH (either acidic, neutral or basic), is placed at both the anode

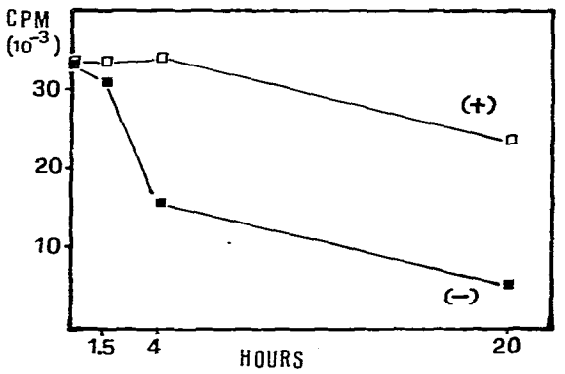


Fig. 6. [$^{14}$C]Glycine migration from the anode (□) and from the cathode (■) during the IEF experiment of Fig. 5. 0.25 *M* Glycine at the electrode strips.

and the cathode. In fact, the original reason why, in IEF, a strong acid was used at the anode and a strong base at the cathode was simply to prevent the carrier ampholytes from coming in contact with the electrodes and thus being modified (especially by anodic oxidation)[21]. As early as 1968, Fawcett[22] carried this concept to its logical conclusion by disposing of strong acids and bases as electrolytes and immersing the platinum wires directly in the same Ampholine solution contained in the focusing gels.

(2) IEF can hardly be regarded as ITP. According to the Kohlrausch autoregulating function[23], it is impossible to have an ITP system in which the same ion acts simultaneously as the leading ion (*i.e.*, the ion having the highest mobility) and as the terminating ion (*i.e.*, the ion having the lowest mobility). Yet, this is just what happens in our "unorthodox" IEF system. Moreover, in ITP the system does not reach a true steady state until the last component of the system has reached a steady state too. In our case, Ampholine reaches a steady state (the pH gradient forms and is stable for the time allotted to the experiment), and macromolecules also reach equilibrium positions (as determined by pH measurements) even though large amounts of amino acids are far from their p*I*.

(3) As a corollary to the above comments, the steady state and transient state seem to coexist in IEF and to be quite insensitive to each other. This was quite a surprising result, yet for all the amino acids tested the pH gradient and the protein patterns form within a few hours, and are stable for several hours, even though, notwithstanding focusing for 20 h, large amounts of amino acids keep moving as two wave fronts from the anode and from the cathode, unsuccessfully trying to reach their p*I* positions.

(4) We had hoped to be able, by using different anolytes and catholytes, to transform a wide pH range (such as Ampholine pH 3.5–10) into a narrow one, but this does not seem to be feasible at present. In fact, there is essentially no difference in pH gradient expansion when using Asp, Gly, Phe or Trp or plain Ampholines as terminators. A somewhat restricted pH range could be obtained only with Lys, but not to the extent of transforming a wide into a narrow pH gradient.

(5) In our IEF system the gel has lost its polarity. Since the same terminator is used at both the extremes of gel length, it is irrelevant which terminator is chosen

as the anode or cathode. Even after the system has reached equilibrium, if the polarity is reversed, all the components move to their new p*I* position. This cannot be done when using acid and base at the anode and cathode, respectively.

Some of our results are still quite puzzling. For instance, we find that the pH of the anolyte and catholyte changes considerably, notwithstanding the type of amino acid used, or the great increase in molarity (from 50 m*M* to 0.5 *M*). In contrast, Nguyen and Chrambach[15] found that the pH of their electrolytes was essentially constant with time, up to 400 h of focusing. They used the following anolyte–catholyte couples: Thr (pH 5.6)–His (pH 7.6); Gly (pH 6.2)–His (pH 7.6); Thr (pH 5.6)–Lys (pH 9.5); Glu (pH 3.1)–His (pH 7.6) and Thr (pH 5.6)–Lys (pH 9.6). In all these cases the pH did not change, even though they generally used 10 m*M* solutions. The only difference between their method and ours is that they still perform "conventional" IEF (*i.e.*, an acid at the anode and a base at the cathode) while in our "unorthodox" system "acids" or "bases" are both placed at either electrode.

Another puzzling result is the fact that the amino acid terminators do not reach their p*I* positions, even upon prolonged focusing. One might argue that Trp, Phe and Gly are "poor" carrier ampholytes and as such are isoelectric over a range of four pH units, which would greatly hinder their migration to the theoretical p*I*. Yet, this behaviour occurs also with Asp and Lys, which are "good" carrier ampholytes in Rilbe's terms. It might be argued that the anolyte and the catholyte do not really belong to the IEF system, which could explain the relative independence of the pH gradient from them. However, in a recent theoretical survey, Rilbe[24] has demonstrated that his IEF theory also applies in full to the anolyte and catholyte. Precipitation or aggregate formation with these amino acids has been excluded by the experiments with 8 *M* urea. Adsorption of the amino acids by the filter-paper strips is also excluded by the experiments in gel cylinders, where no paper strip is used. At the moment we can only postulate the existence, at the gel termini, of a zero-mobility, low-current "constituent barrier", which, presumably through operation of the laws of electroneutrality and mass conservation, prevents the passage of the constituents of the electrolyte reservoir into the adjacent pH gradient (this is just the reversal of the Nguyen and Chrambach hypothesis[15]).

## ACKNOWLEDGEMENTS

This work was supported in part by grants No. CT77.00278.06 and No. CT77.01471.04 from Consiglio Nazionale delle Ricerche (CNR) Rome, Italy.

## REFERENCES

1 H. Svensson, *Acta Chem. Scand.*, 15 (1961) 325.
2 H. Svensson, *Acta Chem. Scand.*, 16 (1962) 456.
3 H. Svensson, *Arch. Biochem. Biophys. Suppl. 1*, (1962) 132.
4 M. Almgren, *Chem. Scripta*, 1 (1971) 69.
5 R. K. Brown, M. L. Caspers, J. M. Lull, S. N. Vinogradov, K. Felgenhauer and M. Nekic, *J. Chromatogr.*, 131 (1977) 223.
6 R. K. Brown, M. L. Caspers and S. N. Vinogradov, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, W. de Gruyter, Berlin, 1977, pp. 87–96.
7 P. G. Righetti and J. W. Drysdale, *Biochim. Biophys. Acta*, 236 (1971) 17.

8 G. R. Finlayson and A. Chrambach, *Anal. Biochem.*, 40 (1971) 292.
9 N. Y. Nguyen and A. Chrambach, *Anal. Biochem.*, 74 (1976) 145.
10 N. Y. Nguyen, D. Rodbard, P. J. Svendsen and A. Chrambach, *Anal. Biochem.*, 77 (1977) 39.
11 N. Y. Nguyen, A. Salokangas and A. Chrambach, *Anal. Biochem.*, 78 (1977) 287.
12 N. Y. Nguyen and A. Chrambach, *Anal. Biochem.*, 79 (1977) 462.
13 M. L. Caspers and A. Chrambach, *Anal. Biochem.*, 81 (1977) 28.
14 N. Y. Nguyen and A. Chrambach, *Anal. Biochem.*, 81 (1977) 54.
15 N. Y. Nguyen and A. Chrambach, *Anal. Biochem.*, 82 (1977) 226.
16 P. G. Righetti and A. B. B. Righetti, in J. P. Arbuthnott and J. A. Beeley (Editors), *Isoelectric Focusing*, Butterworths, London, 1975, pp. 114–131.
17 P. G. Righetti and J. W. Drysdale, *J. Chromatogr.*, 98 (1974) 271.
18 R. W. Blakesley and J. A. Boezi, *Anal. Biochem.*, 82 (1977) 580.
19 P. G. Righetti and F. Chillemi, *J. Chromatogr.*, 157 (1978) 243.
20 P. G. Righetti and A. Chrambach, *Anal. Biochem.*, (1978) In press.
21 H. Haglund, in D. Glick (Editor), *Methods of Biochemical Analysis*, Vol. 19, Wiley-Interscience, New York, 1971, pp. 1–104.
22 J. S. Fawcett, *FEBS Lett.*, 1 (1968) 81.
23 F. Kohlrausch, *Ann. Physic und Chemie*, 62 (1897) 209.
24 H. Rilbe, in N. Catsimpoolas (Editor), *Isoelectric Focusing*, Academic Press, New York, 1976, pp. 14–52.