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CONDUCTIVITY, BUFFERING CAPACITY, CONCENTRATION AND pH PROFILES OF CARRIER AMPHOLYTES FOCUSED IN NARROW-RANGE IMMOBILIZED pH GRADIENTS

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SUMMARY

It is possible to measure pH values in ultra-narrow immobilized pH gradients (IPGs) when the polyacrylamide matrix contains a secondary, carrier ampholyte generated pH gradient. After an IPG run, the buffering ampholytes, contained in 5 mm gel segments, along the focusing axis, are eluted and the following measurements are made: pH, conductivity, buffering capacity and concentration profiles of carrier ampholytes. Local concentration maxima of buffering ampholytes correspond to maximum conductivity and buffering capacity. The same relationship applies to local minima of the same parameters. No single commercial carrier ampholyte mixture ensures smooth conductivity and buffering capacity profiles and even pH gradients over very narrow pH intervals (0.3 pH unit spans). The best profiles are obtained only with mixtures of different commercial carrier ampholytes. Across neutrality, *ca.* 10% total buffering ampholytes should be added to an ultra-narrow IPG gradient to ensure an average buffering power ($\beta_{av.}$) of *ca.* 3 mequiv./l · pH and an average conductivity ($\lambda_{av.}$) of 20–30 μS, while in acidic (around pH 4 and below) and alkaline (above pH 9) pH intervals only one third of this concentration is needed, as the carrier ampholytes exhibit substantially higher λ and β values and the hydrolytic products of water begin to contribute to the buffering capacity and conductivity of the system.

INTRODUCTION

Immobilized pH gradients (IPGs)[1] have been plagued, in the past, by the difficulty of assessing the course of the pH gradient once the buffering ions and titrants have been grafted on to the polyacrylamide matrix and by the slow migration of macro-ions to their p*I* position in narrow and ultra-narrow pH gradients. Based on an original idea developed in our laboratory[2], we have recently described a novel

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approach to pH gradient fractionation of proteins, namely a mixed-type, "carrier ampholyte–Immobiline" gel[3]. In these gels the primary, IPG gradient stabilizes the secondary, carrier ampholyte (CA) gradient. The latter, while increasing background conductivity for quicker focusing of proteins[4,5], should in principle allow easy measurements of the pH gradient profile along the separation axis. In fact, in a recent paper[6], we have demonstrated the feasibility of such an approach: gradients as narrow as 1 pH unit could be read by eluting the CAs superimposed on the primary IPG gradient, with a maximum uncertainty of ± 0.1 pH unit (10% of the pH gradient width) when due precautions were taken to eliminate interference from carbon dioxide and temperature effects on the pH readings. Additionally, we have also shown the possibility of pH assessments in ultra-narrow pH gradients (0.2–0.3 pH unit)[7]. In the latter instance, however, there are two types of problems: determination of the correct amount of CAs to be added to the IPG gel and evaluation of the best commercial buffering ampholyte product ensuring the most even pH profile.

In this work, we dealt with these two aspects by measuring the conductivity, buffering capacity, mass content and pH profiles of isoionic CAs focused against a stationary, immobilized pH gradient.

EXPERIMENTAL

Immobiline buffers and Ampholine CAs were purchased from LKB (Bromma, Sweden), acrylamide monomers, polymerization catalysts and Biolyte buffers from Bio-Rad Labs. (Richmond, CA, U.S.A.), Gel Bond PAG from Marine Colloids (Rockland, ME, U.S.A.), Pharmalyte buffers from Pharmacia (Uppsala, Sweden) and ninhydrin, methanol and all other chemicals of analytical-reagent grade from Merck (Darmstadt, F.R.G.). The isoelectric focusing (IEF) experiments in IPGs were carried out by using the LKB Ultrophor apparatus together with an LKB 2197 constant-power supply and for cooling an LKB 2209 Multitemp. For gel casting an LKB 2117-901 gradient gel kit with a micro-gradient mixer, for pH measurements a pH M64 research pH meter from Radiometer (Copenhagen, Denmark), for differential pH measurements a Delpas ΔpH apparatus from Kontron (Zurich, Switzerland) and for conductimetric measurements a Model 101 conductimeter from Analytical Control Italia (Cinisello, Italy) were used.

IPGs were cast according to published methodologies[8,9]. The gels were 11.5 $\times$ 11 $\times$ 0.07 cm in size. The chambers of the micro-gradient mixer were each filled with 8.0 ml of a solution containing 3.5%T, 4%C* and Immobilines at concentrations calculated to give pH gradients of 7.15–7.45, 4.0–4.3 and 9.3–9.6. For the pH 7.15–7.45 gradient the acidic chamber contained the following Immobilines (each from a stock 0.2 *M* solution): 263 μl of p*K* 7.0 and 115 μl of p*K* 3.6, the corresponding amounts for the basic chamber being 286 μl and 81 μl, respectively. For the pH 4.0–4.3 gradient the acidic chamber contained 252 μl of p*K* 4.6 and 53 μl of p*K* 9.3, the corresponding amounts for the basic chamber being 251 μl and 96 μl, respectively. For the pH 9.3–9.6 gradient the acidic chamber contained 274 μl of p*K* 3.6 and 414 μl of p*K* 9.3, the corresponding amounts in the basic chamber being 197 μl of p*K* 3.6 and 399 μl of p*K* 9.3. The catalysts (8 μl of 40% ammonium persulphate and 4 μl of

* T = [g acrylamide + g N,N'-methylenebisacrylamide (Bis)]/100 ml solution; C = g Bis/% T.

N,N,N′,N′-tetramethylethylenediamine (TEMED) per chamber) were added directly to the gradient mixer immediately before filling the gel into the cassette. After addition of TEMED and prior to the addition of persulphate, the final pH of the solution was adjusted, with standard acid or base, to around neutrality (*ca.* pH 7.5) so as to ensure efficient copolymerization conditions[10] and to avoid destruction of alkaline Immobilines[11]. The carrier ampholyte buffers were added to the IPG gel, after washing and drying, by reswelling it in the desired CA range[12,13]. After focusing at 1000 V, 4 W maximum power, overnight at 10°C, the gels were cut into 8.0 × 0.5 × 0.07 cm strips (a gel volume of 280 μl) and eluted with 2 ml of distilled water overnight. First one aliquot was taken for conductivity measurements, then the remainder was made 100 m*M* in potassium chloride and pH and buffering capacity measurements were performed. On the last aliquot, a spectrophotometric assay for evaluation of the concentration profiles of focused CAs was run.

ΔpH meter

This instrument allows one to measure small pH differences (maximum 0.4 pH unit) between two solutions with the aid of two glass electrodes. The sensitivity is of the order of $5 \cdot 10^{-5}$ pH units[14]. For buffering power (β) measurements, a volume of 300 μl of CAs eluted from IPG gel strips is pumped into one of the two capillary electrodes; 10–30 μl of 0.01 *M* hydrochloric acid or 0.01 *M* sodium hydroxide solution are added to 300 μl of the same CA solution and pumped into the second electrode. The pH difference between the two electrodes is recorded. The intrinsic pH variation, due to spurious electrical signals generated by the electrodes and to external electric interferences, is the ΔpH observed on introducing the same buffer solution in the two electrode chambers. This is of the order of 0.03 pH unit and is determined at the beginning of each measuring cycle and checked periodically every 3–4 pH readings. This value is subtracted from each ΔpH measured. The buffering power (β) expressed as mequiv./l · pH is the ratio between the molar concentration of H^+ or OH^- after addition of hydrochloric acid or sodium hydroxide solution and the ΔpH. In general, 21 β readings are taken for each IPG experiment, corresponding to 5 mm increments along the separation axis. As the gel is 11.5 cm long from anode to cathode, two 5 mm gel segments are discarded, one at each extreme, corresponding to the contact between gel and electrodes. An analogous correction is made to the plot of the theoretical gradient in the various figures.

Carrier ampholyte determination

For ninhydrin assay, 25 ml of degassed 2.08 *M* acetate buffer (pH 5.5) were added to 25 ml of ninhydrin–methanol 3.33% stock solution. This reagent was prepared just before use and kept in the dark. A 500-μl volume of this solution was added to 500 μl of eluate from the above IPG gel fractions (diluted 1:10 or 1:5) and then incubated for 15 min in boiling water. The samples were read at 570 nm in a Varian DMS90 spectrophotometer (Palo Alto, CA, U.S.A.), against a blank of plain acetate buffer. Absorbances were read whilst the solutions were still warm (50°C) so as to prevent precipitation of the hydrindantine complex in highly colored fractions (absorbance *ca.* 3)[15].

RESULTS

Tables I–III give the conductivities, buffering capacities and pH values of stock solutions of different commercial carrier ampholytes diluted to 1% and buffering across neutrality (Table I) or at acidic (Table II) or alkaline (Table III) pH values. These data agree fairly well with published values or with data supplied by the manu-

TABLE I

PARAMETERS OF A STOCK 1% SOLUTION OF DIFFERENT CARRIER AMPHOLYTES BUFFERING ACROSS NEUTRALITY*

Chemical	*Conductivity (μS)*	*Buffering capacity (mequiv./l · pH)*	*pH*
Ampholine pH 6–8	174	9	6.4
Pharmalyte pH 5–8	158	8	6.7
Biolyte pH 6–8	114	11	7.2
Mixture (1:1:1)	132	10	6.8

* Measurements made in stock solutions of different carrier ampholytes having the stated nominal pH interval, diluted to 1% with distilled water.

TABLE II

PARAMETERS OF A STOCK 1% SOLUTION OF DIFFERENT CARRIER AMPHOLYTES BUFFERING AT ACIDIC pH*

Chemical	*Conductivity (μS)*	*Buffering capacity (mequiv./l · pH)*	*pH*
Ampholine pH 3.5–5	196	19	4.4
Pharmalyte pH 2.5–5	222	13	3.9
Biolyte pH 3–5	189	17	4.4
Mixture (1:1:1)	190	16	4.2

* Measurements made in stock solutions of different carrier ampholytes having the stated nominal pH interval, diluted to 1% with distilled water.

TABLE III

PARAMETERS OF A STOCK 1% SOLUTION OF DIFFERENT CARRIER AMPHOLYTES BUFFERING AT ALKALINE pH*

Chemical	*Conductivity (μS)*	*Buffering capacity (mequiv./l · pH)*	*pH*
Ampholine pH 8–10	230	21	9.2
Pharmalyte pH 8–10	129	11	9.2
Servalyte pH 9–11	500	35	9.8
Mixture (1:1:1)	338	21	9.5

* Measurements made in stock solutions of different carrier ampholytes having the stated nominal pH interval, diluted to 1% with distilled water.

facturer. For example, for Pharmalyte pH 5–8 we give β = 8 mequiv./l · pH for a 40-fold diluted stock solution while the manufacturer gives 0.35 equiv./l · pH for the undiluted bottle (it is assumed here that Pharmalytes are also 40% solutions, as the other commercial CAs, but the manufacturer does not give any concentration value for any pH interval; some workers believe that their concentration should be around 32%[16]). For Ampholine pH 6–8 we obtained β = 9 mequiv./l · pH whereas several workers[17–19] give values of 6–8 mequiv./l · pH for focused fractions. It can be seen from the tables that conductance and buffering capacity are two properties that are closely related, as demonstrated by Svensson[20]. If the conductance is good, so is the buffering capacity, and *vice versa*. Minima of β and conductivity (λ) are found for all species across neutrality, while proportionally higher values for both parameters are found at acidic and basic pH values (the extremely high figures we give at alkaline pH values in one instance are due to the fact that, for one of them, we had available only a more alkaline range and a Servalyte instead of a Biolyte product).

We next addressed the question of the behaviour of the different commercial CAs and of the initial levels required in ultra-narrow IPG gradients (0.3 pH unit spans). Across neutrality (IPG pH 7.15–7.45) we used an initial load of 10% CAs, impregnated directly in the IPG matrix. After focusing, gel segments are cut and the following measurements taken: β, conductivity, pH and spectrophotometric assay for relative amounts of CAs focused in a gel fraction. The results of such analyses for Ampholine are shown in Fig. 1 (lower left panel). It can be seen that the conductivity peaks are about midway in the pH 7.15–7.45 span. In agreement with this, there is a major peak of β power and also a broad plateau of concentration of Ampholine species (the latter two parameters show at least 3–4 major peaks). In contrast, Pharmalytes (Fig. 1, lower right panel) show an even conductivity profile associated to a remarkably smooth β power course, although the spectrophotometric assay for carrier ampholytes shows three major peaks. Intermediate between the two is the behaviour of Biolytes (Fig. 1, upper right panel), with an irregular profile in the β distribution. The mixture of the three (Fig. 1, upper left panel) shows fairly even β and conductivity courses, with only two major peaks in the spectrophotometric assay for CAs. The overall parameters of the individual CAs and their mixture are summarized in Table IV, where we report minima and maxima of β and λ and their average values. It can be seen that the remarkable performance of Pharmalytes is associated with considerably smaller values (about half) of β and λ, while the mixture of the three, in addition to showing good β and λ profiles along the pH axis, also has more acceptable average values of these two functions.

From the above, it appears that in ultra-narrow IPG spans (0.3 pH unit) across neutrality an initial input of *ca.* 10% CAs is required to obtain a final gel level of *ca.* 0.5–1% CAs, ensuring reasonable values of buffering capacity ($\beta_{av.}$ = 3 mequiv./l · pH) and of conductivity ($\lambda_{av.}$ = 15–20 μS). We next investigated what happens in acidic pH intervals. We chose a pH 4.0–4.3 IPG interval, equilibrated it with 10% CAs and repeated the above measurements of β power, conductivity, pH and spectrophotometric assay for CAs. As shown in Fig. 2 (lower left panel), this time it is the Ampholines that rank best, giving fairly smooth β power and conductivity profiles, accompanied by a fairly even p*I* distribution of focused CAs, with only one major peak present. Pharmalytes (Fig. 2, lower right panel) exhibit a strong dichotomy with extremely high β value, conductivity and CA distribution in the first half

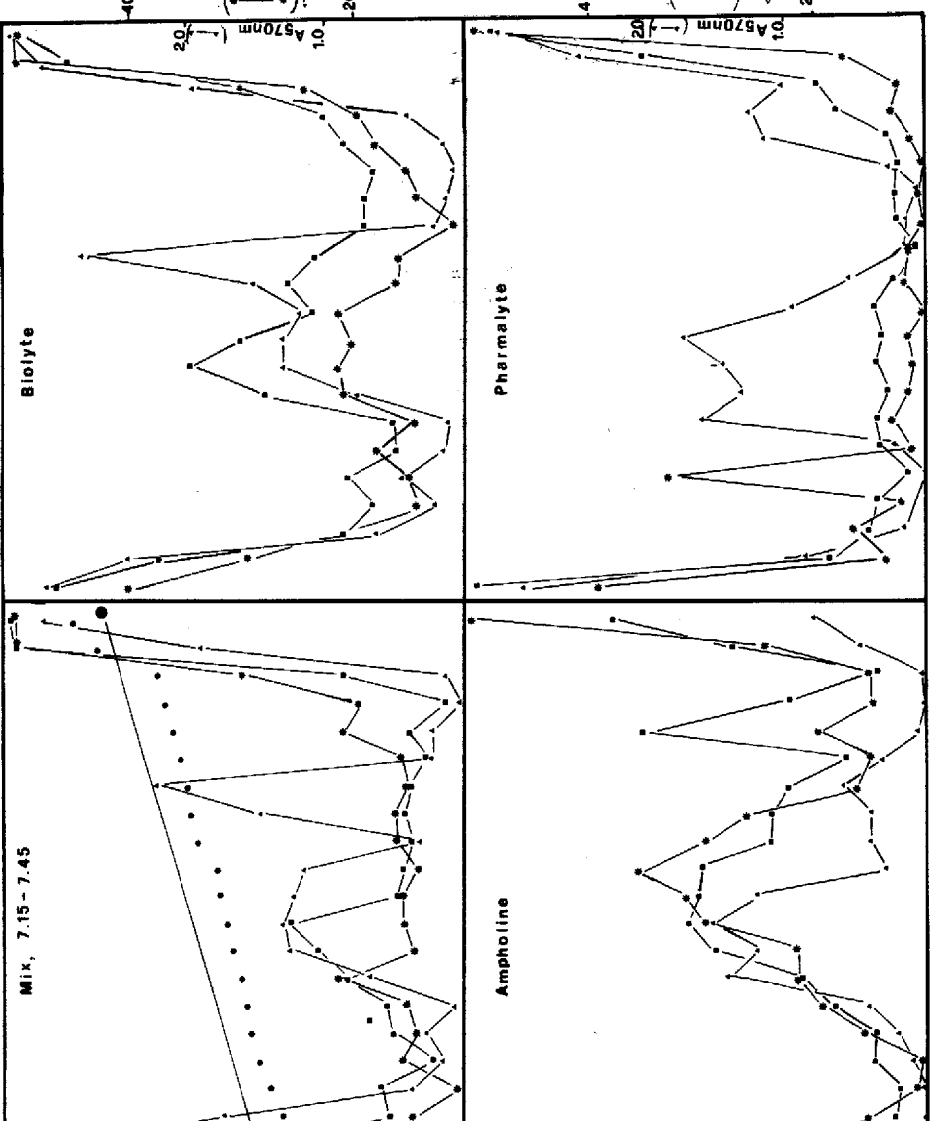

Fig. 1. Conductivity (λ, ★, in μS), buffering capacity (β, ■, in mequiv./l · pH) and spectrophotometric assay for carrier ampholytes (▲, in absorbance units at 570 nm) focused in an ultra-narrow pH 7.15–7.45 IPG gradient. Each single commercial product (Biolyte, upper right; Ampholine, lower left; and Pharmalyte, lower right) was impregnated in the IPG matrix at a 10% concentration. The mixture (upper left panel) contained 3% of each commercial preparation. After focusing, the gel was segmented into 23 slices at 5 mm increments from anode to cathode; one fragment at each extremity was discarded (as it was impregnated with anolyte and catholyte) and the assay was performed in the remaining 21 slices. In the upper left panel, the theoretical pH gradient (solid line between two large dots) is also plotted, together with the experimentally measured pH gradient in each gel slice (line of small dots). These two plots have been omitted from the other panels for the sake of clarity.

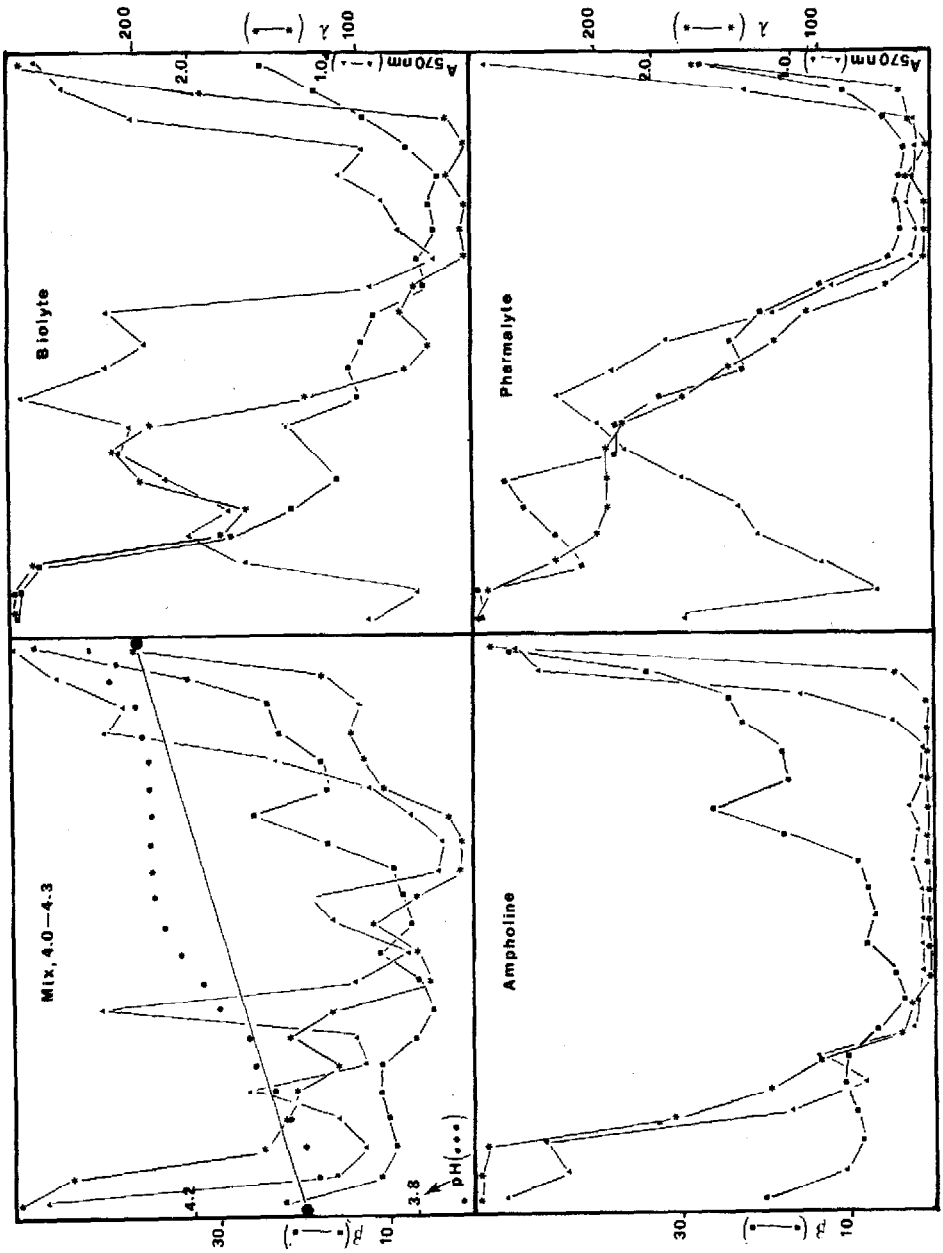

Fig. 2. Conductivity (λ, ★, in μS), buffering capacity (β, ■, in mequiv./l · pH) and spectrophotometric assay for carrier ampholytes (▲, in absorbance units at 570 nm) focused in an ultra-narrow pH 4.0–4.3 IPG gradient. All other experimental details as in Fig. 1.

TABLE IV

CONDUCTIVITY (λ, μS) AND BUFFERING CAPACITY (β, mequiv./l · pH) OF DIFFERENT CARRIER AMPHOLYTES FOCUSED IN AN IPG pH 7.15–7.45 INTERVAL

Parameter	*Ampholine*	*Biolyte*	*Pharmalyte*	*Mixture (1:1:1)*
$\lambda_{min.}$	11	12	10	12
$\lambda_{max.}$	37	24	16	21
$\lambda_{av.}$	21	19	12	15
$\beta_{min.}$	1.3	2.2	1.3	2.0
$\beta_{max.}$	5.0	5.5	3.3	5.0
$\beta_{av.}$	3.3	3.7	1.9	2.8

of the gradient (pH 4.0–4.15) and minima of all these values in the upper half of the pH interval (pH 4.15–4.30). Biolytes (Fig. 2, upper right panel) present a behaviour markedly similar to that of Pharmalytes, with too high values in the acidic region and too low values in the upper, higher pH region of the IPG interval. The 1:1:1 mixture still shows a rugged profile with an improvement of the three parameters compared with Pharmalytes and Biolytes but with a worsening of the profile in comparison with Ampholine. Table V summarizes the relevant parameters for the individual CAs and their mixture: compared with the separation across neutrality (pH 7.15–7.45) it can be seen that the β and λ values are much too high to ensure correct focusing in this region. If we take as reference values those given for the neutral pH interval, in fairly acidic IPG ranges the amounts of CAs added to the system should be one third of that loaded across neutrality, to ensure similar β and λ values.

At this point it appeared crucial to investigate also the alkaline pH range, to see if the same rule for as acidic conditions also applied (in previous work, this had not been taken into consideration[6,7]). As shown in Fig. 3, no single commercial product ensures uniform β and λ courses: Pharmalytes rank best, followed by Servalytes and Ampholines. It is only the mixture, however, that ensures the only acceptable β and λ profiles. As shown in Table VI, as in acidic pH gradients, the average β and λ values appear to be much too high to ensure proper focusing in this region. Again, if we take as acceptable values those obtained across neutral pH (IPG 7.15–7.45), it appears that the amount of CAs to be added to the system should be one

TABLE V

CONDUCTIVITY (λ, μS) AND BUFFERING CAPACITY (β, mequiv./l · pH) OF DIFFERENT CARRIER AMPHOLYTES FOCUSED IN AN IPG pH 4.0–4.3 INTERVAL

Parameter	*Ampholine*	*Biolyte*	*Pharmalyte*	*Mixture (1:1:1)*
$\lambda_{min.}$	38	48	41	55
$\lambda_{max.}$	130	200	220	130
$\lambda_{av.}$	70	116	120	99
$\beta_{min.}$	4.0	4.5	3.9	4.0
$\beta_{max.}$	24	39	51	20
$\beta_{av.}$	12	14	23	12

TABLE VI

CONDUCTIVITY (λ, μS) AND BUFFERING CAPACITY (β, mequiv./l · pH) OF DIFFERENT CARRIER AMPHOLYTES FOCUSED IN AN IPG pH 9.3–9.6 INTERVAL

Parameter	*Ampholine*	*Servalyte*	*Pharmalyte*	*Mixture (1:1:1)*
$\lambda_{min.}$	150	140	45	70
$\lambda_{max.}$	220	340	120	160
$\lambda_{av.}$	200	250	68	100
$\beta_{min.}$	12	6	3	4
$\beta_{max.}$	33	20	10	15
$\beta_{av.}$	18	11	6	11

third of that loaded across neutrality, to ensure similar β and λ values. Hence the addition of CAs to an ultra-narrow IPG gel should follow a bell-shaped profile, with maxima across neutrality and minima at both acidic and alkaline pH values.

DISCUSSION

Quality of carrier ampholytes

It has been stated by Vesterberg (the inventor of CAs)[21] that, by his synthesis, more than 360 homologues and isomers of CA species are obtainable in the pH range 3–11 and that, by isotachophoretic analysis, at least 20 different species per pH unit can be resolved. According to his original patent (used to produce LKB Ampholine), the main ingredient in the synthesis is an oligoamine (pentaethylenehexamine) with its steric isomers, with probably variable amounts of shorter amines, such as triethylenetetramine and tetraethylenepentamine. Unfortunately, this claim might be true: according to Gelsema *et al.*[22], "normal-range Ampholines ($3.5 < pI < 10$) contain a large proportion of ampholytes derived from only a few polyamines, probably the isomers of pentaethylenehexamine". When Pharmalytes appeared on the market, it was stated that over the entire pH interval available (pH 2–11) as many as 5000 different amphoteric buffers could be present, ensuring a very even buffering capacity and conductivity over large portions of the pH scale[23]. When Charlionet *et al.*[24] produced their CA buffers (oligoamines cross-linked with epoxides and then reacted with acrylic acid) they calculated that, solely in the pH 4.3–5.0 zone, "more than 4000 ampholyte species with different isoelectric points and dissociation constants could have been created". Thus, it is seen that there are different commercially available CA preparations with widely different conductivity and buffering capacity profiles owing to (a) the total number of species present and (b) their properties at pH = p*I* ("good" or "poor" CAs, according to Rilbe's original definition)[25]. By testing different batches with carbamylated charge trains, Tollaksen *et al.*[26] concluded that Pharmalytes present the most linear pH profile (implicitly, this must be accompanied by even λ and β courses)[27] over the pH 3–10 interval.

When utilizing ultra-narrow IPG gradients, it can be assumed that, over such narrow pH spans, the λ and β profiles will be extremely even, so that this would represent an ideal milieu for focusing proteins, as there will be uniform migration of the species and no trapping in conductivity gaps or in "hot spots" generated by these

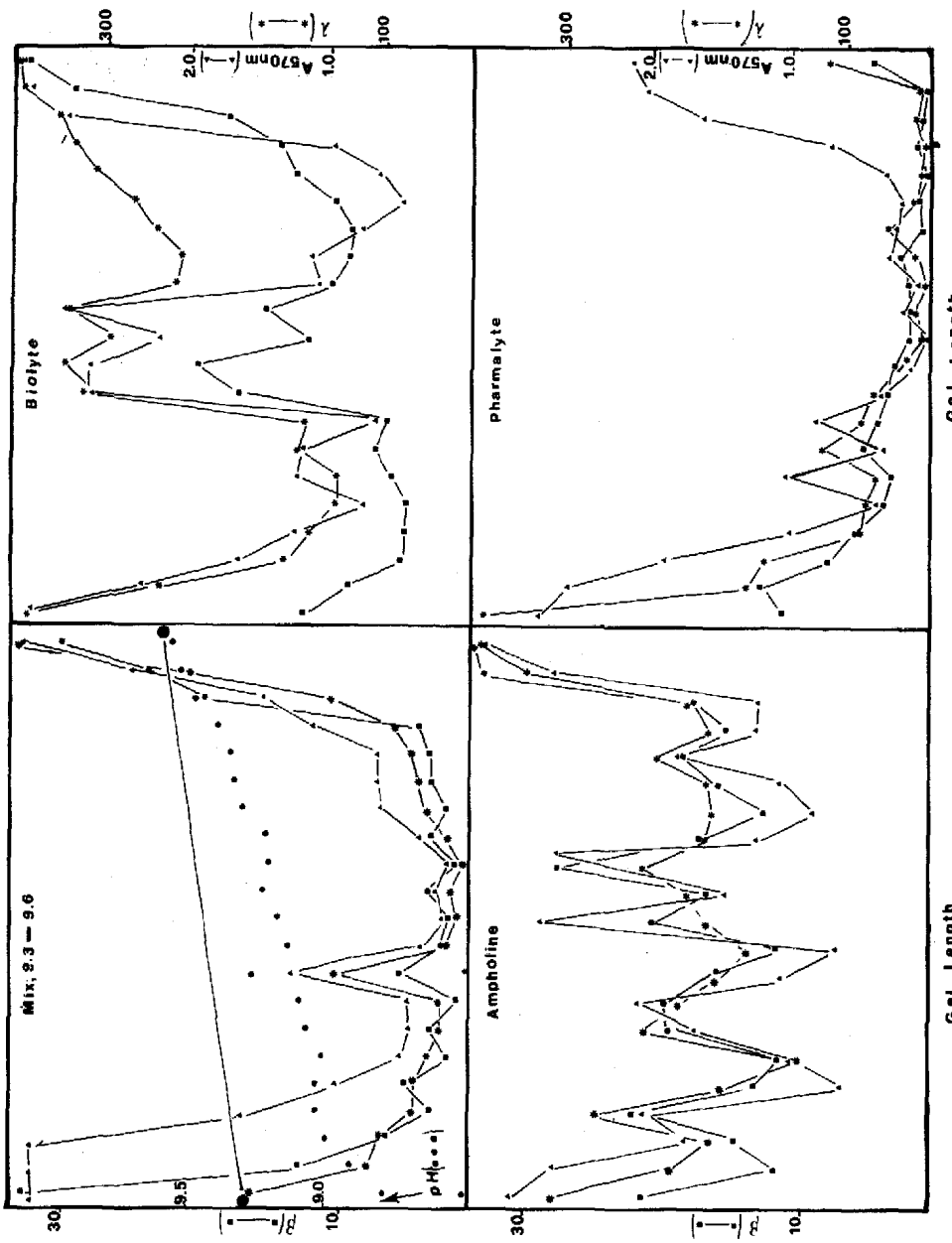

Fig. 3. Conductivity (λ, • in μS), buffering capacity (β, ■, in mequiv./l · pH) and spectrophotometric assay for carrier ampholytes (▲, in absorbance units at 570 nm) focused in an ultra-narrow pH 9.3–9.6 IPG gradient. All other conditions as in Fig. 1. The large discrepancy between theoretical (solid line between two large dots) and measured (line of small dots) pH gradients is due to temperature differences (focusing at 10°C, pH readings at 25°C) and to adsorption of carbon dioxide during gel slice cutting.

gaps. However, owing to the extremely low conductivity of IPG matrices (mostly owing to ionization of water and oscillation of the buffering groups about the chemical bond in the polyacrylamide coils), migration of macromolecules will be severely hampered. It is for this reason that we resorted to mixed "Immobiline–CA" gels[3–5]. The unexpected finding in this work is that, over ultra-narrow IPG ranges, all commercial CA preparations break down, *i.e.*, they present sharp discontinuities and discrete profiles of β and λ. By inspection of the figures it can be seen that, in most instances, the peaks and valleys of β and λ are coincident with the spectrophotometric assay for CAs, which is an indication of local concentration of CAs. Hence it appears that over ultra-narrow ranges CAs show discrete mass distributions accompanied by sharp discontinuities of buffering power and conductivity; in a focusing experiment, this might hamper rather than facilitate the protein migration to its p*I*.

There are two solutions to this problem: one is to mix different commercial carrier ampholytes (in general, as seen in Figs. 1–3, the mixture has smoother β and λ courses); the other is to synthesize highly diversified CA species. On the last point, there is an interesting comment to make on the quality of CAs. It is true that Pharmalytes have smoother β and λ courses but, on an equimolar basis, it appears that, over most of the pH interval (see Tables I and III), they possess about half of the β and λ values compared with Ampholine, Biolyte and Servalyte. This means that, in Pharmalytes, there must be a preponderance of relatively "poor" carrier ampholytes (*i.e.*, species with larger ΔpK, *e.g.*, >3 pH units) over "good" species (*i.e.*, amphoteres with smaller ΔpK, *e.g.*, <3 pH units). The former will focus as broader plateaus with substantially lower β and λ values around the p*I*. Paradoxically, Pharmalytes are "good" in IPGs because they are "poor" whereas Biolytes and Ampholines are "bad" because they are "good" CAs. New generations of CAs will have to account for these findings.

Levels of CAs to be added to IPGs

Our findings suggest a simple strategy for the amount of CAs to be added to ultra-narrow IPG gels: it should be the reciprocal of the β profile (which will also be the λ course) published in the literature for commercial species. As an example, Fig. 4 shows a simple solution for Ampholine: the U-shaped, lower profile is their buffering capacity along the pH axis in the focused state[18]; the bell-shaped upper profile is the amount to be added to ultra-narrow IPG gels, taking as a reference point the 10% level added across neutrality. The result will be an even buffering (and thus conductivity) profile along the pH axis (in other words, we act on the principle of keeping constant the product $C\beta$, *i.e.*, molarity of CAs times their buffering capacity). Even before knowing these results we had in fact suggested that, in extreme IPG ranges (pH 3–4 and 10–11), CAs should not be added at all, on account of the already substantial conductivity due to free H^+ and OH^- in these ranges[28,29]. In fact, the addition of conductivity and electroendosmosis quenchers (strong gradients of small neutral molecules or shallow gradients of free-draining polymers) was suggested.

CONCLUSIONS: A CALL FOR MEDIOCRITY

Rilbe[30] stated that "high-quality as well as low-quality carrier ampholytes, with a preponderance for the high quality in the neutral range, are essential for

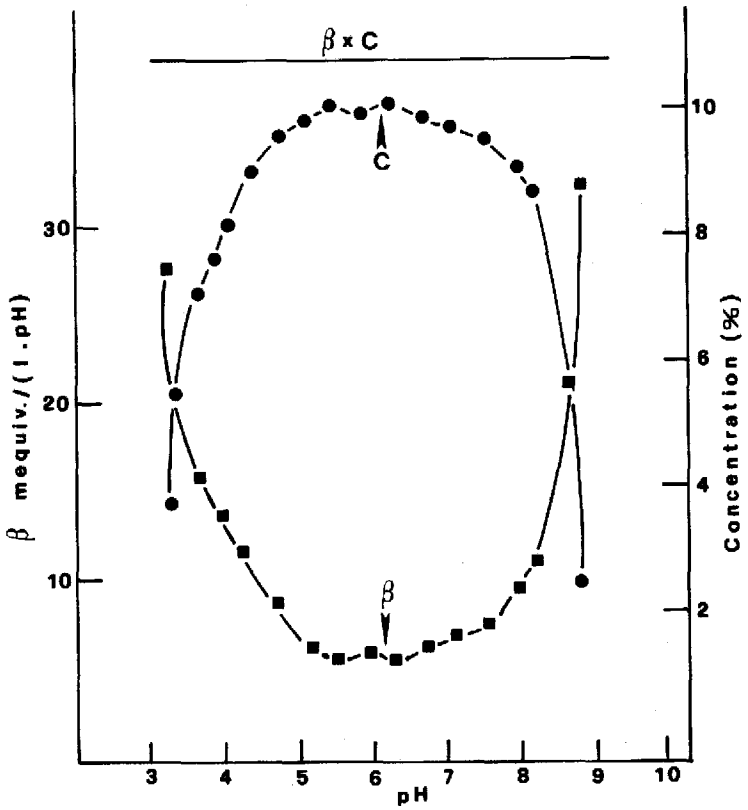

Fig. 4. Plot of buffering power (β, in mequiv./l · pH, ■) of 1% focused Ampholine in a pH 3–10 gradient (experimental data from ref. 18). The superimposed, bell-shaped profile (●) represents the reciprocal amount of carrier ampholytes to be added to ultra-narrow (0.3 pH unit spans) IPG gradients in order to maintain constant the product $C\beta$ (local Ampholine concentration, in % times buffering power) along the pH axis (upper horizontal line).

successful isoelectric focusing". We had always been puzzled by this statement, but now, 10 years later, we fully understand the implications and we regret we have to push it even further: when admixed with ultra-narrow IPGs, only mediocre (low-quality) CAs will ensure flat and broad plateaus on to which proteins can safely land.

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