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PERFORMANCE EVALUATION OF A FOCUSING BUFFER DEVELOPED FOR CHROMATOFOCUSING ON HIGH-PERFORMANCE ANION-EXCHANGE COLUMNS

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SUMMARY

We have developed a single focusing buffer for generation of wide-range pH gradients during chromatofocusing on high-performance anion-exchange columns. Various properties of this focusing buffer have been compared to those of commercially available polyampholyte buffers as well as non-polyampholyte buffers. Relative to the other buffers evaluated, our focusing buffer exhibited lower UV absorbance and only slightly higher conductivity at effective operating concentrations. No other buffer tested was able to produce linear pH gradients (from pH 8.1 to 4) of a quality better than that obtained using our focusing buffer. Using our focusing buffer with 25-cm Bakerbond PEI columns, several purified proteins (10 mg each) were eluted at pH values near to or identical with their elution pH or p*I* values as reported elsewhere. High-performance chromatofocusing with this same system revealed that the surface charge distribution of estrogen receptor proteins (50–100 fmol/mg cytosol protein) in calf uterine cytosol was similar to that reported by us previously. Since our focusing buffer is composed of low-molecular-weight components, in contrast to polyampholytes, these components are more easily separated from proteins and do not interfere with protein stains or dyes. These results confirm and extend the general utility of simple focusing buffer systems as alternatives to the larger polyampholytes for chromatofocusing.

INTRODUCTION

Analytical scale resolution and preparative scale isolation of peptides, proteins and other biological macromolecules are most often achieved by procedures which include exploitation of differences in molecular charge properties. To these ends ion-exchange chromatography is widely used because of its versatility and ease of operation, yet electrophoretic focusing techniques are generally considered as the more discriminating means of affecting high-resolution separations. However, an opportunity to realize the most favorable aspects of both of these techniques in a single chromatographic focusing procedure has been presented by Sluyterman and co-workers[1–5].

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We have employed chromatofocusing principles to develop conditions for the rapid generation of linear, wide-range pH gradients utilizing silica-based high-performance anion-exchange columns[6–8]. These first studies, performed using mixtures of commercially available polyampholytes, resulted in our ability to study variations in the surface charge heterogeneity of receptor proteins which were not apparent when these proteins were analyzed in parallel using ion-exchange chromatography (in salt-gradient elution mode) or electrophoretic focusing[6,7]. However, even though we have demonstrated that commercially available polyampholyte mixtures are able to generate pH gradients of desirable qualities on certain high-performance anion-exchange columns, using these polyampholytes imposes serious theoretical and practical restrictions which prevent more extensive utilization of this technique. Furthermore, their undefined chemical properties inhibit our ability to fully appreciate the separation mechanism(s) involved.

Our experimental evidence fully supports that portion of the theory outlined by Sluyterman which states that the quality and slope of the pH gradient generated internally during chromatofocusing on a given column will be dictated, in part, by the composition and relative concentration of focusing buffer constituents*. Therefore, a chemically defined, simple focusing buffer, in contrast to the uncertainties of a polyampholyte mixture, is better suited for modification to complement the buffering capacity of a given ion-exchange column. Furthermore, the practical contribution of each individual focusing buffer constituent towards the generation of the desired pH gradient can be assessed and ultimately related to its contribution as predicted by theory. Finally, any undesirable effects an individual focusing buffer constituent may have on the structure or biological properties of the sample protein may be identified and presumably avoided. Other advantages and limitations of non-polymeric ampholyte buffers have been discussed by those interested in buffer electrofocusing[9–15].

In a report by Hearn and Lyttle[13] one of the simple buffer mixtures originally tested for buffer electrofocusing[14] was evaluated as an eluent for chromatofocusing on cellulose- and dextran-based ion-exchangers. The resulting pH gradients with acceptable slopes were primarily of the cascade-step type and generally decayed below pH 6. Since focusing buffer inadequacies in pH gradient formation tend to be exaggerated during chromatofocusing attempts on high-performance ion-exchange columns, the need was clear for a thorough investigation into the design of quality focusing buffers for high-performance chromatofocusing.

In our accompanying paper[12], we describe a simple focusing buffer specifically developed to generate linear pH gradients when utilized on either conventional or silica-based, high-performance anion-exchange columns. In this preliminary report we compare various properties of that focusing buffer to other commercially available polyampholyte and non-polyampholyte focusing buffers. We have also evaluated the resolving ability of our simple focusing buffer using several purified marker proteins as well as crude preparations of calf uterine estrogen receptor proteins.

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EXPERIMENTAL

Materials

Bakerbond PEI (polyethyleneimine) anion-exchange columns (250 × 4.6 mm I.D.) were generously supplied by J. T. Baker. [^{3}H]Estradiol-17β (101 Ci/mmol) was purchased from New England Nuclear. Diethylstilbestrol (DES), dithiothreitol, glycerol and marker proteins of known isoelectric points (p*I*) were from Sigma. Prepacked Sephadex G-25 (PD-10) columns (6 × 1.5 cm I.D.), Polybuffer 96 and Polybuffer 74 were obtained from Pharmacia Fine Chemicals. Buffalyte 3–10 and Buffalyte 4–8 were purchased from Pierce and Poly/Sep 47[10] was from PolySciences. All buffer components and other reagents were from Sigma or Research Organics.

High-performance chromatofocusing (HPCF)

The development and details of our HPCF procedures utilizing commercially available polyampholytes are published previously[6–8]. A detailed description of the simple focusing buffer developed for use in these studies is presented in an accompanying paper[12]. Bakerbond PEI high-performance anion-exchange columns were equilibrated to pH 8.0–8.2 using 25 m*M* Tris–HCl buffer containing 20% (v/v) glycerol including 1 m*M* dithiothreitol for HPCF of estrogen receptor proteins. All HPCF was performed in a cold room (3–6°C). Buffer solutions were prepared freshly for each experiment, pH-adjusted at the temperature of chromatography, vacuum filtered through Millipore 0.45-μm HAWP filters and degassed before use. The formation of internal pH gradients was initiated using one of several polyampholyte or non-polyampholyte focusing buffers as indicated at a flow-rate of 0.6 or 1.0 ml/min (Beckman Model 110 A HPLC pumps). The pH of 1.0-min fractions was determined (at 0°C) shortly after elution using a Corning Model 125 pH meter with a microcombination calomel electrode. The elution pH of marker proteins was determined by absorbance at either 260 or 280 nm using a Beckman Model 153 analytical flow-through UV detector (8-μl flow cell) or a Perkin-Elmer Model 3B scanning spectrophotometer. To determine the HPCF elution profile of estrogen receptor proteins, the radioactivity of alternate fractions was monitored (at 30–37% efficiency) by liquid scintillation counting using a Beckman LS 250 scintillation counter.

Preparation of radiolabelled estrogen binding proteins

Uteri from small, immature calves were obtained from a local slaughterhouse. The uterine horns (*ca.* 10 g) were rinsed in ice-cold saline immediately after removal. Approximately 1-g pieces were frozen in liquid nitrogen and stored frozen at −85°C. All procedures were performed in a cold room at 3–6°C. Pieces of frozen uteri were minced and homogenized (Ultra-Turrax) in 2 volumes of 10 m*M* Tris–HCl buffer (pH 7.4 at 0°C) containing 1 m*M* dithiothreitol and 20% (v/v) glycerol. The homogenate was centrifuged at 40 000 rpm in an SW 50.1 rotor for 60 min to obtain cytosol (supernate). Soluble estrogen receptor proteins were labelled for 2–16 h at 0°C with 10 n*M* [^{3}H]estradiol-17β in the presence (non-specific binding) and absence (total binding) of a 100-fold molar excess of the estrogen receptor specific competitor, DES. Immediately prior to chromatofocusing, radiolabelled steroid–protein complexes were quickly (< 5 min) separated from excess free steroid by rapid chromatography through small (9 ml) columns of Sephadex G-25.

RESULTS AND DISCUSSION

We have previously demonstrated the advantages of chromatofocusing on high-performance anion-exchange columns[6–8]. During our comparison of various high-performance anion-exchange columns in a chromatofocusing mode to select those suitable for the separation of structurally labile forms of steroid receptor proteins, we discovered that Bakerbond PEI high-performance anion-exchange columns possess interesting and potentially beneficial properties. We have therefore characterized the chromatofocusing behavior of commercially available polyampholyte and non-polyampholyte focusing buffers on Bakerbond PEI columns.

The pH gradient profile shown in Fig. 1 is representative of those obtained using our focusing buffer[12] during HPCF on Bakerbond PEI high-performance anion-exchange columns. For the same range of pH (*ca.* pH 8 to 3.5), Fig. 2 shows the optimal pH profiles obtained using Bakerbond PEI columns with Polybuffers 96 and 74 (A), Polybuffer 96 alolne (B, top) and Polybuffer 74 alone (B), Buffalyte 3–10 (C), Buffalyte 4–8 (D) or Poly/Sep 47 (E). Each of these profiles reveals both linear and non-linear gradients of pH. While there are certainly regions of acceptable performance, generally, upon initiation and below pH 5 or 6 the gradients can be quite poor. The acidic region appears to be the most difficult region to generate linear changes in pH. Similar results were observed using other high-performance ion-exchange columns. As discussed in our accompanying manuscript[12], most simple buffer mixtures we have designed and/or those evaluated generate more acceptable pH gradients in an electric field (*i.e.* electrofocusing) than during chromatofocusing. As shown here, this seems particularly true during HPCF. This is most probably a reflection of the variable and, to date, poorly defined contribution of the stationary phase (chromatofocusing column) to the generation of pH gradients. The relative contribution of several potential mechanisms for pH gradient formation will require further experi-

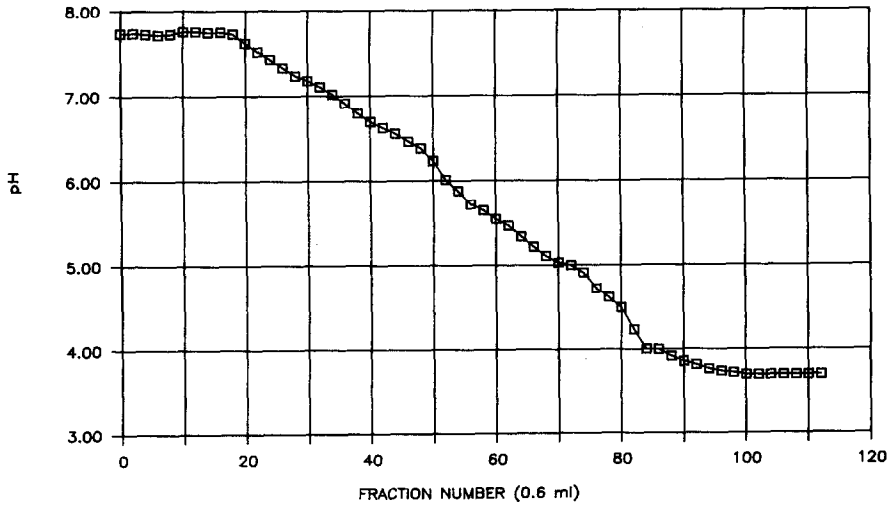

Fig. 1. Development of internal pH gradients during HPCF using our wide-range focusing buffer (pH 3.9) as described in ref. 12.

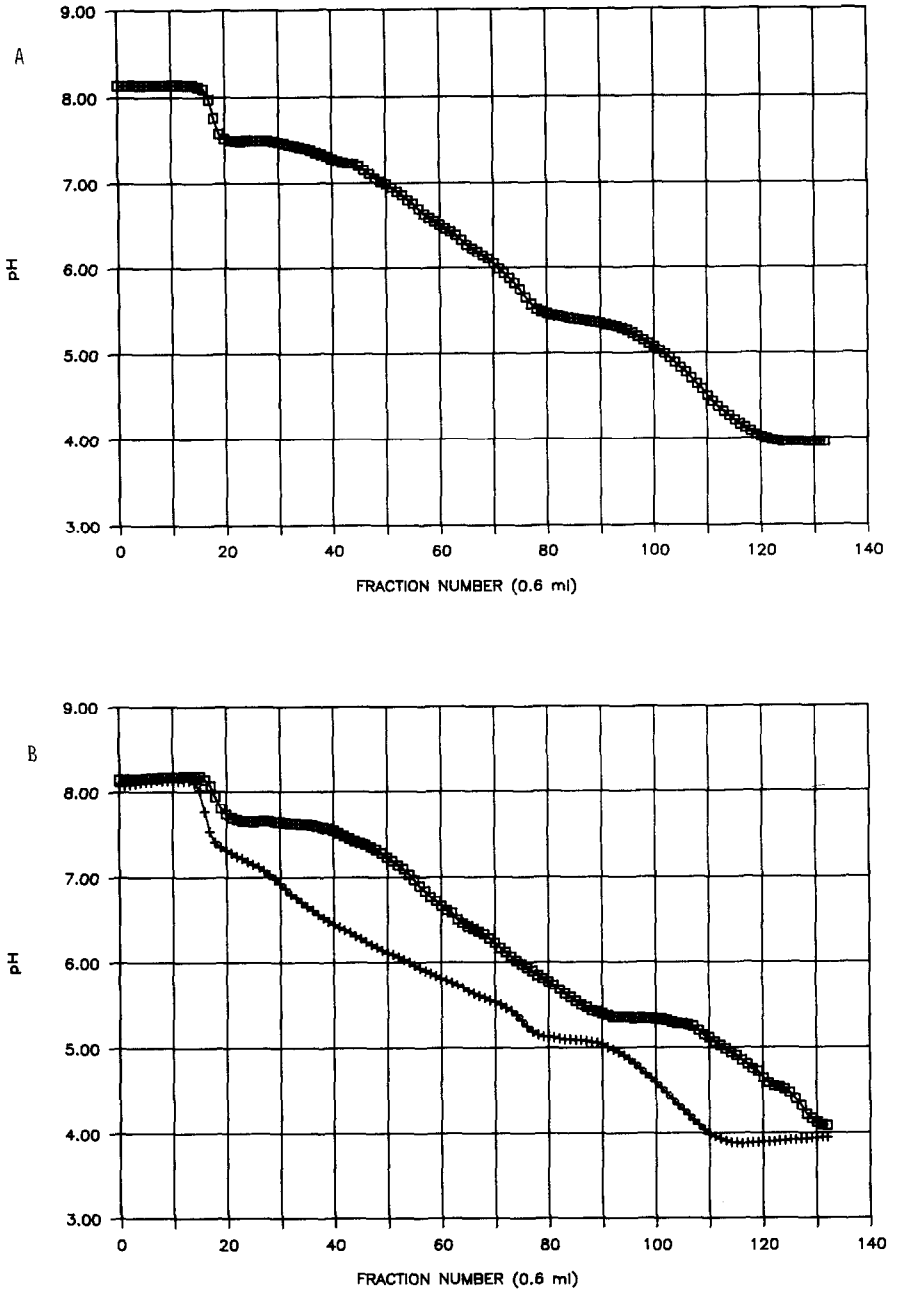

Fig. 2.

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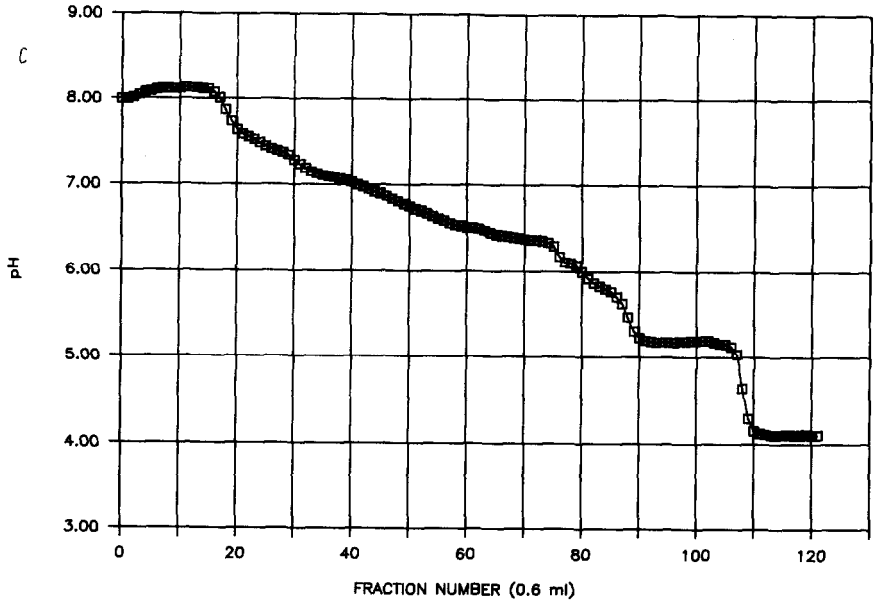
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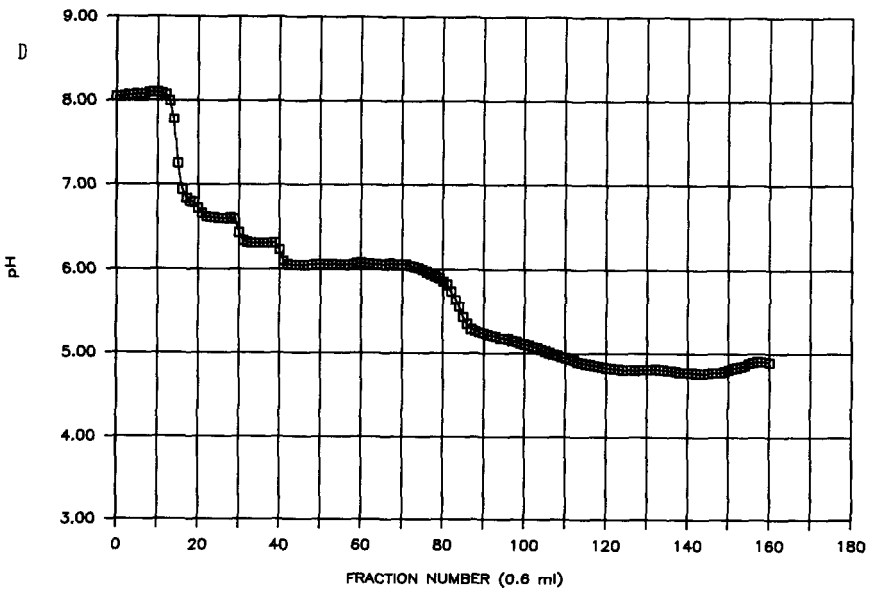
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Fig. 2.

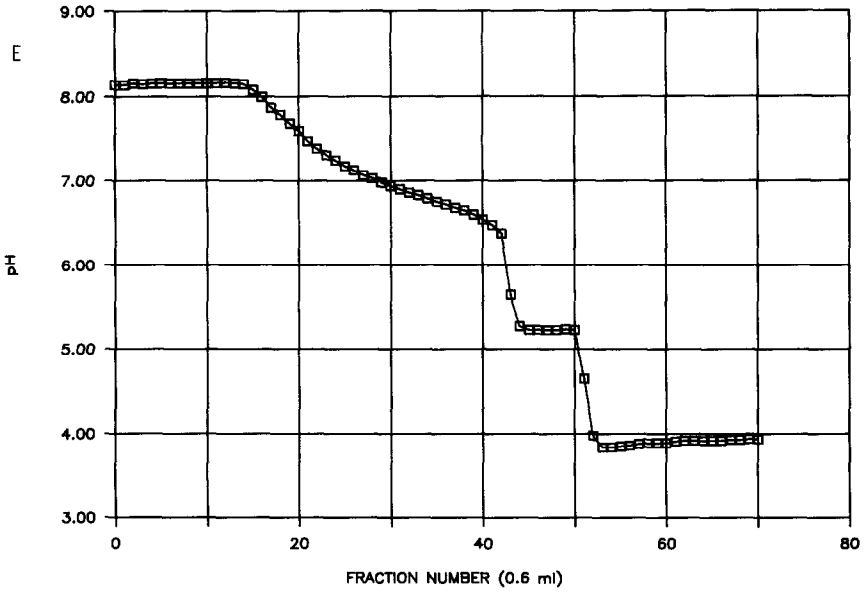

Fig. 2. (A) Development of internal pH gradient during HPCF using an optimal mixture of Polybuffers 96 and 74 (3:7) diluted 1:15 (adjusted to pH 4 with hydrochloric acid). (B) Development of internal pH gradient during HPCF using Polybuffer 96 only (top) Polybuffer 74 only, diluted 1:15 (adjusted to pH 4 with hydrochloric acid). (C) Development of internal pH gradient during HPCF using 2 m*M* Buffalyte 3–10 (adjusted to pH 4 with hydrochloric acid). (D) Development of internal pH gradient during HPCF using 2 m*M* Buffalyte 4–8 (adjusted to pH 4 with hydrochloric acid). (E) Development of internal pH gradient during HPCF using 2 m*M* Poly/Sep 47 (adjusted to pH 4 with hydrochloric acid).

mental evaluation. The use of chemically defined, non-polymeric buffers with a variety of well-characterized ion-exchange columns should facilitate this process.

Theoretically, lower ionic strength focusing buffers should enhance chromatofocusing resolution on high capacity ion-exchange materials[2], however, the practical limitations of this effect are not known. Table I compares the conductivity of

TABLE I

COMPARISON OF FOCUSING BUFFER CONDUCTIVITIES AT EFFECTIVE WORKING CONCENTRATIONS DURING HPCF

Manufacturer	*Focusing buffer*	*Concentration or dilution*	*Conductivity (μMho)*
Pierce	Buffalyte 3–10	1–2 m*M*	17–31
	Buffalyte 4–8	1–2 m*M*	10–18
Polysciences	Poly/Sep 47	1:10–1:20	58–116
Pharmacia Fine Chemicals	Polybuffer 74	1:10–1:15	12–16
	Polybuffer 96	1:10–1:15	10–14
	Polybuffers 96/74 (30:70)	1:10–1:15	15–20
The authors	As described in ref. 12	2 m*M*	40

TABLE II

MARKER PROTEINS: COMPARISON OF CHROMATOFOCUSING ELUTION pH VALUES WITH REPORTED ISOELECTRIC POINTS

Protein	*Observed elution pH by chromatofocusing with our focusing buffer*	*Reported pI by buffer electrofocusing*[15]	*Reported pI by isoelectric focusing in polyampholytes*	*Ref.*
Ovalbumin (chicken egg)	4.1 and 4.5	4.7	4.5–5.0	15
			4.7	16
Ferritin (horse spleen)	4.5	–	4.27–4.57	16
			4.1–4.6	17
			4.1–5.1	18
Transferrin (human)	5.6–5.7	–	5.9, 6.0	17
			5.2–6.1	18
Catalase (bovine liver)	5.6	5.6 and 6.4	5.4, 6.3	15
Hemoglobin (human)	7.3 and 7.5*	7.5 (bovine)	6.95–8.12	16
			6.9–7.4	17
Estrogen receptor (calf uterus)	6.7 region**	–	5.6–7.8	7, 8
Myoglobin (horse skeletal muscle)	>8***		6.8–7.8	16

* Four other minor peaks consistently resolved using this sample.

** Peaks at pH 6.6–6.7 and pH 6.3–6.4 normally represent the major peaks of receptor activity observed under these conditions[7]. Minor peaks are also observed eluting at pH 7.8, 7.3, 7.1, 5.9–6.0 and 5.3 under these conditions[7].

*** Two major peaks and one minor peak were resolved (isocratically) under these conditions immediately before initiation of the pH gradient. Similar results ($pI > 8$) are reported by Hearn and Lyttle[13].

the various focusing buffers tested. The ionic strength of our focusing buffer was found to be above the average of those tested. However, at the same effective working concentrations, the focusing buffer prepared by us had among the lowest UV absorption properties of any tested down to wavelengths of 240 nm (Fig. 3). Poly/Sep

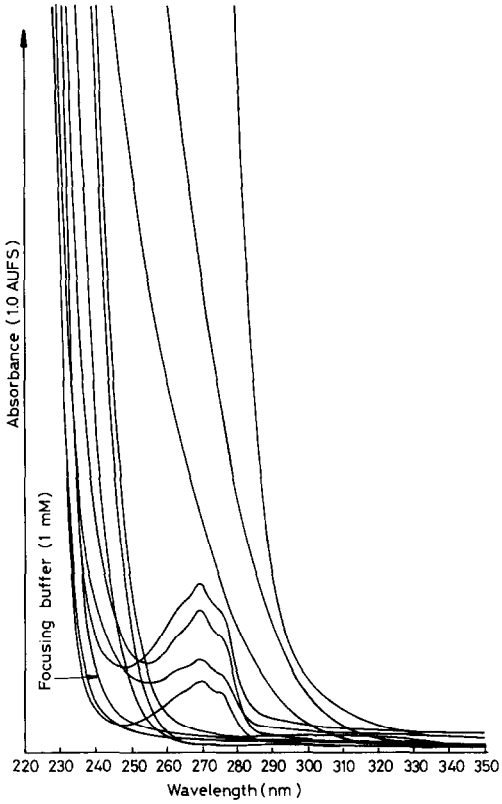

Fig. 3. UV absorbance profiles of polyampholyte and non-polyampholyte focusing buffers at their effective working concentrations as outlined in Table I and the legends to Figs. 1 and 2.

47 began absorption at wavelengths below 310 nm, and Buffalytes 3–10 and 4–8 had minor absorption maxima in the 280–260 nm region.

We next investigated whether a well-characterized group of marker proteins (10 mg each) would be eluted according to their reported p*I* or elution pH values during HPCF on the Bakerbond PEI columns with our focusing buffer as the mobile phase. As shown in Table II, each of these proteins was eluted at or within the range of its reported p*I*. This result was irrespective of whether the p*I* was determined by buffer electrofocusing or (more commonly) electrophoresis in polyampholytes.

Finally, Fig. 4 helps demonstrate both the resolving ability and reproducibility of HPCF using the focusing buffer described here. These profiles represent separate preparations of estrogen receptor proteins in cytosols from different calf uteri. It is instructive to compare the similarity of these profiles with those we published earlier using different high-performance anion-exchange columns with polyampholyte mix-

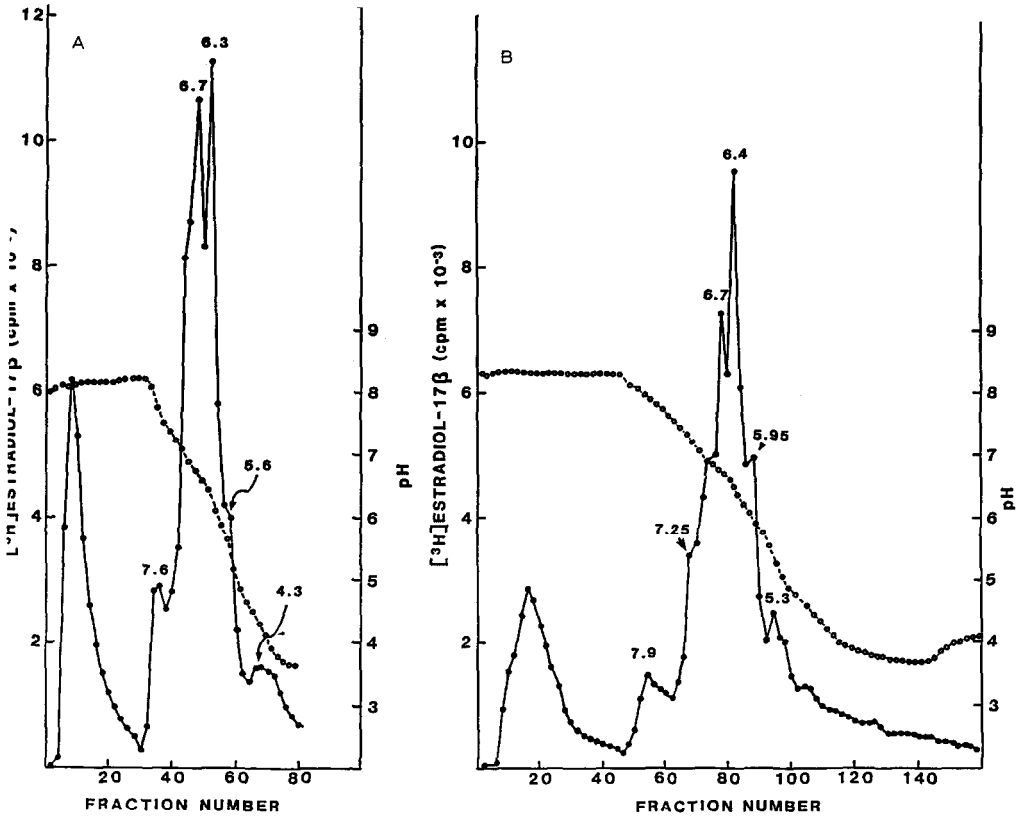

Fig. 4. Surface charge heterogeneity of calf uterine estrogen receptor proteins demonstrated by HPCF using our wide-range focusing buffer to generate the internal pH gradient. (A) The receptor concentration of this sample was separately determined to be 80 fmol/mg of cytosol protein. A 500-μl aliquot of cytosol (17 mg/ml) was eluted at 0.6 ml/min. (B) The reproducibility of internal pH gradient formation during subsequent HPCF using a different preparation of focusing buffer and estrogen receptor from that used for the experiment in A. Radiolabelled estrogen receptors present in calf uterine cytosol were used because of their heterogeneous surface charge properties. The profiles shown here can be compared to those generated by us previously using different ion-exchange columns with polyampholytes to develop pH gradients[6–8].

tures[6–8]. The pH values of individual elution peaks are indicated only for ease of comparison among these and other HPCF receptor profiles[6–8]. While shallower pH gradients have been used previously to fully separate major estrogen receptor species in the region of pH 6.3–6.7[7], aside from results presented in Fig. 4, this focusing buffer has only been used to produce similar results for receptor analyses in two other experiments. Importantly, the pH profiles shown in Fig. 4 show no evidence of gradient perturbation even though 8–10 mg of protein were loaded. This was also true for the pH profiles generated during collection of data for Table II.

Because of the known effects of molybdate (reversible) on the structural stability and surface charge heterogeneity of most steroid receptor proteins in (what is possibly) their native configuration, it is important to point out that the focusing

buffer described here is compatible with the inclusion of 10 m*M* sodium molybdate[12]. No interference with the pH gradient formation during HPCF has been observed under those conditions. The insolubility of molybdate in the polyampholytes at acidic pH values often precludes its effective use.

These results help confirm and extend the general usefulness of well-defined simple buffer systems for chromatofocusing and related procedures. The limits of this procedure are not obvious. We are presently investigating long term stability of both the mobile and stationary phases as well as complications which may be associated with larger scale (preparative) applications.

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