Journal of Chromatography, 334 (1985) 71–82
Chromatographic Reviews
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHREV 191

ISOELECTRIC FOCUSING IN NON-AMPHOTERIC BUFFERS

CATASTROPHE AND NON-CATASTROPHE THEORIES

PIER GIORGIO RIGHETTI* and ELISABETTA GIANAZZA
Chair of Biochemistry, Faculty of Pharmaceutical Sciences and Department of Biomedical Sciences and Technology, University of Milan, Via Celoria 2, Milan 20133 (Italy)
(Received November 14th, 1984)

CONTENTS

1 INTRODUCTION

Modern isoelectric focusing (IEF) is still based on the rigorous theoretical treatment of Svensson-Rilbe[1,2] who more than 20 years ago, derived the fundamental equations governing the system at the steady state and defined the minimum requirements for correct functioning of the technique. Central to his theory is the concept of "carrier ampholytes". the buffers used in IEF have to be amphoteric, so that they also would seek a stationary position in the system; moreover, they have to be "carriers". This is a more subtle concept, but just as fundamental: they have to be "carriers" of buffering power, so that they would prevent local pH changes, and of conductivity, so as to ensure an unhindered flow of current through the focusing cell. Over about 25 years, the technique has proved very suitable for this job[3].

Is it possible, or is there a need, to break away from such a well constructed and much explored methodology? A need definitely arose, as conventional IEF had begun to show signs of age, such as (a) cathodic drift; (b) lack of even conductivity and buffering capacity; (c) extremely low and unknown ionic strength (I) and (d) limited load capacity, mostly due to isoelectric precipitation caused by the low I environment. As for the possibility, this is a complex story: over the years, several groups have tried to break away from Svensson's theory, by resorting to different approaches of what has been generally termed "non-amphoteric buffer isoelectric focusing" (NAB-IEF) (let us recall here that, around 1955, this concept had been amply described by Kolin[4] with his "artificial" pH gradients).

0378-4355/85/$04.20

2. THEORY AND RESULTS

2.1. Chrambach's theory

According to this theory, IEF can be viewed as a particular form of isotachophoresis (ITP), where a separation within a mobile pH gradient, as in ITP, is replaced by a separation within a stationary pH gradient, typical of IEF (for a review, see, ref. 5; this concept had been proposed long ago by Routs[6]). While this can be easily accepted for conventional IEF in amphoteric buffers, it is not immediately apparent for NAB-IEF. how can non-amphoteric buffers form a stationary (and also a "natural") pH gradient? They have to be arrested, and this blocked "stack" or "train" of buffers is obtained by a neutralization process, *i.e.*, a protonation with acidic species and a deprotonation mechanism with basic compounds. There is a simple way of achieving this: free acids and free bases are deprived of counter ions (*i.e.*, of titrants) and are allowed to migrate into a pH region where they will have only the hydrolytic products of water as counter ions. As an example, we report in Table 1 and Fig. 1 the data of Chrambach and Hjelmeland[5] on the generation of a "stationary" and "natural" pH gradient between pH 10.29 and 12.18 solely with the use of six free bases, with pK values ranging from 6.88 to 10.35. While we agree that such a system can generate a pH gradient, as long ago demonstrated by Schumacher[7] and subsequently by Pettersson[8] and Stenman and Grasbeck[9] for free acids, we hesitate to call it "stationary", a fundamental prerequisite of true IEF. We could give a number of reasons, but there is some obvious macroscopic, negative evidence: how

TABLE 1

PREDICTED PROPERTIES OF AN ISOELECTRIC FOCUSING SYSTEM COMPOSED OF SIX NON-AMPHOTERIC BASES

From Chrambach and Hjelmeland[5] r = Ionic mobility relative to Na^+, φ = flux (mol cm^{-2} sec^{-1}), $\bar{r}$ = relative net mobility; κ = specific conductance (10^{-6} Ω^{-1} cm^{-1}), v = boundary displacement rate (cm^3 C^{-1}), v = boundary velocity (cm per day)

Parameter	*Ethanolamine*	*Morpholine*	*N-Ethyl-morpholine*	*N-(2-Hydroxy-ethyl)-morpholine*	*Lutidine*	*Bis-Tris*
r*	0 86	0 73	0 62	0.61	0.60	0 38
pK_a*	10 35	8 85	8 03	7.19	7.00	6 88
Concentration (M)	1 00	0 87	0 76	0.75	0.74	0 49
pH	12 18	11 40	10 96	10 53	10.43	10 29
φ	1 50 10^{-2}	2 85 10^{-3}	1 19 10^{-3}	4 55 10^{-4}	$3.68 \cdot 10^{-4}$	3 93 10^{-4}
$\bar{r}$	1 29 10^{-2}	2 08 10^{-3}	7 37 10^{-4}	2 78 10^{-4}	$2.21 \cdot 10^{-4}$	1 45 10^{-4}
κ	1 91 10^{-3}	3 08 10^{-4}	1 09 10^{-4}	$4.11 \cdot 10^{-5}$	$3.27 \cdot 10^{-5}$	2 21 10^{-5}
v	1 85 10^{-3}	1 85 10^{-3}	1 85 10^{-3}	$1.85 \cdot 10^{-3}$	$1.85 \cdot 10^{-3}$	1 85 10^{-3}
v**	6 85 10^{-6}	6 85 10^{6}	6 85 10^{-6}	$6.85 \cdot 10^{-6}$	$6.85 \cdot 10^{-6}$	6 85 10^{-6}
w (cm)	9 33 10^{-3}	4 29 10^{-3}	1.67 10^{-3}	4 08 10^{-3}	1 74 10^{-3}	

* Values at 0°C

** 1 mA per 0 27 cm^2 of gel

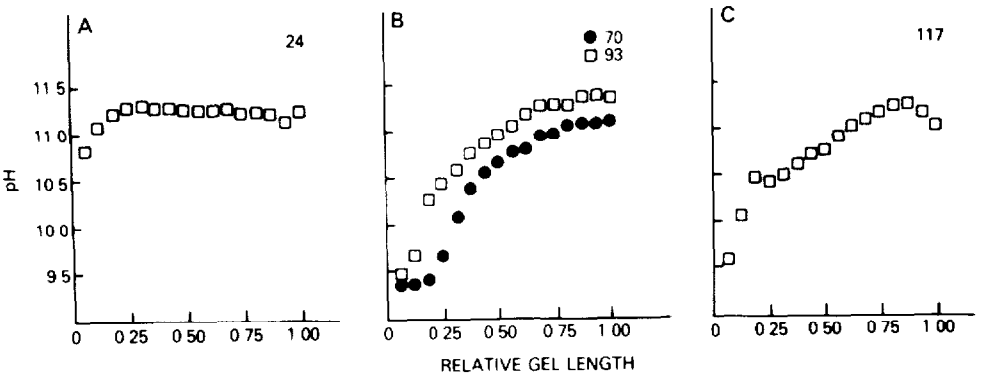

Fig 1 Experimental pH gradient formed by the moving boundary system of six non-amphoteric bases having p*K*s and molarities as defined in Table 1 A, measured after 24 h of electrophoresis; B, after (●) 70 and (□) 93 h, C, after 117 h (From Chrambach and Hjelmeland[5])

can a buffer possibly have any buffering capacity (and also contribute to the conductivity) when it is confined to a pH region more than 3 pH units removed from its p*K* (and on its deprotonated side also)? We therefore re-simulated their computer simulations with the program we have developed for a very peculiar kind of NAB-IEF, namely immobilized pH gradients(IPG)[10–12], in order to demonstrate how to use correctly these buffers for IEF. Our computer was required to simulate and optimize what we believe are the fundamental physico-chemical parameters of an IEF separation· the slope of the pH gradient (and its optimization in terms of minimal deviation from linearity) and accompanying buffering power (β) and ionic strength (I). When we took the six buffers in Table 1, with their relative molarities, and asked the computer to calculate the β and I values along the pH 10.29–12.18 potential gradient, it could not do so, since in our computer program both a buffer and a titrant are required. We had to calculate these values manually, by assuming that the six bases are sequentially distributed along the pH gradient, starting with Bis–Tris at pH 10.29 and ending with ethanolamine at pH 12.18 and occupying "boxes" with lengths proportional to their respective absolute amounts present in the system. The

TABLE 2

BUFFERING POWER (β) AND IONIC STRENGTH (I) OF CHRAMBACH'S ARRESTED STACK OF FREE BASES

Parameter	*pK*					
	6 88	*7 00*	*7 19*	*8 03*	*8 85*	*10 35*
Concentration (m*M*)	490	740	750	760	870	1000
pH_m*	10.4	1.65	10.95	11 25	11.6	12 0
I (mequiv l^{-1})	0 148	0 166	0.130	0 458	1.544	21 9
β (mequiv. l^{-1} pH^{-1})	0 34	0 38	0 30	1 05	3 55	49.3

* pH at mid-point of each "isotachophoretic" zone, having a length proportional to the total amount present in the system.

results are reported in Table 2. It can be seen that the β and I values are much too low to ensure any buffering power and conductivity for the system, and as such this cannot be included among the natural pH gradients characteristic of true IEF, where the concept of "carrier" buffers is indissolubly linked to the generation of a pH gradient (in IPGs, we give as acceptable average values β = 3–4 mequiv. pH^{-1} l^{-1}

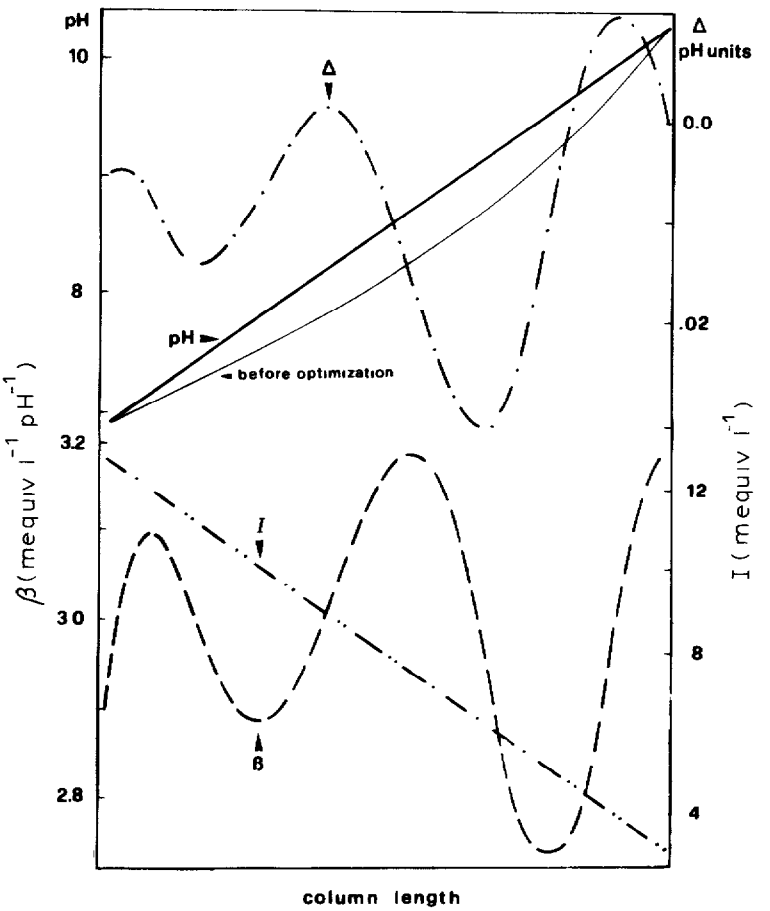

Fig. 2 pH gradient, deviation from linearity (Δ), buffering power (β) and ionic strength (I) of Chrambach's system (Fig 1) when titrated between pK (min) (pH 6 88) and pK(max) (pH 10 35) It has been assumed that the same system of six non-amphoteric bases as in Fig 1 are Immobilines and they have been titrated in the correct pH range with an acidic counter ion (Immobiline of pK 0 8). If the six bases are used in the same molarity ratios as in Fig 1, a bow-shaped pH gradient is obtained ("before optimization" line) When the molarity ratios of the six buffers are changed according to the optimization algorithm of ref 12, the upper, linear pH gradient is generated. The Δ, β and I values refer to the "optimized system". The concentrations of each base were as follows pK 6 88, 2 09 mM, pK 7 00, 1 66 mM, pK 7 19, 0 80 mM; pK 8.03, 1 85 mM, pK 8.85, 3 82 mM, pK 10 35, 5 00 mM. For the titrant (Immmobiline of pK 0 8): 13 mM in the acidic chamber and 2 6 mM in the basic reservoir These concentrations are adjusted so as to give an average buffering power of 3 mequiv l^{-1} pH^{-1} (note that in the original system in Fig. 1 the molarities of the buffering ions are 200–500-fold higher, ranging from 490 to 1000 mM!). This "immobilized pH gradient" was generated with the aid of a two-vessel gradient mixer, according to the "same concentration" formulation, as explained in ref 11

and I = 4–5 mequiv. l^{-1}). Certainly such β and I values could be obtained even in this system of six bases, but by utilizing enormous molarity values (> 10 M), definitely incompatible with protein integrity and with an electrophoretic process. That this system cannot be stationary as well is amply demonstrated by the data of the authors who claim it to be so (see Fig. 1C). There is another reason why these gradients will not have any focusing action on proteins: the conductivity from pH 10.29 to 12.18 will be dictated primarily by the free OH^- in solutions (and the equivalent amount of protonated base), which means that it will increase substantially towards the cathode, leaving behind a high field strength at the anodic side. Over much of the pH gradient (above pH 11) there will be too little voltage drop to ensure any focusing power.

However, if we now assume these buffers to be Immobilines, and we titrate them with an acidic counter ion between pK (min) (pH 6.88) and pK (max) (pH 10.35), the situation is definitely brighter: it is possible to generate a decent pH course with acceptable β and I values (Fig. 2, thin solid line) Even in the correct pH range, however, the system does not behave well when using for the simulation the concentrations of the various buffers given in Table 1. After extensive modification of the relative ratios by our optimization algorithm, a very smooth pH course may be generated (Fig. 2, thick solid line).

2.2. *"Chemically" immobilized pH gradients*

There is not much to be said here, except that the system works, it allows full control of the experimental parameters (pH gradient width and slope, β and I courses)[10,12], complete choice of any pH gradient (from ultra-narrow ranges, *e.g.*, 0.01 pH unit/cm separation distance up to the widest possible span, pH 3–10) and, of course, generates indefinitely stable pH gradients. IPGs are, in a way, a particular case of NAB-IEF: as non-amphoteric buffer focusing, in reality, would never work, the problem has been solved by resorting to non-amphoteric, but bifunctional buffers. These chemicals are acrylamide derivatives, with pK values spaced at regular intervals along the pH scale [pK (min) = 3.6, pK (max) = 9.3] and they are utilized according to "canonical" principles (the "carrier" concept of Svensson!), *i.e.*, for generating pH gradients by titration with a counter ion around their respective pK values (ideally 0.5 pH unit above and 0 5 below the pK) where they exhibit maximum buffering power and substantial conductivity The gel slab is cast with the aid of a gradient mixer, the two vessels being titrated one to pH (min) (*e.g.*, in the dense solution) and the other to pH (max) (*e.g.*, in the light solution). Therefore, when the gel cassette is filled up, it already contains a linear pH gradient, existing prior to the electrophoretic process itself: this system appears to have all the prerequisites of the "artificial" pH gradients according to Kolin[4]. However, a moment later, as the monomers polymerize to form the gel network, the non-amphoteric buffers are covalently linked to the matrix, and therefore the entire pH gradient is insolubilized (copolymerized, grafted, immobilized, etc.), this system is indefinitely stable to the flow of electric current and thus now exhibits all the prerequisites of the natural pH gradients according to Svensson[1,2]. Thus, by burning at the stake Kolin and Svensson, from their ashes this new "arab phoenix" was born, with the virtues of both and none of their defects. That IPGs are the new highway (or star port) to the year 2000 is already amply documented[13].

2.3. *"Physically" immobilized pH gradients*

Yet another approach to NAB-IEF comes from Bier's group, who has extensively developed highly sophisticated theories and models over the years. Their results were summarized in a recent paper by Bier *et al.*[14] and are exemplified in Fig. 3, taken from the same paper. They were finally able to generate "quite stable pH gradients formed using a simple system of a weak acid and a weak base (around neutrality), mixed in the proportion required to cover the desired pH range". In the simple case of Fig. 3, a gradient of a buffering acid (cacodylate, pK 6.2), varying linearly from 4 to 2 mM (anode to cathode), is titrated with a reciprocal linear gradient of buffering base (Tris, pK 8.3) ranging from 2 to 4 mM (anode to cathode). Bier *et al.* stated that their system defies ready classification in terms of conventional modes of electrophoresis as it is not isoelectric focusing, because none of the components of the buffer is isoelectric, and it is not IPG, because their buffers are not immobilized. However, close scrutiny of their data reveals that they have some of both attributes. The similarity with IPGs is striking. An analogous situation occurs in IPGs in the pH region 4.4–6.2, with Immobilines of pK 4.4 (a weak acid) and pK 6.2 (a weak base), where the system is used under conditions such that the two components act simultaneously as a buffer and as a titrant. As can be seen in Fig. 3A, the concentrations of the two are reciprocal, symmetrical linear gradients, generating a pH gradient from 6.21 (the pK of cacodylate, because here the [cacodylate]/[Tris] ratio is 2:1) to pH 8 3 (the pK of Tris, because here the [Tris]/[cacodylate] is 2:1). However, the pH gradient is not linear, but slightly sigmoidal, because ΔpK = 2.09, as predicted by our computer modelling[11]. Such a situation was indeed fully predicted by our general theory on IPGs[15]. We have in fact re-simulated their data with our computer algorithm, by assuming the two species to be Immobilines. As shown in Fig. 4, we in fact obtain the same results: the expected pH gradient is identical in the two instances, except that the deviation from linearity is high (the

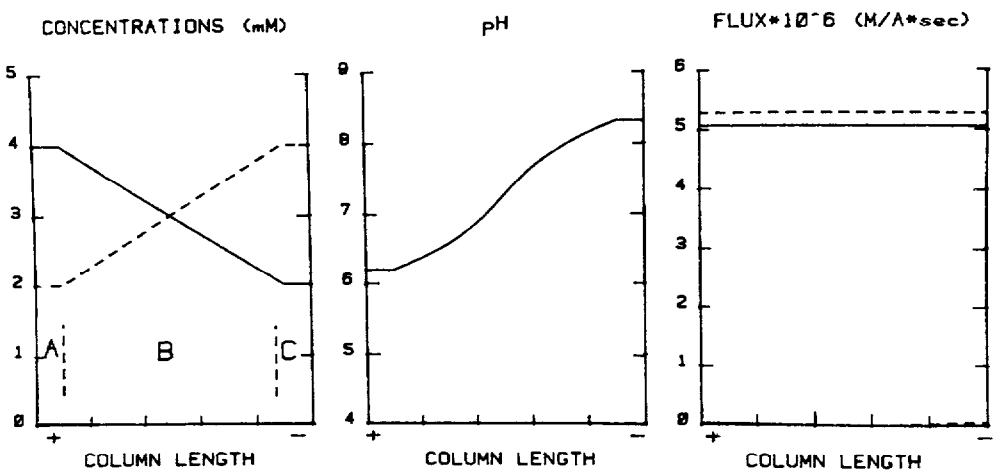

Fig 3 Schematic representation of the courses of the concentration of Tris–cacodylate and their respective fluxes and of the resulting pH gradient Microcomputer-calculated flux values are also plotted, assuming for Tris (dashed lines) a mobility of 2.42 cm^2 V^{-1} sec^{-1} and a pK of 8 3, and for cacodylate (solid lines) a mobility of 2 31 cm^2 V^{-1} sec^{-1} and a pK of 6 21 (From Bier *et al*[14])

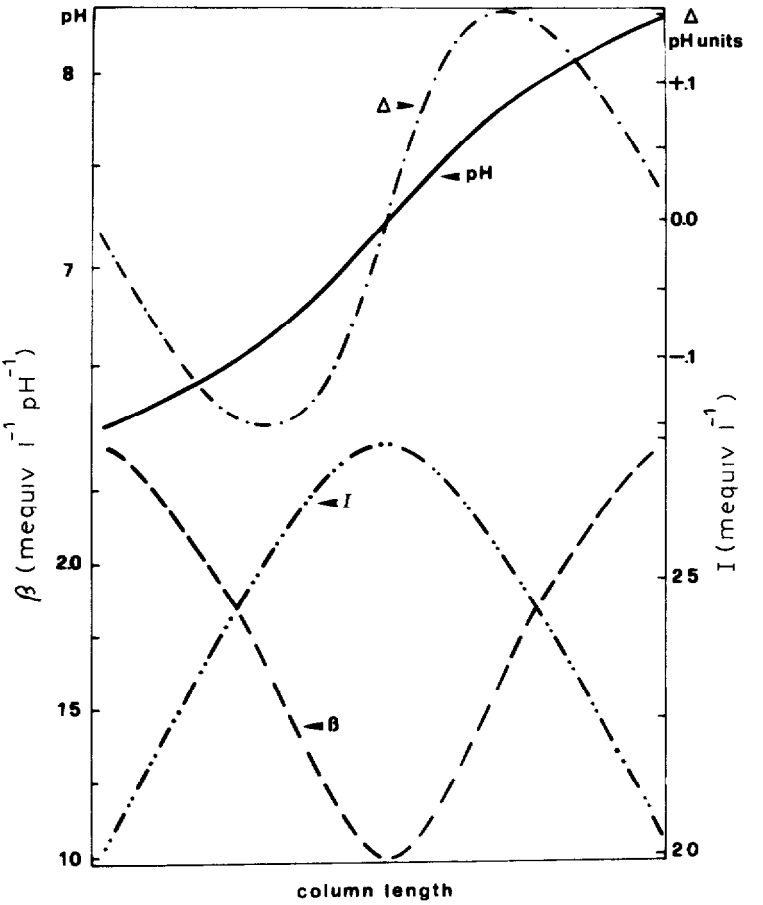

Fig 4 pH gradient, deviation from linearity (Δ), buffering power (β) and ionic strength (I) of Bier *et al.*'s system in Fig 3 Their data were re-calculated by using the same molarity and pK values and the same pH interval as in Fig 3, except that Tris and cacodylate were assumed to be two Immobilines (*i.e* , with mobility = 0, flux = 0, diffusion coefficient = 0) Note that the shapes of the expected pH gradient are identical in the two different computer models Our computer simulation predicts that Bier *et al* 's gradient is fully compatible with a well functioning IEF system (ideally, however, we prefer to have an average β = 3 mequiv l^{-1} pH^{-1}, so that the concentrations of the two species should be approximately doubled)

maximum excursion, positive + negative, is 0.3 pH unit, *i.e.*, 15% of the stated pH interval; for typical Immobiline gradients, both with narrow and with extended ranges, the deviation is contained within less than 1% of the generated pH span). We could also simulate the β and I courses (Fig. 4, lower part); as expected, they appear as two reciprocating, bell-shaped functions, with a minimum of β power half-way between the two peaks, corresponding to a maximum of ionic strength (because, by titrating the two species "inside" the two pKs, we ensure conditions of maximum ionization of the buffering groups). While, with true Immobilines, we can arrange for smoother β and I courses, the physico-chemical parameters of Bier *et al.*'s system are acceptable and compatible with a well functioning IEF set-up. While it is true that the buffers here are not "chemically immobilized", they nevertheless

ensure substantial stability of the system because they are immobilized by physical laws. The fundamental requirement of Bier *et al.*'s gradients is that the flux of the two species is constant across the whole length of the column. In fact, they introduced a parameter ρ, which predicts the stability of the system, defined as

$$\rho = \frac{M_a^{A-} M_b^{C+}}{M_b^{A+} M_a^{C-}}$$

where M is the concentration of the acid (a^-) and base (b^+) in reservoirs A and C. Only when $\rho = 1$ would we have perfect migrational stability, and this is one of the weaknesses of the system: as shown in Fig. 3, the flux of Tris is slightly higher than the flux of cacodylate, and eventually the system is bound to decay.

2.4. Steady-state rheoelectrolysis

Another approach to the generation of useful and stable pH gradients from simple, two-component, non-amphoteric buffers was proposed in 1978 by Rilbe[16]. The idea is that it should be possible to create a stable pH course in a suitable electrolyser by balancing the internal electrical and diffusional mass flows by external mass flows generated by the pumping of anolyte to the catholyte and *vice versa* (hence the term rheoelectrolysis, *i.e.*, external hydrodynamic flow coupled to internal electrophoretic transport). In this system the steady state would generate a useful pH gradient provided the following criteria were fulfilled: (i) the transference numbers and the diffusion coefficients of the buffer ions are constant in the pH interval chosen; (ii) the compositions of the buffer system at the ends of the electrolyser are kept constant with time; (iii) no net liquid flows through the electrolyser are present; and (iv) losses of buffer constituents due to anodic oxidation, cathodic reduction, evaporation or precipitation are negligible. The system was further developed by Jonsson and Fredriksson[17] and the theory expanded by Rilbe[18–20].

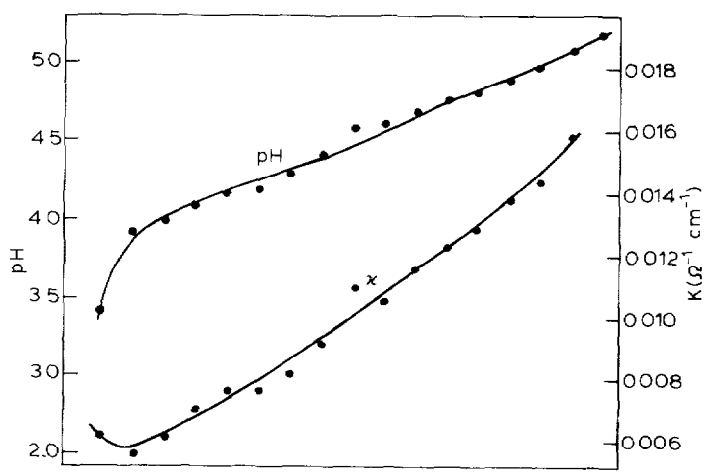

Fig. 5. Steady-state rheoelectrolysis of an acetate buffer solution. Experimental pH and conductivity courses in the electrophoretic cell at the steady state (From Rilbe[15]).

Fig. 5 shows one of Rilbe's experiments for generating a linear, pH 3.9–5.2 course by steady-state rheoelectrolysis of an acetate buffer solution [three cases have been considered. (a) a buffer composed of a weak acid and its salt with a strong base (as shown in Fig. 5); (b) a buffer composed of a weak base and its salt with a strong acid, and (c) a buffer containing a salt of a weak acid and a weak base]. By the same reasoning, assuming acetate to be an Immobiline with pK 4.6, we re-simulated Rilbe's data in our computer system As shown in Fig. 6, a smooth pH gradient is obtained in the pH 3.9–5.2 region with a maximum deviation from linearity of about 6% of the generated pH interval and with substantial average β and I values, compatible with a well performing IEF system (note that the slopes of Rilbe's conductivity profile in Fig. 5 and of our ionic strength curve in Fig. 6 are very similar, as the quantities are interchangeable)

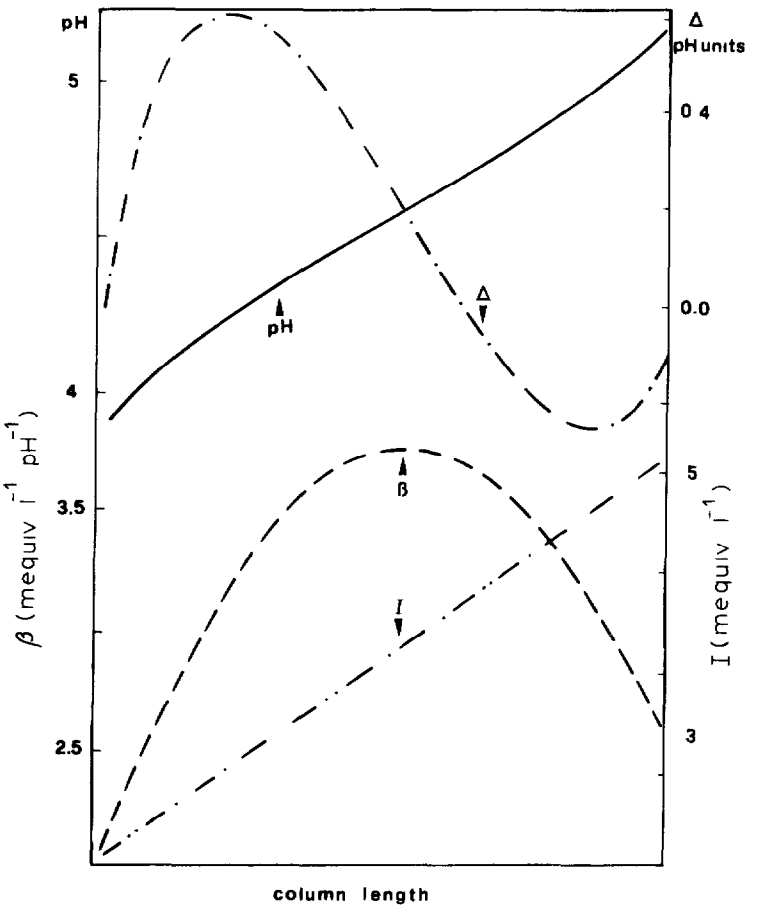

Fig 6 pH gradient, deviation from linearity (Δ), buffering power (β) and ionic strength (I) of Rilbe's system in Fig 5 His data were re-calculated with our computer program by assuming acetate to be an Immobiline with pK 4 61 as follows buffering ion = 6.52 mM in both chambers, titrant (Immobiline of pK 12), 1.06 mM in the acidic chamber and 5 19 mM in the basic chamber The acetate concentration was selected so as to give an average β value of 3 mequiv l^{-1} pH^{-1} Again, our computer simulation predicts that Rilbe's gradient is fully compatible with a properly behaving IEF system.

3 DISCUSSION

Modern IEF became a reality when buffers became available that met Rilbe's fundamental requirements. (a) of being amphoteric, so that they could reach a steady state by being titrated to a pH level ensuring "zero net-charge"; and (b) of being "carriers", *i.e* , of being good conducting and good buffering species at their p*I* values. After about 25 years of conventional IEF, the four modes of non-amphoteric buffer IEF that we have summarized and compared here clearly demonstrate that the system no longer depends on amphoteric species. However, a properly performing IEF system still has as an absolute requirement the concept of a "carrier", which requires that the chemicals used to generate and stabilize the pH gradient behave as "good buffers" and "good conductors". The systems discussed in sections 2.2–2.4 fulfill this fundamental requirement; in these three instances, in fact, quasi-linear pH gradients are generated by titrating weak acids or weak bases symmetrically around their respective p*K* values, where they automatically provide the much needed buffering capacity and conductivity In the Chrambach "arrested stack", the system breaks down because the potential buffers are allowed to be stripped electrophoretically of counter ions and thus to collect in strongly acidic anodic layers or strongly basic cathodic zones, where they are deprived of their buffering and conducting powers. While it is true that in Chrambach's system a natural pH gradient can still form (it is in fact a pH gradient generated by a stack of moving boundaries, like in isotachophoresis), it can hardly be controlled (for lack of buffering power) and it can never be assumed to become stationary (for lack of immobilization). In fact, the so called "arrested stack" created by protonation of acids and deprotonation of bases, is in fact never completely arrested; it could only be so when the current in the system becomes zero, but at this point it would be meaningless still to speak in terms of "electrophoresis" (which, by definition, requires a current to be flowing through the system). As for the other systems, although all three are based on sound and correct hypotheses, they are markedly different in operational terms. Bier *et al.*'s system is subject to two inherent disturbances: (a) migrational instability (the parameter ρ will very rarely be unity); and (b) diffusional instability (decay of the boundaries). The two instabilities are additive and will ensure ultimate decay of the pH gradient. As for Rilbe's system, here again what theory predicts and what practice can achieve rapidly come into conflict: the predicted pH courses are only established in the absence of an internal liquid flow (*i.e.*, inside the electrophoresis cell); unfortunately, there is always a net liquid flow within electrolysers, and this induces exponential, and slowly decaying, pH gradients

About 10 years ago, Catsimpoolas[21] predicted that conventional IEF had the potential for measuring the diffusion coefficients (D) and mobility slopes (du/dpH) of focused proteins. However, the strong discrepancies between theoretical and experimentally measured values induced him to call for the synthesis of "second generation ampholytes", which could not only provide highly stable pH gradients, but also uniform conductance and concentration distribution courses throughout the separation path. Perhaps present-day IPGs represent just the ideal system envisaged by Catsimpoolas: they have certainly overcome all the drawbacks of conventional IEF and they truly provide for a medium of unlimited stability However, we prefer to end with a note of caution: even such a highly refined and extremely successful

method as IPG, while superbly fit for performing IEF, will still not allow proper measurements of D and $du/d(pH)$, because the protein will form a salt with the matrix as it begins to move away from the pI zone (possible even in the pI region).

4 SUMMARY

Four potential modes of isoelectric focusing in non-amphoteric buffers are evaluated: (a) "stack" or "train" of free bases or acids "arrested by a deprotonation or protonation mechanism", respectively (Chrambach); (b) "chemically bonded" (immobilized) pH gradients (Righetti *et al.*); (c) "physically bonded" or "quasi-immobilized" pH gradients (Bier *et al.*); (d) steady-state rheoelectrolysis (Rilbe). The first is based on a "catastrophe" theory, *i.e.*, it confines the buffers in a pH region where they can create a pH gradient by an isotachophoretic mechanism, but where they do not have sufficient buffering capacity to stabilize it; no true isoelectric focusing can ever be achieved with this system. The last three are based on sound and well defined theories; however, at present, only system (b) (immobilized pH gradients) has proved to be a simple and reliable technique, easily transplantable in any laboratory. Bier *et al.*'s and Rilbe's approaches require complex and elaborate experimental set-ups and strict adherence of laboratory practice to a set of physical laws governing the system. In practice, owing to the divergence of experimental approaches from idealized physical equilibria, the last two approaches appear still to be far away from daily laboratory work.

5 ACKNOWLEDGEMENTS

We thank Drs. M. Bier, R. A Mosher and W. Thormann (Tucson, AZ, U.S A.) for much enlighting discussion and criticism. This work was supported by a grant from the Consiglio Nazionale delle Ricerche (CNR, Rome, Italy), Progetto Finalizzato Chimica Fine e Secondaria, Sottoprogetto Metodologie di Supporto.

REFERENCES

1 H Svensson, *Acta Chem Scand*, 15 (1961) 325–341
2 H Svensson, *Acta Chem Scand*, 16 (1962) 456–466
3 P. G Righetti, *Isoelectric Focusing Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983
4 A. Kolin, *Proc. Nat Acad Sci U S*, 41 (1955) 101–110
5 A Chrambach and L M Hjelmeland, in H Hirai (Editor), *Electrophoresis '83*, Walter de Gruyter, Berlin, 1984, pp. 81–97.
6 R J Raouts, *PhD Thesis*, University of Eindhoven, 1972
7 E Schumacher, *Helv Chim. Acta*, 40 (1957) 221–228
8 H Pettersson, *Acta Chem Scand*, 23 (1969) 2631–2635
9 U H Stenman and R Grasbeck, *Biochim. Biopohys. Acta*, 286 (1972) 243–251.
10 G Dossi, F Celentano, E Gianazza and P G Righetti, *J Biochem Biophys Methods*, 7 (1983) 123–142
11 E Gianazza, G Dossi, F Celentano, B Bjellqvist and P G. Righetti, *Electrophoresis*, 5 (1984) 88–97
12 E Gianazza, G Dossi, F. Celentano and P G Righetti, *J Biochem Biophys Methods*, 8 (1983) 109–133
13 P. G Righetti, *J Chromatogr*, 300 (1984) 165–223
14 M Bier, R A. Mosher, W Thormann and A Graham, in H. Hirai (Editor), *Electrophoresis '83*, Walter de Gruyter, Berlin, 1984, pp 99–107

15 B Bjellqvist, K Ek, P G Righetti, E Gianazza, A Gorg, W Postel and R Westermeier, *J Biochem. Biophys Methods*, 6 (1982) 317–339
16 H Rilbe, *J Chromatogr*, 159 (1978) 193–205
17 M Jonsson and S Fredriksson, *Electrophoresis*, 2 (1981) 193–203
18 H Rilbe, *Electrophoresis*, 2 (1981) 261–267
19 H Rilbe, *Electrophoresis*, 2 (1981) 268–272
20 H Rilbe, *Electrophoresis*, 3 (1982) 332–336
21 N Catsimpoolas, *Separ Sci*, 10 (1975) 55–106