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DEVELOPMENT OF FOCUSING BUFFER SYSTEMS FOR GENERATION OF WIDE-RANGE pH GRADIENTS DURING HIGH-PERFORMANCE CHROMATOFOCUSING

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SUMMARY

Our application of chromatofocusing principles to high-performance anion-exchange columns has resulted in greatly increased resolution of certain protein isoforms. However, commercially available polyampholyte mixtures used to generate the internal pH gradient required during chromatofocusing impose ambiguities and restrictions which prevent us from utilizing the full potential of this separation technique. Therefore, one goal of this investigation has been the development of a single focusing buffer of defined composition for the internal generation of both narrow- and wide-range (pH 8.5 to 3.5) pH gradients during high-performance chromatofocusing. Different focusing buffers were formulated and tested utilizing both conventional anion-exchange materials (Polybuffer Exchanger 94) and high-performance anion-exchange columns (Bakerbond PEI). For any given focusing buffer, the slope and other qualities of the pH gradient varied with the column type utilized. A focusing buffer is described which can produce good quality, linear pH gradients (in the region from pH 8.1 to 3.5) with slopes of 0.05 pH units/ml or greater. Similar results were obtained in both the presence and absence of 6 *M* urea. Equations describing the relationship of column and focusing buffer variables to the theoretical and observed pH gradients formed during chromatofocusing are discussed.

INTRODUCTION

Since Kolin first described the "isoelectric condensation" of ampholyte ions in an electric field and demonstrated the potential of this technique for separation of protein mixtures[1–3], several investigators have made significant contributions towards the improvement of this and other protein focusing techniques. Svensson (Rilbe) provided theoretical calculations for using the buffering capacity of "carrier" ampholytes to create "natural" pH gradients in an electric field[4,5]. However, the paucity of suitable ampholytes (with p*I* values between 4 and 7) and the cumbersome process of experimentally determining the appropriate mixtures of simple ampholytes limited the general usefulness of isoelectric fractionation until Vesterberg[6] reported the synthesis of polyampholytes with the necessary isoelectric properties. Isoelectric focusing

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utilizing an electric field-dependent pH gradient developed rapidly thereafter and remains one of the most sensitive means available for separation of proteins by surface charge. Useful and improved variations of this technique have continued to evolve[7,8].

The application of isoelectic fractionation principles has been extended in theory and practice by Sluyterman and co-workers[9–13] who first described the focusing of proteins during generation of "internal" pH gradients on ion-exchange columns. Commercially available synthetic polyampholytes were found best suited to produce the pH gradients (mostly 2–3 pH units) investigated during experimental verification[11–13].

Our development of high-performance chromatofocusing techniques for the characterization of steroid receptor surface charge heterogeneity has resulted in the ability to rapidly separate various forms of steroid receptor proteins not resolved by ion-exchange chromatography or isoelectric focusing during electrophoresis[14–16]. However, the continued use of commercially available polyampholytes for extensions of these studies imposes both theoretical and practical limitations. These considerations and our recognition of a general need for a more definitive approach to the preparation of alternative chromatofocusing buffers has prompted these studies*.

We report here some of the end results from our investigation into the development of simple focusing buffers for the internal generation of wide-range, linear pH gradients using both conventional and, particularly, high-performance ion-exchange columns.

EXPERIMENTAL

Materials

Bakerbond PEI (polyethyleneimine) anion-exchange columns (approximately 0.1 mequiv. pH^{-1} for 1 g PEI silica) were generously supplied by J. T. Baker. Polybuffer Exchanger (PBE) 94 and Polybuffers 96 and 74 were obtained from Pharmacia Fine Chemicals. Ultra-pure urea was from Bethesda Research Labs. All buffer components listed in Table I are readily available from Sigma and/or Research Organics, and were not purified further before use.

Chromatofocusing on conventional and high-performance anion-exchange columns

The development and details of our high-performance chromatofocusing (HPCF) techniques employing commercially available polyampholytes (Pharmacia Polybuffers 96 and 74) were described previously[14–16]. Both the high-performance anion-exchange columns (250 × 4.6 mm I.D.) and the small (5–9 × 0.7 cm I.D.) open columns of PBE 94 used for these studies were equilibrated to pH 8.0–8.2 using 25 m*M* Tris–HCl buffer containing 10% or 20% (v/v) glycerol. Chromatography was performed in a cold room (4–6 °C) and at room temperature (20–25°C). Buffer pH was adjusted at the temperature of chromatography. All buffer solutions were vacuum filtered through Millipore 0.45 μm HAWP filters and degased before use.

* Preliminary portions of this work were presented at the *Eighth International Symposium on Column Liquid Chromatography, New York, NY, U.S.A., May, 1984.*

The formation of internal pH gradients was initiated using either polyampholytes (Polybuffers 96 and 74) or our focusing buffer (Table I) at a flow-rate of 0.6 or 1.0 ml/min. The pH of collected fractions (1.0 min) was determined (at the temperature of chromatography) immediately upon elution using a Corning Model 125 pH meter equipped with a microcombination calomel electrode. The pH values determined for collected fractions allowed to stand several hours were not measurably altered.

RESULTS

During our approach to the results presented here, we have designed a series

TABLE I

FOCUSING BUFFER

Group	*Buffer*		*Abbreviation*
	No.	*Composition*	
D	1	γ4-Amino-*n*-butyric acid (piperidic acid)	GABA
	2	Glycine	GLY
	3	3-(Cyclohexylamino)-1-propanesulfonic acid	CAPS
	4	2-Aminoethanesulfonic acid (taurine)	TRN
	5	2-(N-Cyclohexylamino)ethanesulfonic acid	CHES
	6	Asparagine	ASN
	7	Tris(hydroxymethyl)methylaminopropanesulfonic acid	TAPS
	8	Tris(hydroxymethyl)aminomethane	TRIS
C	9	Glycylglycine	GLYGLY
	10	N,N-Bis(2-hydroxyethyl)glycine	BICINE
	11	N-Tris(hydroxymethyl)methylglycine	TRICINE
	12	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	TES
	13	Piperazine-N,N′-bis(2-hydroxypropanesulfonic acid)	POPSO
	14	3-(N-Tris(hydroxymethyl)methylamino)-2-hydroxypropanesulfonic acid	TAPSO
	15	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid	HEPES
B	16	3-(N-Morpholino)propanesulfonic acid	MOPS
	17	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid	BES
	18	2-(2-Amino-2-oxoethyl)aminoethanesulfonic acid	ACES
	19	N-(2-Acetamido)-2-iminodiacetic acid	ADA
	20	Histidine	HIS
	21	3-(N-Morpholino)-2-hydroxypropanesulfonic acid	MOPSO
	22	Piperazine-N,N′-bis(2-ethanesulfonic acid)	PIPES
	23	Bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane	BIS-TRIS
	24	2-(N-Morpholino)ethanesulfonic acid	MES
A	25	Succinic acid	SA
	26	Citric acid	CA
	27	Propionic acid	PA
	28	Acetic acid	AA
	29	Lactic acid	LA
	30	Formic acid	FA
	31	Aspartic acid	ASP

of individual focusing buffers composed of simple, chemically-defined buffer constituents that collectively participate in the generation of internally developed pH gradients during chromatofocusing on ion-exchange columns. One particular goal of this investigation has been to obtain a clearer understanding of the precise mechanism(s) involved during the formation of these "internally" developed pH gradients. This requires understanding the properties of the mobile phase (focusing buffer) as well as the stationary phase (ion-exchanger and support), both separately and together. Therefore, the various focusing buffers we created ranged in complexity from 4 to 34 constituent buffer reagents (in variable relative proportions) and, on a given anion-exchange column, generated a spectrum from simple step gradients through the nearly linear gradients shown below. In a separate more comprehensive report we plan to detail the results of our exploratory investigations into such pH gradient--forming variables as: (1) the relative complexity and concentration of focusing buffer constituents, (2) buffering capacity of focusing buffer relative to column charge (ligand) and density, (3) initial column pH and final focusing buffer pH, (4) alterations in temperature and eluent flow-rates, and (5) focusing buffer viscosity, dielectric constant and ionic strength. The following are select results from our overall efforts to design focusing buffers which will generate wide-range, linear pH gradients during chromatofocusing on both conventional and high-performance anion-exchange columns.

Table I lists the individual buffer reagents used to construct a focusing buffer with several desirable characteristics. The focusing buffer constituents shown in Table

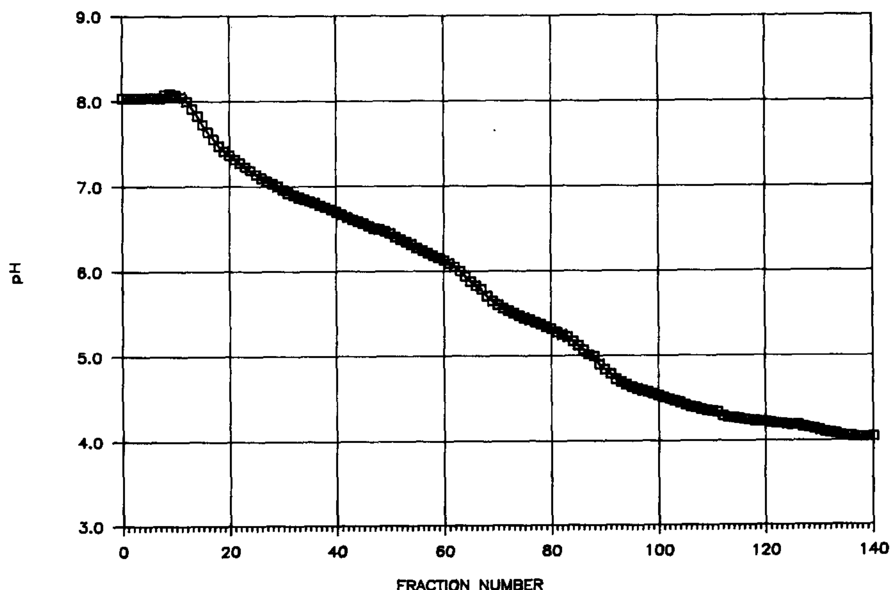

Fig. 1. Generation of internal pH gradient by chromatofocusing on PBE 94 using our focusing buffer outlined in Table I. The constituents from groups B, C and D were present at 2 m*M* each. Group A constituents were present at 0.5 m*M* each. The intrinsic pH of this focusing buffer was 3.9 and was not adjusted further. The average gradient slope (ΔpH/ml) from pH 8 to 4 is 0.07. Chromatography was performed at 20–22°C. Details are described in the text.

I are non-polymeric, chemically-defined reagents. The physicochemical properties of these components have been described by the manufacturers (see *Materials*) and in several recent publications[17–20]. Titration of this focusing buffer with acid or base yields a nearly straight line from pH 3 to 8, indicating an even buffering capacity[21] throughout the entire pH range of interest (approximately 0.0117 mequiv./pH/ml). The profile shown in Fig. 1 is representative (n = 8) of the pH gradient generated using our focusing buffer (Table I) on columns of PBE 94. The pH gradients presented in Figs. 2 and 3 reveal that the performance of this focusing buffer is relatively unaffected by inclusion of either molybdate or urea, both routinely used by us for analyses of steroid receptor protein structure[14–16]. The conditions used were different from those described for Fig. 1 only by the inclusion of either 10 mM sodium molybdate (Fig. 2) or 6 M urea (Fig. 3) in both the column equilibration and elution (focusing) buffer. In each case the pH gradients generated remain fairly linear. Inclusion of molybdate only slightly alters gradient slopes in the acidic pH region. This phenomenon has been observed using polyampholytes as well[14,16]. The observed effect of 6 M urea to increase the slope of the resulting pH gradient is quite reproducible (n = 10). Care was taken during preparation of fresh urea solutions to prevent the formation of cyanate[22]. Furthermore, the urea effects were observed using exactly the same experimental approach proposed by Ui[23] and therefore probably do not represent the same kind of systematic errors in pH measurements as described for isoelectric focusing of polyampholytes in 6 M urea[23,24]. The mechanism for this effect is not clear but may likely reflect the altered pK values of both mobile and

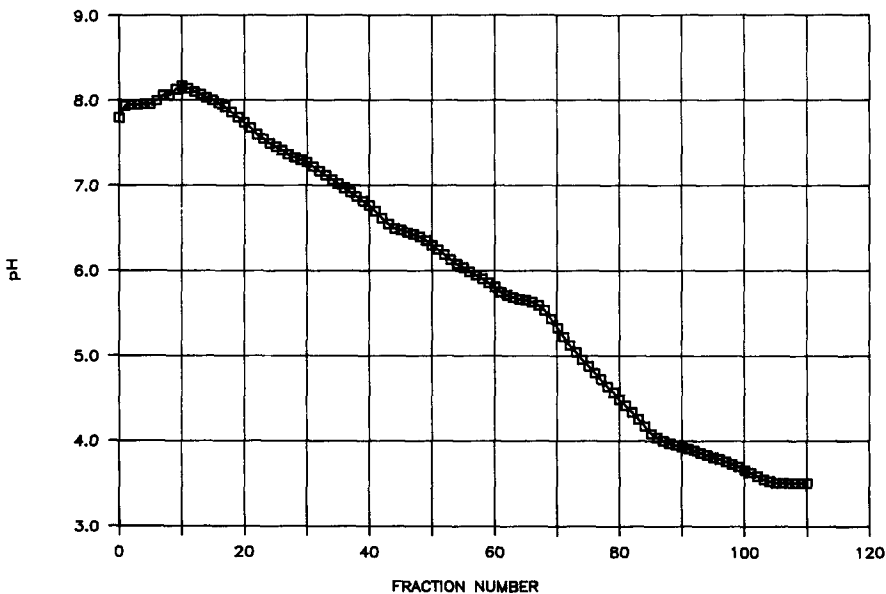

Fig. 2. Generation of internal pH gradient by chromatofocusing on PBE 94 using our focusing buffer (Table I) in the presence of molybdate. Both the column equilibration buffer and focusing buffer contained 10 mM sodium molybdate, otherwise all conditions, including buffer composition, were exactly as described for Fig. 1. The average gradient slope (ΔpH/ml) from pH 8 to 4 is 0.085.

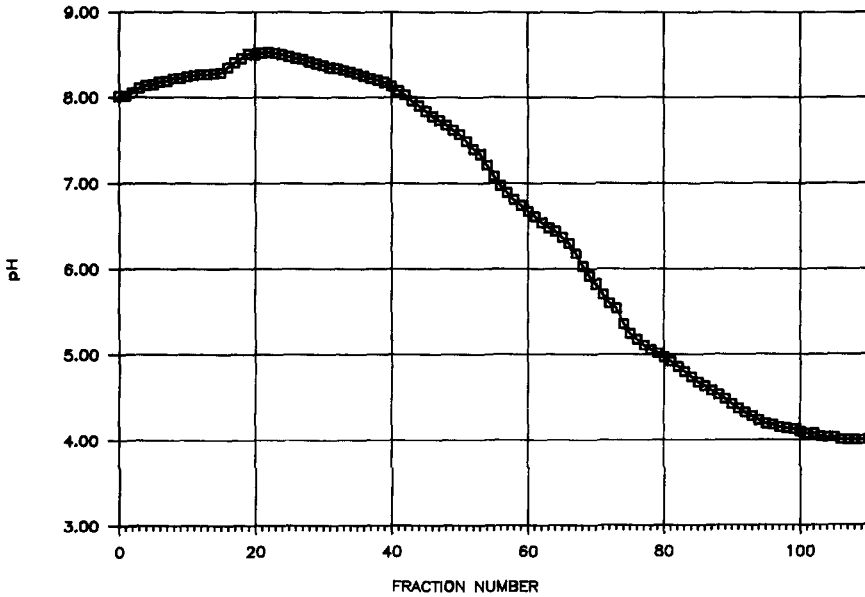

Fig. 3. Generation of internal pH gradient by chromatofocusing on PBE 94 using our focusing buffer (Table I) in the presence of urea. Both the column equilibration buffer and focusing buffer contained 6 *M* urea, otherwise all conditions, including buffer composition, were exactly as described for Fig. 1. The average gradient slope (ΔpH/ml) from pH 8 to 4 is 0.126.

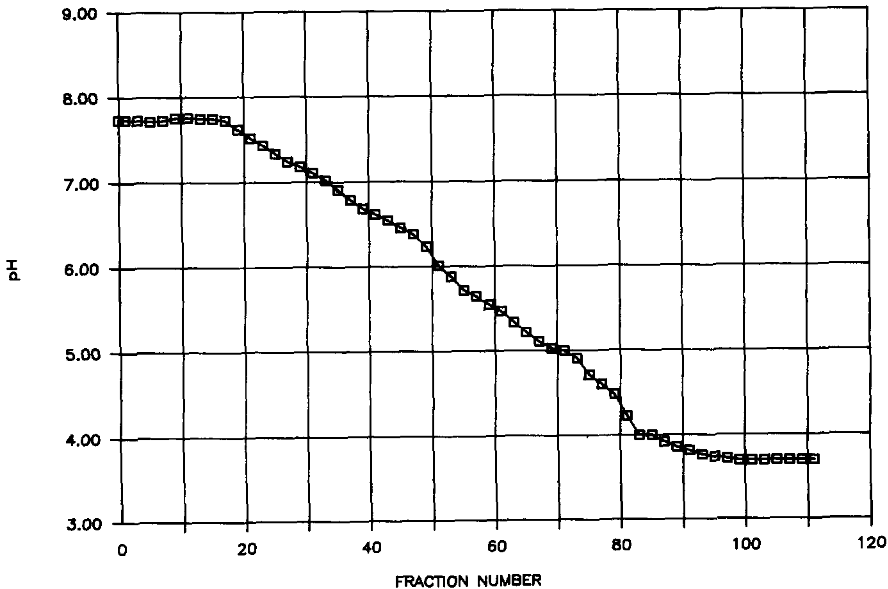

Fig. 4. Generation of internal pH gradient by HPCF on a Bakerbond PEI high-performance anion-exchange column using our focusing buffer outlined in Table I. The composition of focusing buffer and all other conditions were exactly as described for Fig. 1. The average gradient slope (ΔpH/ml) from pH 8 to 4 is 0.10.

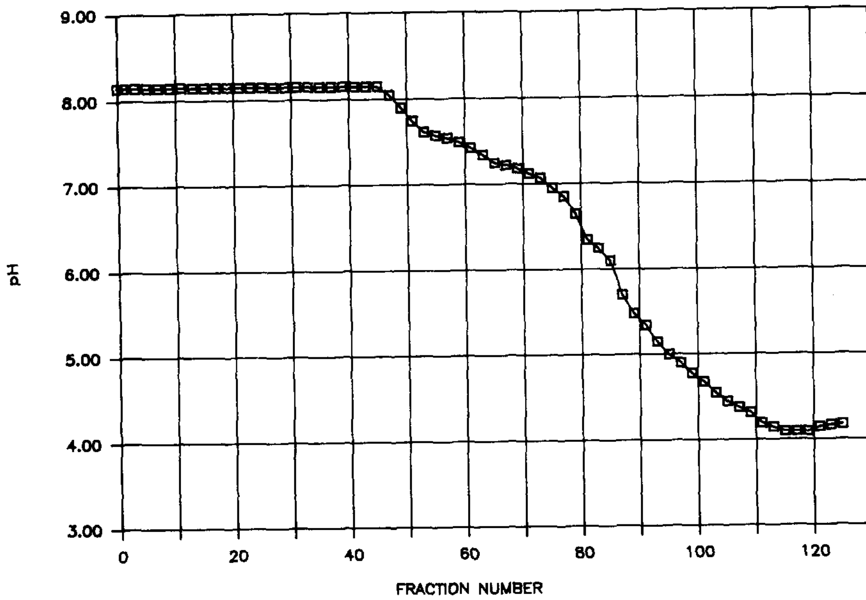

Fig. 5. Generation of internal pH gradient by HPCF on Bakerbond PEI high-performance anion-exchange column using our focusing buffer (Table I) in the presence of 6 *M* urea. The composition of focusing buffer and all other condition were as described for Fig. 3. The average gradient slope (ΔpH/ml) from pH 8 to 4 is 0.10.

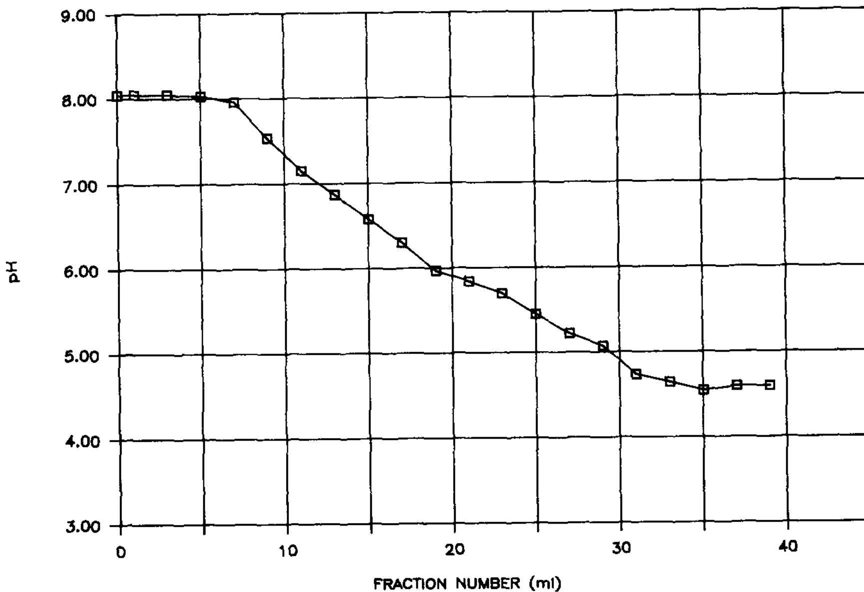

Fig. 6. Generation of internal pH gradient by HPCF on a Bakerbond PEI high-performance anion-exchange column using altered focusing buffer. The constituents from groups B, C and D were present at 4 m*M* each. Group A constituents were present at 1.25 m*M* each. All other conditions were as described for Fig. 4. The average gradient slope (ΔpH/ml) from pH 8 to 4 is 0.18.

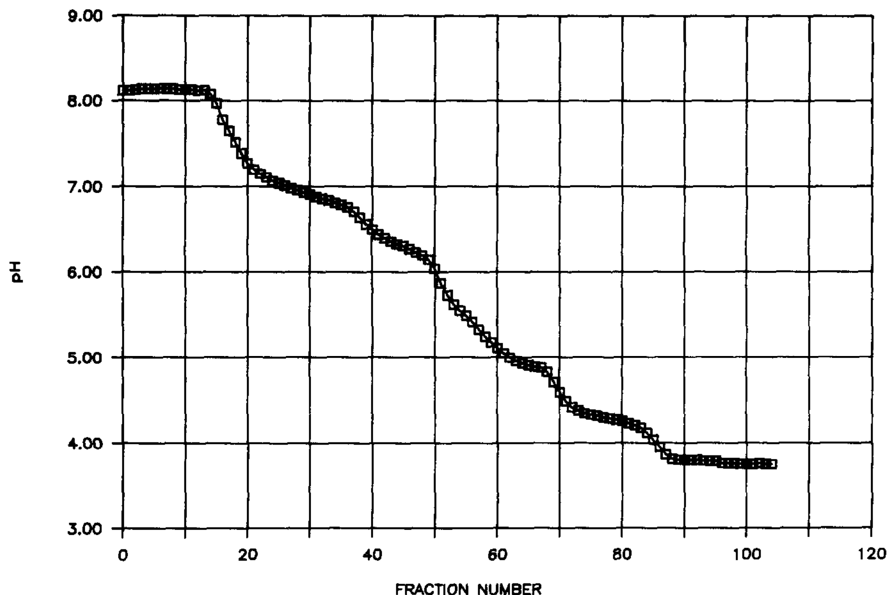

Fig. 7. Internal pH gradient produced during HPCF on Bakerbond PEI high-performance anion-exchange columns. The composition of focusing buffer and all other conditions were as described for Figs. 1 and 4. See text for details.

stationary phase buffering groups in the presence of the high concentration of urea.

The profiles shown in Figs. 4–6 demonstrate the ability of the same focusing buffer (Table I) to form linear pH gradients when used with the silica-based Bakerbond PEI high-performance anion-exchange columns as the stationary phase. The defined focusing buffer generates quite similar pH gradients on these columns both in the absence (Fig. 4) and presence (Fig. 5) of 6 *M* urea. This finding is in contrast to results obtained using PBE 94 (Figs. 1 and 3) and suggests that urea differentially effects the stationary phase functional groups. To generate the pH gradient shown in Fig. 6 the concentrations of focusing buffer constituents were altered. In this manner, minor differences in the performance between different HPLC columns can be corrected. As shown in Fig. 7, occasionally, certain columns will generate pH gradients less smooth than those shown in Figs. 4 and 5. We have found that our focusing buffer can often be used to generate equal or better quality pH gradients during HPCF than are observed when polyampholyte mixtures are employed[25]. This is an interesting finding since we generally find the polyampholyte mixtures more accommodating of disparities in the column exchange (buffering) capacity than the focusing buffers we have designed.

DISCUSSION

The enormous compilation of data in recent reviews[7,26] is an attestation to the importance of synthetic polyampholytes for the electrophoretic separation and purification of proteins. The ready availability of synthetic polyampholytes also led to the development of ampholyte displacement chromatography[27] and was important

for the experimental verification of chromatofocusing[11]. However, there are limitations with each of these techniques imposed by the size and chemical properties of synthetic polyampholytes, as well as their cost. Polyampholytes have been prepared by a number of synthetic routes from a variety of polyamines and acids (for a review see ref. 7). These polyampholytes (by design) are quite heterogeneous and there is considerable controversy as to the molecular weight distribution of a given preparation. As reviewed by Righetti[7], conservative estimates suggest that most preparations have an average molecular weight of 700–800 with varied proportions or subfractions reported to be above 1000. Some of the estimates reviewed were found less prone to the usual criticisms but still emphasized the possibility of molecular weights distributed up to 15 000. Attempts at molecular weight estimates have been complicated by the known propensity of polyampholytes to form self-aggregates due to either electrostatic and/or hydrophobic interactions[7]. More importantly, such aggregations have been shown to occur even during electrophoresis[7]. Indeed, it is these same properties that contribute to the well-documented interaction of polyampholytes with a variety of proteins, nucleic acids, polysaccharides, protein stains and dyes[28–38]. In the specific case of polyampholytes synthesized for chromatofocusing by Pharmacia (Polybuffers 96 and 74) we have found no direct estimates of average molecular size or size distribution. However, it has been indicated by Pharmacia[39] that columns of Sephadex G-75 longer than 20 cm are necessary to achieve base-line separations of Polybuffer from proteins of molecular weight less than 25 000. In one recent report[28], the authors claim of difficulty in removing Polybuffer from synexin (47 000 daltons) prompted the use of alternative elution mechanisms from the chromatofocusing media. There is clearly a need for alternative (non-polymeric) focusing buffers.

An investigation into the use of simple buffer mixtures (both amphoteric and non-amphoteric) to generate "natural" pH gradients during electrophoresis was "reintroduced" by Chrambach and co-workers[40–43] in an attempt to help clarify apparent disparities between the theoretical and observed "focusing" mechanism(s) involved. These studies have advanced the general appreciation for isotachophoretic mechanisms that result in pH gradient formation due to the relative mobility of constituent species (for a review see ref. 20). Subsequently, using a similar type of buffer system already known to form pH gradients under electrofocusing conditions[18], Hearn and Lyttle investigated the generation of internal pH gradients on conventional cellulose- and dextran-based ion-exchange columns[44]. The resulting pH gradients were not of the quality as had been observed during buffer electrofocusing and appear largely unsatisfactory for general applications due to either excessive slope, narrow pH range and/or non-linearity. In fact, whether during buffer focusing in an electric field or by chromatofocusing on ion-exchange columns even the most ambitious simple buffer mixtures previously described[19], tend to produce pH gradients more irregular and of lesser quality (*i.e.* slope and linearity) than those generated using synthetic polyampholytes. We have found this to be particularly true during chromatofocusing on high-performance ion-exchange columns[25]. In fact, several of the focusing buffers developed by us during this investigation generated linear pH gradients in an electric field but not during chromatography on ion-exchange columns (data not shown).

We have demonstrated here that it is possible to generate linear pH gradients

(pH 8.1 to 3.5) from both conventional and "high-performance" anion-exchange columns in an isocratic elution mode (internally) using a single, chemically-defined buffer system composed of simple (low-molecular-weight) constituents. The relative contribution of several variables towards the mechanism by which this occurs will require further modelling and experimental verification to ascertain with any degree of certainty. It seems clear, however, that relative to electric field-dependent buffer electrofocusing[20], generation of linear, wide-range, pH gradients on ion-exchange columns is more complex. This is a reflection of the variable and, as yet, poorly defined contribution of the stationary phase to the pH gradient-forming process.

The initial assumptions and first approximation equations originally outlined by Sluyterman (eqns. 1–3 on p. 18 of ref. 10) form a logical approach to the underlying principle still used to explain the generation of internally developed pH gradients during chromatofocusing. When the buffering capacity per unit (*e.g.* of column length) of the mobile phase or focusing buffer (a_m) and the stationary phase (a_s) are assumed equal, the following equation for the final eluted pH upon mixing the two phases:

$$\mathrm{pH_f} = \frac{a_m \mathrm{pH_m} + a_s \mathrm{pH_s}}{a_m + a_s}$$

becomes

$$\mathrm{pH_f} = \frac{\mathrm{pH_m} + \mathrm{pH_s}}{2}$$

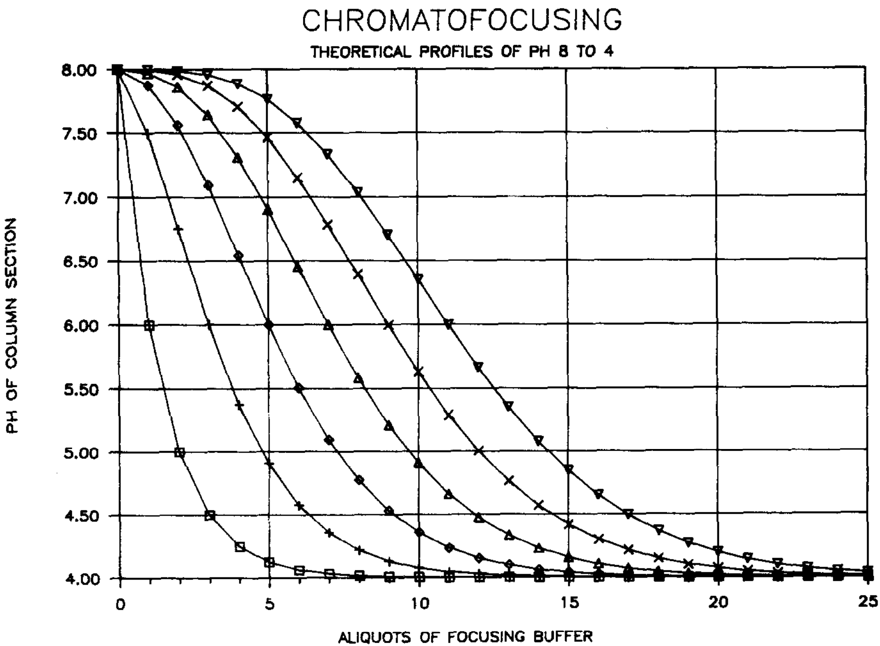

Fig. 8. Theoretical pH profiles generated from pH 8 to pH 4 as a function of focusing buffer (volume) for each of 25 aliquots as described in the text. The pH for column sections 1, 3, 5, 7 and 9 (from left) are shown during development of the pH gradient. The pH gradient of the final column eluate is also shown (far right). An equal buffering capacity of focusing buffer and stationary phase is assumed per unit volume.

To best illustrate the consequences of this assumption, Fig. 8 shows the theoretical pH profiles generated using this equation for the initial condition of $pH_m = 4$ and $pH_s = 8$. In this case, column pH is followed sequentially as 25 aliquots of focusing buffer at pH 4 (each aliquot equal in buffering capacity to one section of a ten-section column) migrate through the column. Ignoring the extremes at either end of the process, a linear pH gradient is finally developed. To demonstrate the important relationship between column capacity (*i.e.* number of sections) and focusing buffer volumes, the curves shown in Fig. 8 illustrate the change in the pH (gradient slope) in column sections 1, 3, 5, 7 and 9 as a function of focusing buffer volume (aliquots). In a practical sense, however, we have found that matching the buffering capacity of the focusing buffer and the stationary phase (ion-exchanger) does not insure the development of linear pH gradients, irrespective of slope. Universal buffers with even buffering capacities from pH 3–11 have been known for many years[45–47]. However, many universal-type buffers do not work well at all during chromatofocusing (data not shown). It is probable that when the stationary and mobile phases are mixed during chromatography, polyelectrolyte effects alter the functional pK values (and therefore the buffering capacity) of the stationary and soluble buffering groups involved. Furthermore, since buffering capacities (β) are additive[21], an unequal distribution (equilibrium) of focusing buffer constituents between the mobile and stationary phases may cause transient variations in column buffer capacity and eluted pH.

Commercially available, synthetic, polyampholyte mixtures have been used to accomplish both ampholyte displacement chromatography[27] and chromatofocusing[9–13]. Discussions have been presented which theoretically and practically attempt to distinguish the two processes mechanistically[39,48,49], however, uncertainties remain regarding the precise separation mechanism(s) in either case. It seems probable that under conditions thus far employed for "chromatofocusing", the distinction may be more apparent than real since these processes do not appear to be mutually exclusive. Gradients of pH may be generated according to the relative mobility or stacking of buffer constituents during chromatography. We have observed cascade-step gradient elutions using three- to five-component buffers where neither the plateaus nor inflexion points were simply correlated with constituent pK_a or pI values (data not shown). This appears in contrast to the case for buffer electrofocusing (see Fig. 1 of ref. 18).

Despite the theoretical uncertainties involved, we have shown that chemically-defined mixtures of simple buffer reagents can be used with both conventional and high-performance anion-exchange columns to generate pH gradients of a quality at least comparable to those generated with polymeric ampholytes. We are continuing our efforts to further simplify these focusing buffer formulations, to make them less specific for individual columns and to verify their practical application[25].

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