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SPACER PERFORMANCE IN THE CATIONIC ISOTACHOPHORESIS OF PROTEINS

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SUMMARY

Performance of narrow range ampholytes and a discrete spacer mixture is evaluated for improved protein separations by cationic isotachophoresis. A spacer mixture containing 22 cations is developed and relative step heights of components are presented. Different ampholytes and the discrete spacer give unique results for test mixtures of model proteins. While no spacer mixture can be universally recommended, discrete spacers offer the possibility of optimizing separations based on component selection. An example of optimizing a separation of five model proteins is presented.

INTRODUCTION

In a previous paper[1], the potential for cationic isotachophoresis (ITP) separations of proteins in potassium acetate–acetic acid electrolytes was demonstrated. Cationic ITP of proteins is characterized by wide applicability, low μg/ml detection limits and good quantitative linearity. In addition, complete resolution of proteins is seen if relative step height differences (mobility differences) are greater than *ca.* 10%. One difficulty with the method is the visualization of protein resolution from UV signals. While wide range ampholytes are effective for spacing cationic protein mixtures, use of these spacers results in sample/spacer mixing, reduced sensitivity and broadened zone profiles.

Advantages of discrete spacers for protein ITP in anionic systems have been discussed, both for serum[2] and model protein[3] separations. This work investigates the use of narrow range ampholytes and discrete spacer mixtures for improved resolution in cationic protein ITP. A mixture of 22 cations is used for discrete spacing and advantages *vs.* ampholytes are seen. Spacer performance is seen to be mixture-dependent, but optimized separations can be obtained using the flexibility inherent in discrete spacing.

EXPERIMENTAL

Proteins, amino acids, 2-amino-2-methyl-1,3-propanediol (ammediol), galactosamine, tris(hydroxymethyl)aminomethane (Tris) and lyophillized human serum were obtained from Sigma (St. Louis, MO, U.S.A.). Triethanolamine, potassium

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acetate and glacial acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Other amines or salts were obtained from either Aldrich (Milwaukee, WI, U.S.A.) or Eastman Kodak (Rochester, NY, U.S.A.).

The 22-ion discrete spacer mixture was prepared by diluting appropriate amounts of the compounds or salts to a final concentration of *ca.* 50 μg/ml of each cation in water. Ampholyte solutions (1%) were prepared by diluting 5 μl LKB Ampholines® (Bromma, Sweden) with 200 μl water. The leading electrolyte was 10 m*M* potassium acetate adjusted to pH 4.5 with 10% acetic acid, and the terminating electrolyte was 10 m*M* acetic acid. Distilled, de-ionized water was used for all spacer and electrolyte dilutions. Model protein mixtures were prepared by diluting weighed amounts in the leading electrolyte. Human serum was reconstituted by diluting 20 mg lyophillized powder with 200 μl water.

Cationic ITP was run on an LKB 2127 Tachophor isotachophoresis instrument using a 200 mm × 0.8 mm PTFE capillary and an LKB 2127-140 conductivity–UV detector. UV detection was performed at 280 nm. Separation currents were 250 μA for 10 min and detection currents were 50 μA. UV and conductivity signals were recorded on a Kipp and Zonen (Delft, The Netherlands) BD-41 strip chart recorder at a chart speed of 1.2 cm/min.

RESULTS AND DISCUSSION

Narrow range ampholytes and discrete spacers were evaluated for their performance in cationic ITP. For discrete cationic spacing, a mixture of 22 alkylammonium ions and amino acids or derivatives thereof was tested. Discrete spacer components and their relative step heights (rsh) are listed in Table I. Numerous compounds tested as spacers were not included in the final mix. Several components failed to

TABLE I

PERCENT RELATIVE STEP HEIGHTS OF SPACER CATIONS AND PROTEINS IN pH 4.5 POTASSIUM ACETATE–ACETIC ACID ELECTROLYTE

	rsh(%)		*rsh(%)*
Tetraethylammonium	42	Dodecylamine	72
Ammediol	47	Dodecyltrimethylammonium	74
Triethanolamine	50	Tributylamine	76
Tris	52	Carbonic anhydrase (CAN)	85
Lysozyme (LYS)	52	Trypsinogen (TRP)	85
Cytochrome *c* (CYC)	53	ε-Aminocaproic acid	87
Lysine	56	8-Aminocaprylic acid	88
Creatinine	57	Tetrabutylammonium	90
Myoglobin (MYO)	57	γ-Aminobutyric acid	94
Histidine	59	Cetyltrimethylammonium	95
Tripropylamine	62	Conalbumin (CAL)	95
Galactose amine	63	Tetrapentylammonium	98
Tetrapropylammonium	67	β-Lactoglobulin B (BLB)	103
Glycylhistidine	68	β-Lactoglobulin A (BLA)	105
Arginine	70	β-Alanine	124
Ribonuclease A (RNA)	71	Ovalbumin (OVA)	150

migrate in the pH 4.5 electrolyte (glycine, glycylglycylglycine, tricine, N-acetylhistidine, betaine). Other cations had high mobilities, which made them inappropriate for spacing proteins (Li^+, ethylenediamine, choline, pyridine, 4-methylmorpholine). Finally, UV absorbance at 280 nm ruled out other potential spacers (diphenylguanidine, 4-hydroxypyridine, adenine).

The final 22-spacer mixture contains components at rsh 42–124%. Spacer mobilities are distributed across this range, but several gaps occur at rsh 76–87 and 98–124%. Relative step heights were determined from injections of single spacer cations, and no attempt was made to assess their separability.

A comparison of mobility distributions for the different spacer solutions tested is shown in Fig. 1. Conductivity traces are given for the electrolyte blank, four ampholyte solutions (pH 3.5–10, 3.5–5, 5–8 and 7–9) and the discrete spacer mixture. As expected, pH 3.5–5 ampholyte contains predominantly low-mobility species while pH 7–9 contains predominantly high-mobility species. The discrete spacer mixture shows a mobility distribution similar to pH 7–9 ampholytes, but with more distinct zones observed on the conductivity trace.

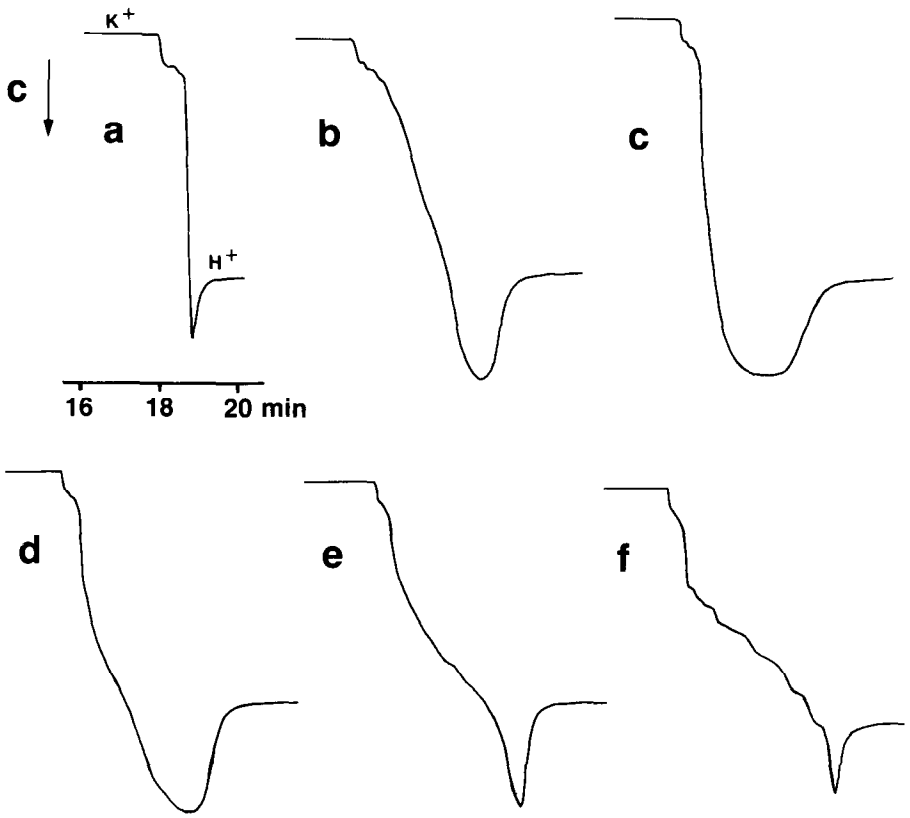

Fig. 1. Isotachopherograms for (a) electrolyte blank and injections of 0.5 μl 1% ampholytes pH, 3.5–10 (b), 3.5–5 (c), 5–8 (d), 7–9 (e) and 5 μl (f) discrete spacer mixture. K^+ = leader, H^+ = terminator. C axis is decreasing conductivity.

TABLE II

SPACER DISTRIBUTION (%) IN DIFFERENT STEP HEIGHT RANGES

rsh(%)	*Ampholyte pH range*				
	3.5–10	*3.5–5*	*5–8*	*7–9*	*Discrete*
<50	17	3	3	9	16
50–100	25	9	26	53	65
>100	58	88	71	38	19

Close inspection of mobility distributions reveals that all ampholytes tested have low mobility components, even pH 7–9 ampholytes. Table II lists the percent of total spacer zones at different rsh values. Narrow range ampholytes have components in all mobility ranges. The polymeric nature of these materials means that low effective mobilities are possible even though all ionized groups are positively charged.

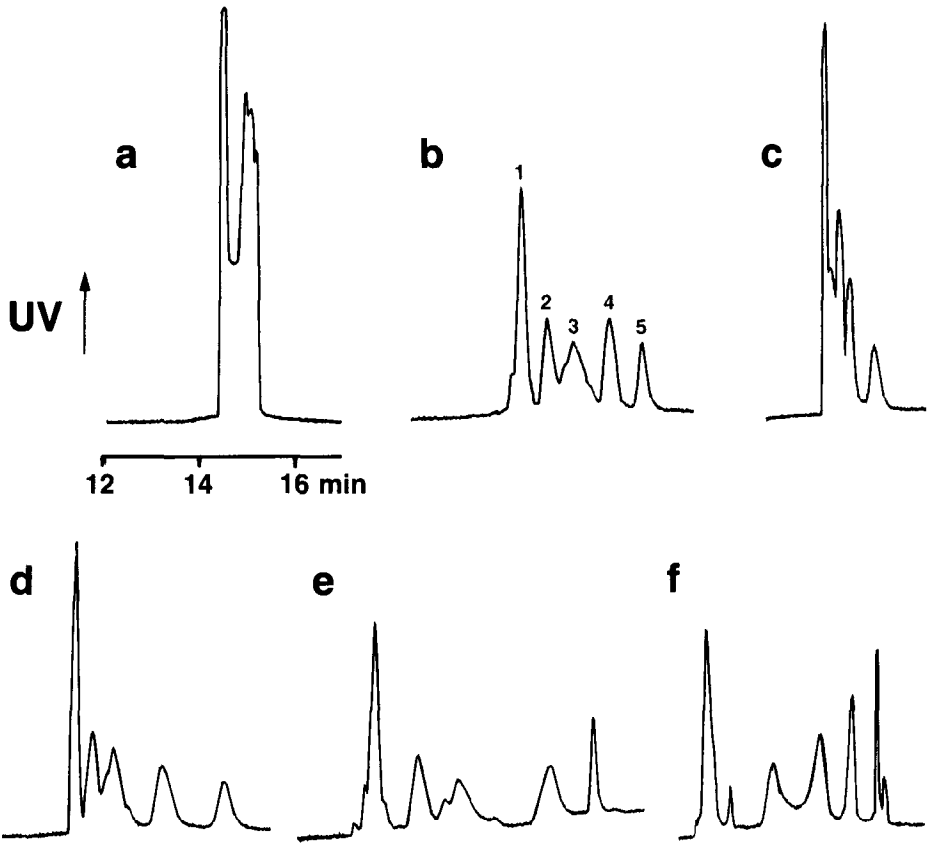

Fig. 2. Isotachopherograms for a mixture of 1 mg/ml LYS (1), 2.2 mg/ml RNA (2), 1.0 mg/ml TRP (3), 1.0 mg/ml BLA (4) and 1.3 mg/ml OVA (5). Spacers injected were (a) none, (b) 2 μl pH 3.5–10, (c) 2 μl pH 3.5–5, (d) 2 μl pH 5–8, (e) 2 μl pH 7–9 and (f) 10 μl discrete spacers. Volume protein mixture injected is (a) 4 μl, and (b)–(e) 2 μl. UV axis is increasing absorbance at 280 nm.

To test the performance of the different spacer solutions, a mixture of model proteins with disparate mobilities at pH 4.5 was separated. Fig. 2 shows the UV traces obtained from injections of a mixture of LYS, RNA, TRP, BLA and OVA. Good resolution of these five proteins is obtained with all spacers except pH 3.5–5 ampholytes. This narrow range spacer has insufficient high mobility components to space proteins other than OVA. Wide range ampholytes also give good spacing, but with considerable dilution and broadening of the peaks. pH 5–8 ampholytes give less broadening of LYS, but small impurity peaks seen with other spacers are unresolved.

Best resolution of this mixture is obtained with pH 7–9 ampholytes or discrete spacers. With pH 7–9 ampholytes, three impurity peaks near LYS and two near TRP are resolved. Little broadening is seen for well resolved OVA. Discrete spacers also give good resolution of the five proteins, with BLA and OVA showing less broadening than with ampholytes. However, RNA and TRP show distinct mixing. The apparently resolved impurity trailing OVA in Fig. 2f is a spacer impurity.

Fig. 2 reveals several important points regarding the use of these spacers for cationic ITP. Narrow range pH 7–9 ampholytes contain sufficient low mobility components to yield high overall resolution. Since the p*I* range of proteins separated is 4.8–11, this narrow range ampholyte can space proteins with p*I* values much less than the pH range indicated for isoelectric focusing. Similar observations of the utility of narrow range ampholytes at pH values different from the indicated p*I* range were made for anionic spacing[4]. Secondly, sharp peaks can be obtained with a discrete spacer mixture *vs.* ampholytes. Such separations may be useful for obtaining the desired resolution of a single component or for maximizing sensitivities to specific proteins.

The effect of increasing the volume of discrete spacers on the above model protein separation is shown in Fig. 3. A minimum of 10 μl spacers gives good resolution of the five proteins. A 20-μl volume allows separation of some impurity peaks near LYS, but both LYS and TRP show extreme broadening at this spacer loading.

Unspaced cationic ITP showed sensitivities in the 50 ng range for model proteins[1]. Higher resolution separations with wide range ampholyte spacing gave decreased UV sensitivities due to spacer/sample mixing. Fig. 4 shows separations of the above model protein mixture at different loadings to assess sensitivity with discrete

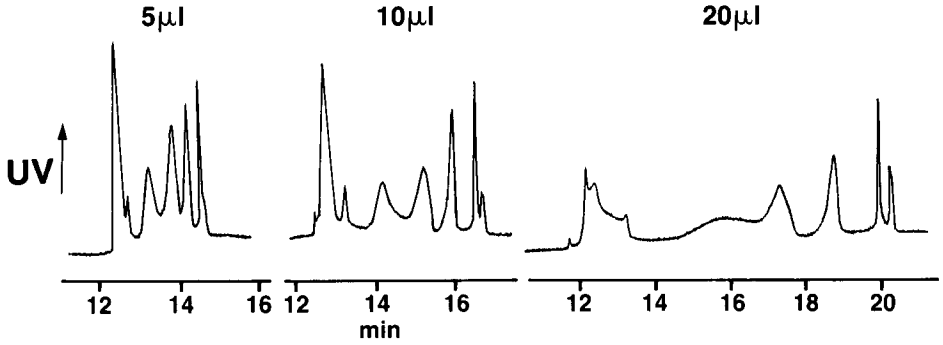

Fig. 3. Isotachopherograms for 2 μl protein mixture from Fig. 2. Volume discrete spacers added is noted in the figure. UV axis same as in Fig. 2.

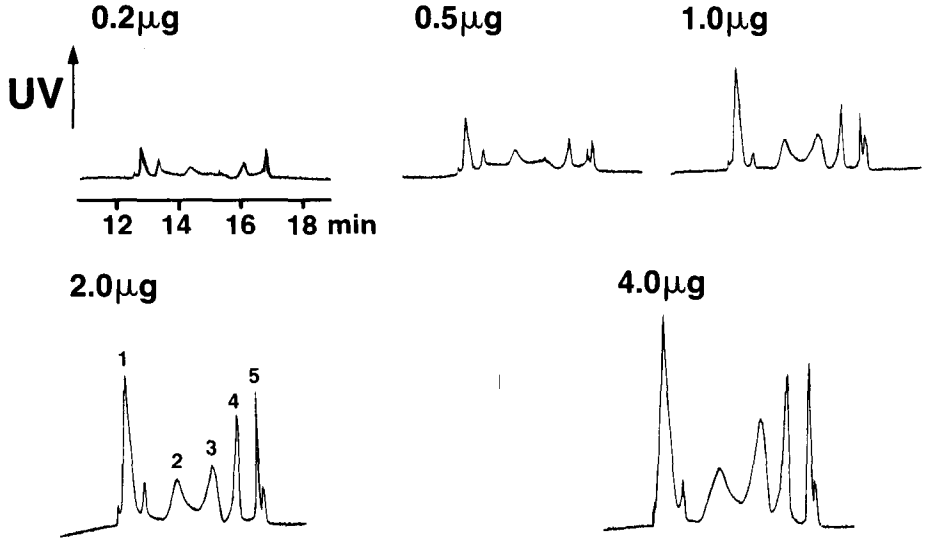

Fig. 4. Isotachopherograms for protein mixture from Fig. 2 with 10 μl discrete spacers. Approximate μg of each protein injected is noted in the figure. UV axis same as Fig. 2.

spacers. Estimated detection limits are 100–500 ng for individual proteins. Approximate quantitative linearity is observed on the basis of peak area over the concentration range studied. Some decrease in resolution is seen with increasing sample load. For example, injection of $\leqslant 2$ μl of the protein mixture is necessary to obtain baseline resolution of BLA and TRP.

To determine if the relative performance of the different spacers depends on sample composition, a second mixture of MYO, CYC, CAN, CAL and BLB was studied. Fig. 5 shows the separations obtained without spacers, with pH 3.5–10 and

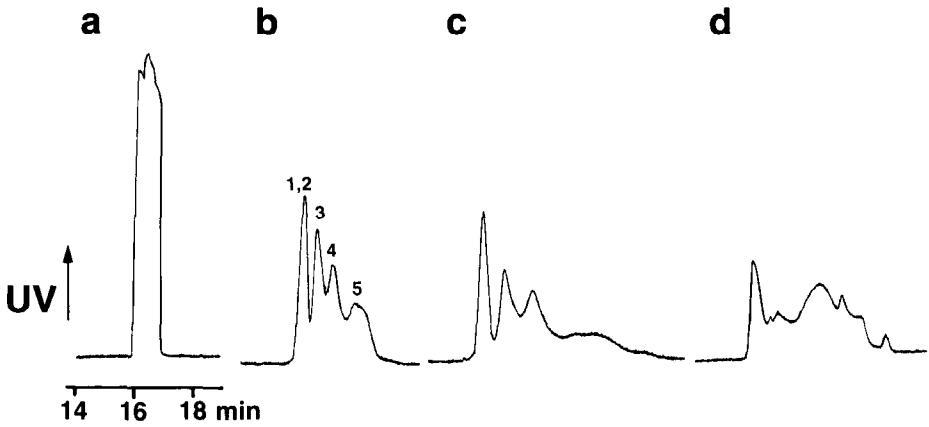

Fig. 5. Isotachopherograms for a mixture of 1.0 mg/ml MYO (1), 1.2 mg/ml CYC (2), 1.3 mg/ml CAN (3), 1.9 mg/ml CAL (4) and 1.1 mg/ml BLB (5). Spacers injected were (a) none, (b) 2 μl pH 3.5–10, (c) 2 μl pH 7–9 and (d) 10 μl discrete spacers. Volume protein mixture injected is (a) 4 μl, and (b)–(d) 2 μl. UV axis same as Fig. 2.

7–9 ampholytes and with discrete spacers. Poorer spacing performance is seen than in the previous mixture, with MYO and CYC unresolved with any spacer used. Differences also are noted concerning the relative performance of the spacers. pH 7–9 ampholytes give considerable dilution of BLB, rendering it less useful than pH 3.5–10 ampholytes. Poorer performance of discrete spacers also is seen, despite the resolution of an impurity between CAL and BLB. Thus, relative spacer performance is mixture-specific, and no spacer can be generally recommended.

Cationic ITP was tested on reconstituted serum and the results are shown in Fig. 6. Unspaced serum gives essentially a single zone with little UV resolution. Wide range ampholytes yield only two, poorly defined peaks that are considerably diluted. The discrete spacers separate four distinct peaks, with the trailing zone being particularly sharp. No attempt was made to identify the resolved peaks.

Fig. 6d shows a separation of reconstituted serum with a larger amount of spacer applied. The broad leading peak seen in Fig. 6c is not detected, but several sharp leading peaks are seen. These peaks have heights and relative positions that are not particularly reproducible, suggesting a non-steady-state situation. Preliminary serum separations shown in Fig. 6 compare poorly with similar anionic separations. However, the potential for cationic serum separations is seen, and specific components may benefit from cationic *vs.* anionic analysis.

It is apparent that for complex mixtures of proteins, separate runs with different spacer solutions are desirable to assess ultimate separability. Kenndler and Reich[5] used cluster analysis to indicate the best 2 or 3 electrolyte systems for testing anionic ITP separations of organic and inorganic acids. A similar analysis of different discrete spacer mixtures and ampholytes with a variety of protein mixtures could yield a small, optimum set to test protein separability.

One advantage of discrete spacers is the ability to fine-tune spacer compositions to achieve desired separations. Judicious selection of spacer components can yield better performance, as shown in Fig. 7 for an optimized separation of LYS, RNA, TRP, BLA, and OVA. Despite preliminary knowledge of spacer mobilities (Table I), such an optimization is largely a trial-and-error operation. For instance, determina-

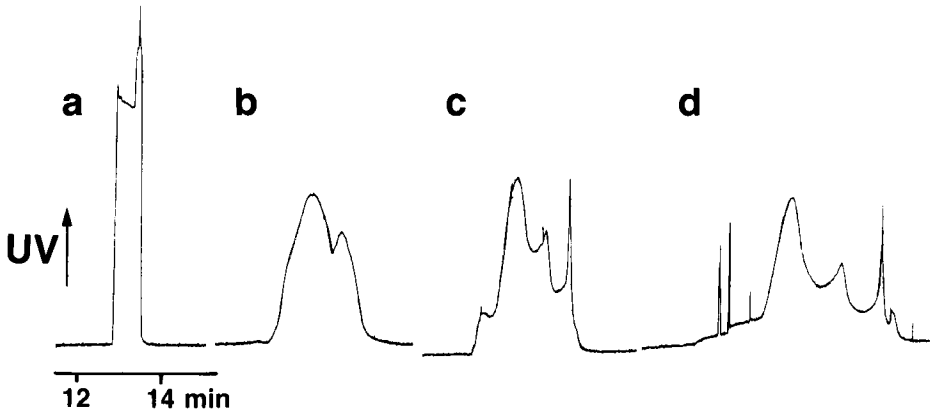

Fig. 6. UV isotachopherograms of 1 μl reconstituted human serum diluted 1:1 with leading electrolyte. Spacers injected are (a) none, (b) 2 μl pH 3.5–10, (c) 10 μl and (d) 20 μl discrete spacers. UV axis same as Fig. 2.

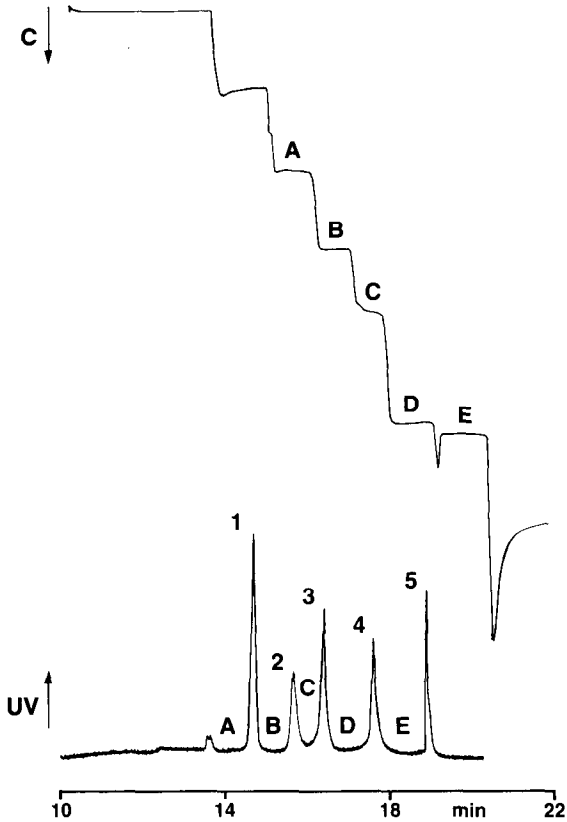

Fig. 7. Isotachopherograms for 2 μl of protein mixture from Fig. 2 with optimized, discrete spacing. Spacers are 2 μg each (A) tetraethylammonium, (B) tripropylammonium, (C) tributylammonium, (D) tetrapentylammonium and (E) γ-aminobutyric acid. C axis of upper trace is decreasing conductivity and UV axis is increasing absorbance at 280 nm. Time offset in conductivity and UV signals is due to physical separation of detectors in the capillary.

tion of single cationic mobilities gave no indication that BLA is enforced by γ-aminobutyric acid or that OVA is enforced by β-alanine. To date, no spacers can be recommended for separating the following proteins with pH 4.5 potassium acetate–acetic acid electrolytes: LYS, MYO and CYC; TRP and CAN; and BLB and BLA.

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