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USE OF ELECTROPHORETIC TITRATION CURVES FOR PREDICTING OPTIMAL CHROMATOGRAPHIC CONDITIONS FOR FAST ION-EXCHANGE CHROMATOGRAPHY OF PROTEINS

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SUMMARY

The charge characteristics of a number of proteins were determined over a broad range of pH using the "electrophoretic titration" technique recently introduced by Rosengren and others. The chromatographic behavior of these proteins was then characterized by cation- and anion-exchange chromatography to determine if electrophoretic and chromatographic characteristics could be correlated. The results indicate that retention, in terms of salt concentration required for elution, is generally dependent upon the charge density of a protein. Exceptions to this dependence were found, and most of these exceptions were probably due to asymmetry in shape, or charge inhomogeneity within the protein.

INTRODUCTION

Proteins are polyelectrolytes, and many of their fundamental physical characteristics, such as solubility, are profoundly influenced by the numbers and types of amino acids in the polymer. Some of the earliest studies of proteins, in the 1900's, were carried out by acid–base titrations[1]. Actual titrations of proteins are difficult; it is easier to measure the free electrophoretic mobility of a protein which is closely correlated with its charge[2]. However, such experiments at multiple pH values are tedious and not very commonly employed.

Recently, Rosengren *et al.*[3], Bossio *et al.*[4] and Ek and Righetti[5] described a much more convenient technique for obtaining this information. In this method, isoelectric focusing is performed in one dimension of a slab gel, composed of a low percentage of polyacrylamide gel. Sample is applied in a narrow trough in the center of the gel throughout a pH gradient, followed by electrophoresis at right angles to the pH gradient. Upon staining, an "electrophoretic titration curve" of the protein is produced, which reveals the electrophoretic mobility of the protein throughout the

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gradient typically pH 3–10. This method has numerous applications for the characterization of proteins[6,7] and is especially powerful in quickly characterizing complex mixtures of crude proteins.

It has been suggested that "electrophoretic titration" could be effective in predicting optimal conditions for the ion-exchange chromatography of proteins[8]. Theory predicts that the pH value, or values, at which the electrophoretic mobility of a protein differed as much as possible from those of contaminating proteins, would be optimal for an ion-exchange separation. Chromatofocusing generally would be expected to perform well if the proteins exhibited large differences in their isoelectric points (the point of zero mobility on the titration map), particularly if the curves had steep slopes at the point of zero mobility (indicating a high charge-density change with change in pH). Recently, electrophoretic titration was shown to be useful in predicting the best conditions for purifying creatinine kinase from a chicken muscle extract[8] and for purifying a sample of carbonic anhydrase[9].

In this work, we examined the correlation between electrophoretic titration and chromatographic behavior of proteins in a systematic manner. Establishing chromatographic behavior over a wide range of pH, as planned, would be tedious if it were not for the introduction of new chromatographic materials and systems optimized for the fast, high-resolution chromatography of proteins[9]. In our experiments, we used columns packed with macroporous, hydrophilic, monodisperse beads (Monobeads™) modified with strongly acidic or strongly basic ion-exchange groups. Because of this strongly acidic or basic character of the packings, changes in chromatographic behavior of proteins as a function of pH can largely be ascribed to charge changes only in the proteins, and not the ion exchanger. Likewise, since the matrix is porous to molecules up to about 10^7 daltons, large proteins can be chromatographed without exclusion properties of the matrix becoming significant[10]. Chromatofocusing[11] experiments were conducted using Mono P, which contains wide-pH-range ion-exchange groups on the Monobead matrix[12,13]. The charged groups on the Mono P column are the same as those on Pharmacia's chromatofocusing media PBE 94.

In our experiments, mixtures of generally well-defined proteins were analyzed electrophoretically to produce "electrophoretic titration curves". Correspondingly, chromatography of the same proteins on cation and anion exchangers produced "chromatographic retention maps" over a range of pH. The objective was to determine if conditions for optimal pH of chromatography, as predicted by the electrophoretic method, were obtained in practice, as previously described.

MATERIALS AND METHODS

Proteins

All proteins were available commercially and are listed with their suppliers in Table I. Most of these proteins have been extensively studied in assembling molecular-weight and isoelectric-point (p*I*) marker kits (Pharmacia). The p*I* and molecular weight values were determined or confirmed within the laboratory and differ little from published values.

Lactic dehydrogenase isoenzymes were prepared by a quick-freeze, slow-thaw method[14]. Aliquots of 100 μg of the isoenzyme mixture were dispensed into small vials, lyophilized in sucrose and stored at 4°C.

TABLE I

PROTEINS USED IN THIS STUDY

Protein	*Supplier*	*Isoelectric point*	*Molecular weight*
L-Amino acid oxidase (*Crotalus* venom)	Sigma	5.6, 5.7, 5.87	135,000
Amyloglucosidase (*Aspergillus niger*)	Boehringer	3.55	97,000
Carbonic anhydrase (bovine erythrocyte)	Pharmacia	5.8	30,000
Chymotrypsinogen A (bovine pancreas)	Pharmacia	9.6	25,000
Cytochrome *c* (horse heart)	Sigma	10.2	12,200
α-Lactalbumin (bovine milk)	Sigma	5.2	14,400
Lactate dehydrogenase (bovine heart)	Boehringer	5.2	140,000
Lactate dehydrogenase (bovine muscle)	Boehringer	8.4	140,000
β-Lactoglobulin A (bovine milk)	Sigma	5.2	35,000
Lentil lectin	Pharmacia	8.4	52,000
Ovalbumin (eggwhite)	Pharmacia	4.7	45,000
Phosphorylase *b* (rabbit muscle)	Pharmacia	6.35	370,000
Ribonuclease A (bovine pancreas)	Sigma	9.3	13,700
Serum albumin (bovine)	Pharmacia	4.9	67,000
Trypsin inhibitor (soy bean)	Sigma	4.55	20,000

Chromatography materials

Buffers and reagents were of reagent grade. All buffers were filtered through 0.22-μm filters and degassed under vacuum. Buffers used in the isoenzyme study are listed in Table II. A Pharmacia FPLC chromatography system, used throughout the study, consisted of two P-500 dual-piston pumps, a GP-250 gradient programmer, a V-7 injection valve, a UV-1 monitor with HR flow cell and a REC-482 chart recorder. Conductivity was monitored with a flow-through monitor from Chromatronix (Berkeley, CA, U.S.A.). Absorbance was monitored at 280 nm, and peak widths and retention volumes were stored and recovered from the memory of a Pharmacia FRAC-100 fraction collector.

Chromatographic columns from Pharmacia Fine Chemicals used in this study included Mono Q, a strong anion exchanger; Mono S, a strong cation exchanger; and Mono P, an anion exchanger designed for chromatofocusing. Chromatofocusing columns were developed with Polybuffer electrolyte solutions. Mono Q and S columns were 1 ml in volume (5 × 50 mm), while Mono P columns were 4 ml in volume (5 × 200 mm).

Unless otherwise indicated, all ion-exchange experiments were conducted at

TABLE II

BUFFER SYSTEMS USED IN ISOENZYME STUDY

No.	*Description*
1	20 m*M* 1,3-diaminopropane
2	20 m*M* piperazine
3	20 m*M* ethanolamine
4	20 m*M* diethanolamine
5	20 m*M* N-methyldiethanolamine
6	20 m*M* tris(hydroxylmethyl)methane
7	20 m*M* triethanolamine–HCl
8	20 m*M* Bis-TRIS-propane*
9	20 m*M* Bis-TRIS**

* 1,3-Bis[tris(hydroxymethyl)methylamino]propane.
** Bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane.

room temperature at 1 ml/min with a linear gradient of sodium chloride and a total gradient volume of 20 ml.

Materials for titration curves

Silane 174, Pharmalyte 3-10, Agarose IEF, Sephadex G-200 Superfine and Gel Bond were obtained from Pharmacia Fine Chemicals. Coomassie Blue R-250 was obtained from Eastman-Kodak (Rochester, NY, U.S.A.), and Page Blue 83 from BDH (Poole, Great Britain). Other chemicals and buffers were of reagent grade. Focusing and electrophoresis were performed on a Pharmacia Flatbed Apparatus, FBE 3000, with an ECPS 3000/150 power supply and VH-1 volt-hour integrator. A circulating bath from Haake (Saddle Brook, NJ, U.S.A.) was used for temperature control and cooling. The gels were formed in a custom-made casting frame, which forms a trough for sample application. Commercially produced troughs are available and are satisfactory for polyacrylamide gels.

Gel preparation

Glass plates, with the same dimensions as the casting frame, were treated with Silane 174, rinsed and dried. Polyacrylamide gels were prepared from a stock solution (10% acrylamide, 3% bisacrylamide) deionized with Amberlite MB-3. A solution sufficient for two gels contained 22.5 ml acrylamide stock solution, 12 ml 50% glycerol and 3 ml Pharmalyte 3-10. Water was added to a volume of 45 ml, and the solution was filtered through Whatman No. 1 paper and degassed. To polymerize the gel 100 µl of 60 mg/ml ammonium peroxydisulfate was added. The solution was quickly injected into the casting frame and allowed to polymerize at room temperature for 90 min. The gels were stored in a moist environment at 40°C after removal from the casting frame.

Titration curves containing agarose–Sephadex were prepared from a solution containing 0.45 g Agarose-IEF, 0.75 g Sephadex G-200 SF and 4.5 g D-sorbitol. The solution was diluted to 45 ml with hot water, boiled and cooled to 70°C before 3 ml Pharmalyte 3-10 were added. Gel Bond, cut to the size of the glass plates, was allowed to adhere to the plates (hydrophobic side to the glass) and the plates were clipped to

the casting frame. The solution was injected into the casting frame, prepared as described above. After they had hardened at room temperature for 45 min, the gels were removed and stored as described above.

Electrophoretic and staining conditions

Both types of gels were electrophoresed on the flatbed apparatus, cooled at 12°C, with the sample trough perpendicular to the electrodes. The anode strip was soaked in 1 *M* phosphoric acid for agarose gels, or 0.04 *M* aspartic acid for polyacrylamide gels. Cathode strips were always soaked in 1 *M* sodium hydroxide solution. To form the pH gradient, agarose gels were electrophoresed at 7 W constant power for 750 volt-hours, and polyacrylamide gels were run at 15 W for 750 volt-hours. For electrophoresis in the second dimension, the gels were rotated a quarter turn and the wicks soaked again in their respective solutions. Approximately 50 μl of sample (usually 2.5 mg/ml) were applied to the trough with a microsyringe. Agarose gels were electrophoresed at 1000 V for 100 volt-hours, and polyacrylamide gels were electrophoresed at 1000 V for 150 volt-hours.

Both types of gels were fixed for 30 min in 10% trichloroacetic acid–5% sulfosalicylic acid, and destained by two consecutive washes with 35% methanol–10% acetic acid. Agarose gels were then dried by blotting for 30 min, followed by drying in a hot air stream until the gels were dry to the touch. Both gel types were stained with 0.2% Coomassie Brilliant Blue R-250 or 0.2% Page Blue 83 in 35% methanol–10% acetic acid. Agarose gels were stained for 10 min. Polyacrylamide gels were stained for 6–18 h. Both types were destained in 35% methanol–10% acetic acid.

RESULTS

Generally, polyacrylamide electrophoretic titrations were conducted to predict chromatographic behavior of proteins. Polyacrylamide gels usually produced tighter, better-resolved bands than agarose–Sephadex, although the gels required more time for preparation, electrophoresis and staining. For proteins ranging in size from 15,000 to 100,000 daltons, the results obtained with the two gels were generally equivalent, although all proteins migrated more slowly in polyacrylamide than in agarose–Sephadex under equivalent conditions. For proteins over 150,000 daltons, agarose–Sephadex was the matrix of choice because sieving effects of polyacrylamide began to predominate. With larger proteins such as phosphorylase *b* and ferritin, agarose–Sephadex was definitely the matrix of choice because these proteins would hardly migrate at all in the second dimension in polyacrylamide. 1% Agarose gels have also been used, but agarose–Sephadex produced better resolution and had superior mechanical properties[15].

A typical agarose–Sephadex titration curve of a large protein, phosphorylase *b*, is shown in Fig. 1. When complex mixtures of proteins were electrophoresed, the pH profile was generally internally calibrated, since the isoelectric points (zero mobility point) of most of the proteins were known. In the titration curve shown in Fig. 1, phosphorylase *b* produced a fuzzy pattern below its isoelectric point of 6.35. A number of relatively high-molecular-weight proteins also exhibited this behavior (catalase, arginine decarboxylase, apoferritin, isocitrate dehydrogenase, aldehyde dehydrogenase and thyroglobulin). Correspondingly, these proteins produced fuzzy

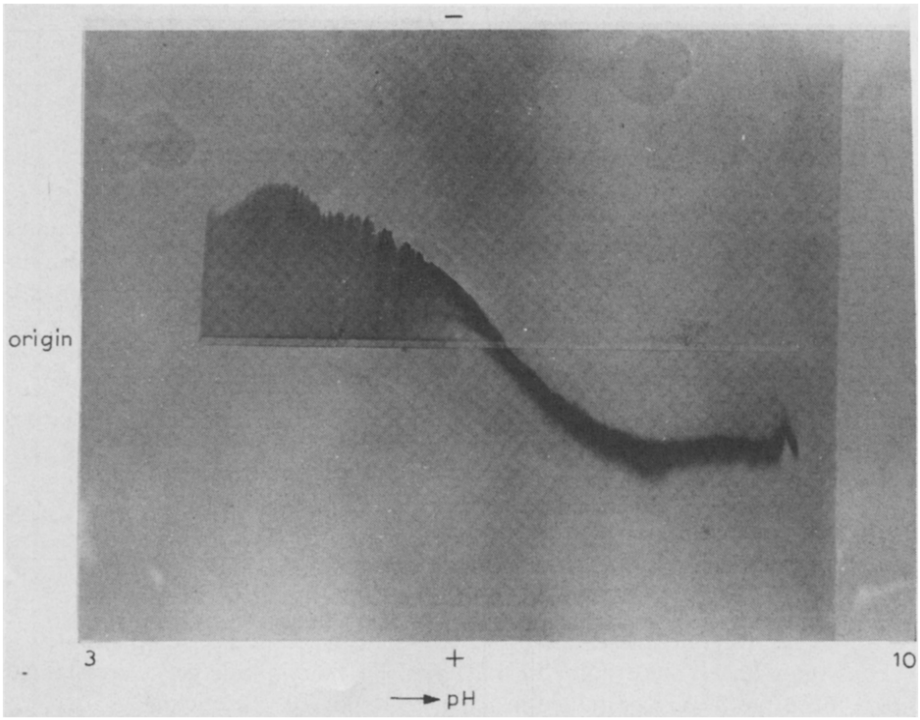

Fig. 1. Electrophoretic titration of phosphorylase in agarose–Sephadex.

elution bands or low yields upon cation-exchange chromatography. This behavior appears to be due to the intrinsic low solubility of these proteins at low pH, since many tended to precipitate upon standing at low pH.

Electrophoretic titration of beef lactic dehydrogenases

Lactic dehydrogenases are a family of structurally related proteins, all containing four subunits and having a native molecular size of about 140,000. While lactic dehydrogenases, as a family, are similar in size and shape, they differ in amino acid composition, isoelectric points, catalytic reactions and immunological reactions[16]. Because of the physical similarity between these isoenzymes, the relationship between electrophoretic titration curves and chromatographic behavior should not be complicated by considerations of size or shape, but be determined primarily by differences in surface charge.

Test mixtures contained beef heart lactic dehydrogenase (H_4), muscle lactic dehydrogenase (M_4) and hybrids containing subunits from both types (H_3M, H_2M_2, HM_3).

Electrophoretic titrations were produced in both polyacrylamide (Fig. 2a) and agarose–Sephadex (Fig. 2b). While the patterns obtained were similar, the bands were narrower in polyacrylamide than in agarose–Sephadex. Mobilities of all the bands were higher in agarose–Sephadex, but remained in about the same proportions to one another. In agarose–Sephadex, M_3H and M_2H_2 bands were actually split into two

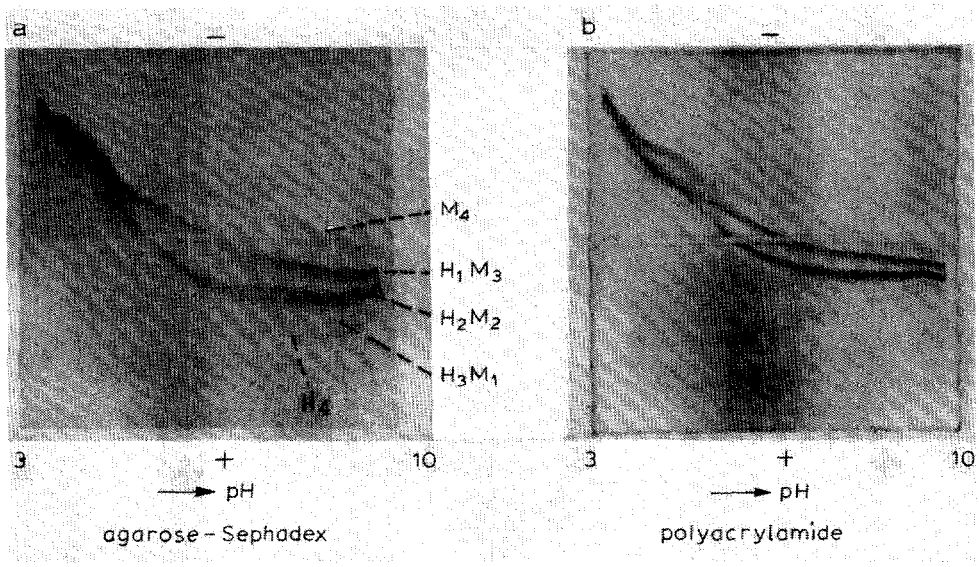

Fig. 2. Electrophoretic titration of lactic dehydrogenase isoenzymes in agarose–Sephadex (a) and in polyacrylamide (b).

bands, but this splitting could just barely be discerned by visual inspection of the polyacrylamide gels. Protein bands appeared fuzzy on the cationic side of the titration curves (positively charged protein) especially in agarose–Sephadex, suggesting that the proteins were poorly soluble or unstable in this pH region.

Retention maps of beef lactic dehydrogenases

Retention maps of the isoenzyme mixtures were prepared by chromatographing them at various pH values on a high-performance, strongly basic anion exchanger, Mono Q. In Fig. 3, the average retention volume, based upon chromatography at a given pH (in some cases with two or more different buffers) was plotted for each isoenzyme at 0.5 pH unit intervals.

The correlation between the ionic characteristics of the isoenzymes and the expected chromatographic behavior was excellent. In general, when we started at low pH and increased the pH by 0.5 units for each successive experiment, each of the isoenzymes began to exhibit weak retention on the Mono Q column at a pH about 0.5 units above its p*I*. For example, MH_3 exhibited zero mobility about pH 5.8 on the electrophoretic titration curve, but exhibited retention on the Mono Q column at around pH 6.5. M_4, with a p*I* of approximately 9, failed to be retained on Mono Q up to pH 10. The lines in the retention map (Fig. 3) resembled those of the bottom part of the titration curve (anionic side) —the lines diverged from each other progressively from high pH to neutrality.

Between pH 5 and 10 (but not at pH 10 itself) two well-resolved M_2H_2 peaks were obtained upon anion exchange of lactic dehydrogenase (Fig. 4). This separation was much better than expected on the basis of the electrophoretic titration curve in polyacrylamide and better than expected on the basis of the agarose–Sephadex

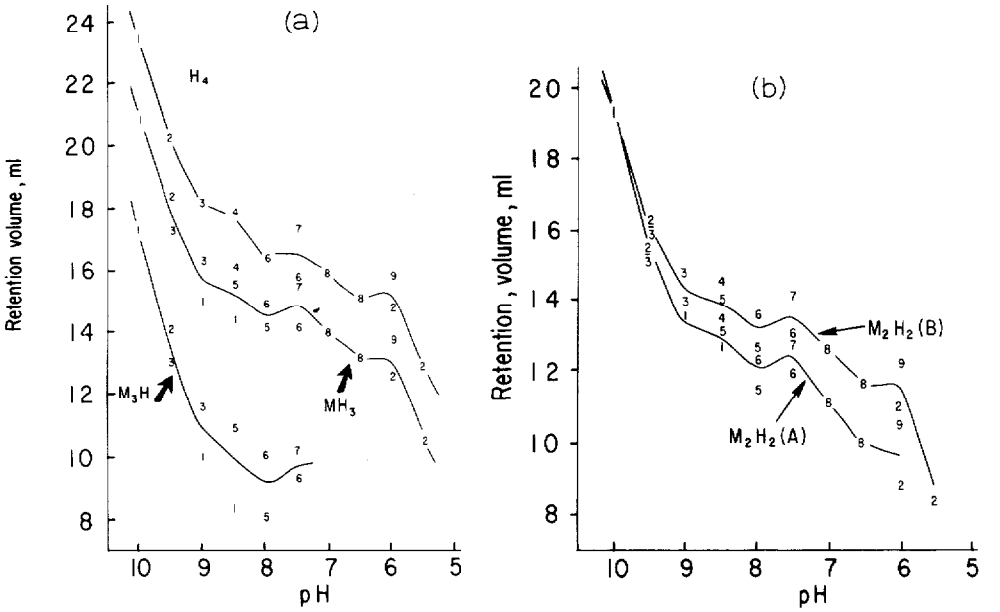

Fig. 3. Retention map of lactic dehydrogenase isoenzymes on an anion exchanger, Mono Q. Isoenzymes were prepared and chromatographed as described in Materials and methods. The start of the gradient was at 5 ml, and the end (0.35 *M* sodium chloride) at 25 ml. Multiple buffer systems were tested at each pH value: numbers in the graph refer to buffers in Table II. Isoenzyme M_4 failed to bind at all pH values tested. a, Retention values for H_4, Θ_3 and M_3H species; b, separation of two M_2H_2 species, M_2H_2 (A) being eluted before M_2M_2 (B).

titration curve. The two M_2H_2 isoenzymes were well resolved at pH 8.5, the separation being maximal at pH 6.5 (Fig. 3).

A more detailed examination of the results from retention mapping of the isoenzymes is shown in Fig. 5. Here, the relative separation, R_s★, of adjacent pairs of isoenzymes was plotted *vs.* the eluent pH. Because the R_s term incorporates both differences in elution volumes and peak widths, it is a good indicator of separation efficiency. Several buffer systems were used, but sodium chloride was always employed as the eluting agent. For all experiments with Mono Q the limit buffer concentrations was 0.35 *M* sodium chloride and for Mono S it was 0.43 *M*.

As shown in Fig. 5, for each isoenzyme pair there was a trend for maximal resolution at lower pH values, *i.e.*, at pH values approaching the isoelectric points of the enzymes. However, the effect of the buffer system itself on resolution was often dramatic, altering the R_s at a given pH by a factor as great as two. In analyzing the effects of elution volume and differences in bandwidths separately, two generalizations became apparent. In general, increased resolution at different pH values was

★ Resolution, $R_s = \dfrac{2\,(V_{e_1} - V_{e_2})}{W_1 + W_2}$

where V_{e_1} and V_{e_2} are the elution volumes of Components 1 and 2 and W_1 and W_2 are the widths of the eluted peaks of those components expressed in ml.

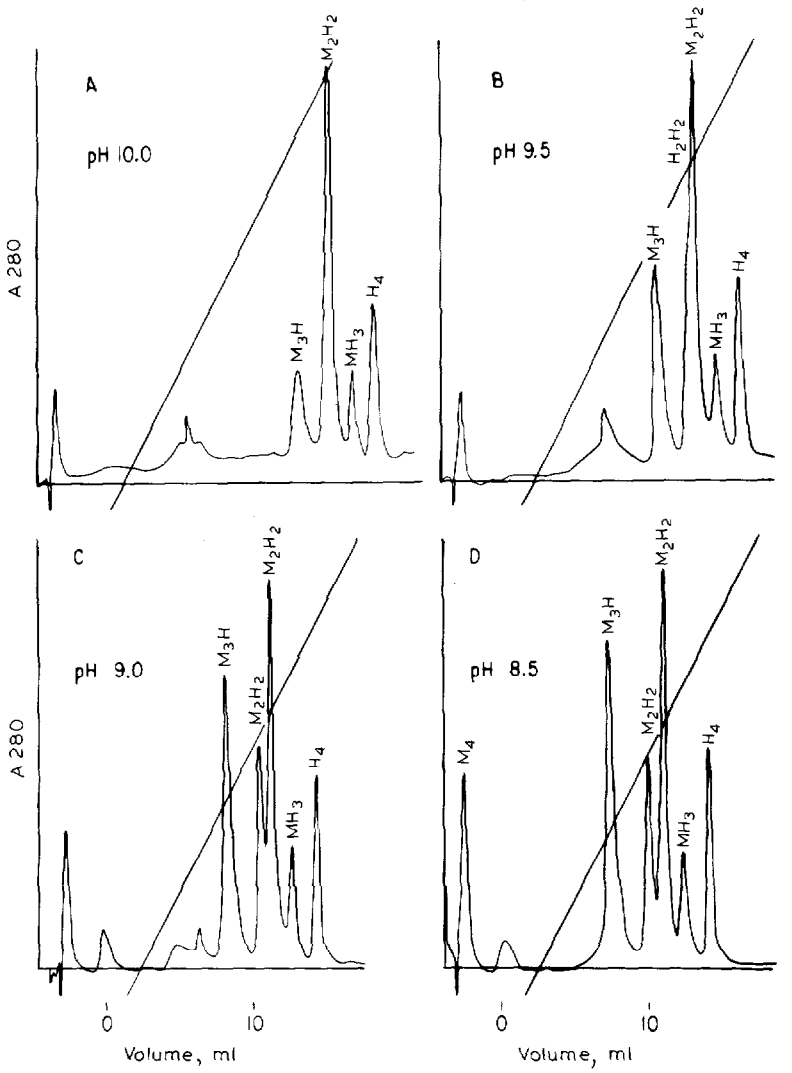

Fig. 4. Anion exchange of lactic dehydrogenase isoenzymes on Mono Q, at different pH values. 100 μg of isoenzyme mixture were chromatographed in the indicated buffers as described in Materials and methods. In each case, the gradient spanned from 0–0.35 *M* sodium chloride in a 20-ml gradient, starting at 5 ml and ending at 25 ml. Buffers: A, 20 m*M* 1,3-diaminopropane, pH 10; B, 20 m*M* piperazine, pH 9.5; C, 20 m*M* ethanolamine, pH 9.0; D, 20 m*M* diethanolamine, pH 8.5. Flow-rate: 1 ml/min.

due to increased differences between the two elution volumes of an isoenzyme pair. On the other hand, at any given pH, the differences in performance of two or more buffers was directly related to bandwidths. In short, the best-performing buffers yielded narrow bands rather than different elution volumes.

These results indicate that chromatography of a mixture of proteins with similar size and shape correlate well with the electrophoretic characteristics of the proteins as described by an electrophoretic titration curve. However, even with such

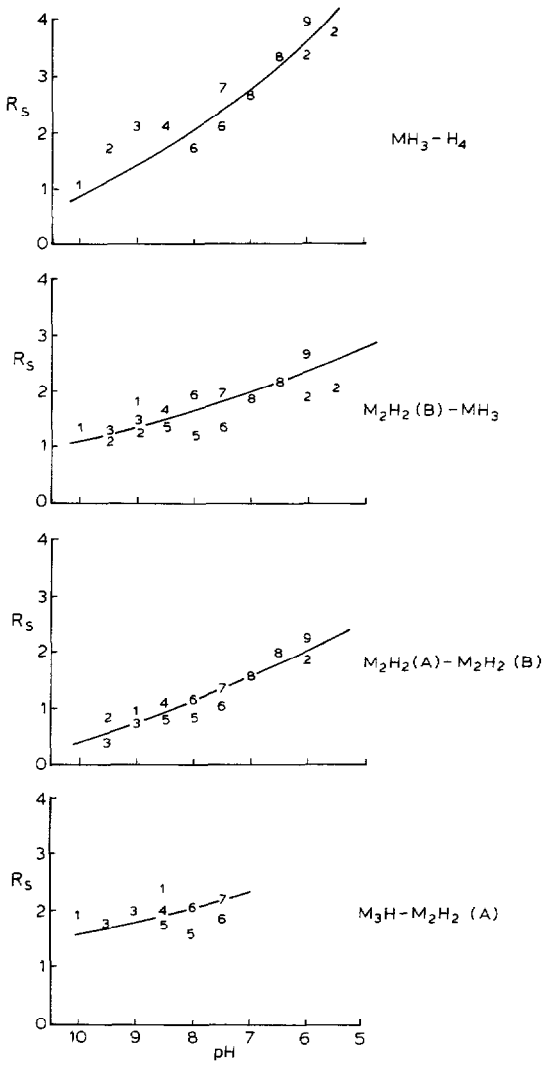

Fig. 5. The relationship between pH in anion-exchange chromatography and resolution of lactic dehydrogenase isoenzymes. R_s (relative separation) values for various isoenzyme pairs chromatographed at different pH values in different buffers were calculated. Numbers within the graph refer to buffer systems employed (see Table II).

mixtures, resolution in ion-exchange chromatography can be greatly affected by the choice of buffer or eluent[10].

Proteins with unexpected chromatographic behavior

While many mixtures of unrelated proteins that were tested exhibited good correlations between their electrophoretic characteristics and their chromatographic retention, some interesting exceptions were evident. For example, polyacrylamide electrophoretic titration of a number of small, basic, globular proteins (otherwise not

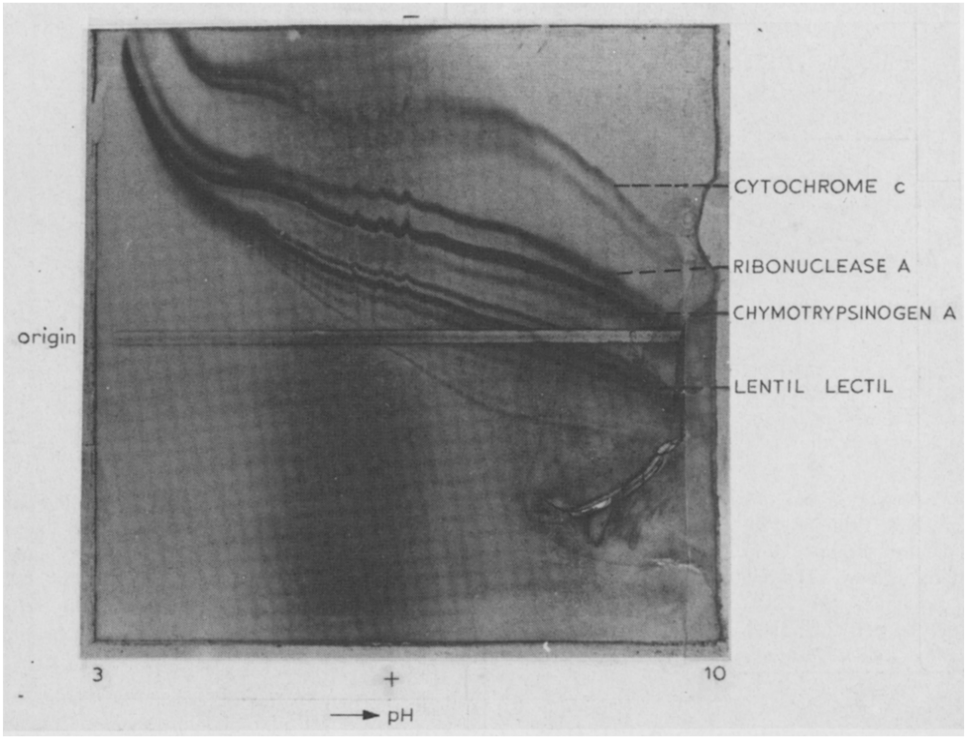

Fig. 6. Polyacrylamide electrophoretic titration of lentil lectin, cytochrome *c*, chymotrypsinogen A, and ribonuclease A.

closely related) produced a set of non-intersecting curves (Fig. 6). A straightforward prediction from this plot would be that the relative elution order of these proteins on a cation exchanger would be invariant with pH. However, the retention curves produced by lentil lectin and chymotrypsinogen (Fig. 7) on Mono S crossed at pH 4.5. Also surprisingly, the chromatographic retention curves of ribonuclease and chymotrypsinogen crossed, ribonuclease A exhibiting significantly stronger retention than chymotrypsinogen below pH 6.5. These data suggest that the mode of separation on the cation exchanger was influenced by some parameters other than the charge density of the proteins. The reason for the discrepancy between electrophoretic and chromatographic behavior is unclear, since precise data on shape and charge characteristics of even these well-defined proteins are hard to obtain.

Chromatography of a protein with unusual charge distribution

It was felt that asymmetric charge distribution could be one parameter expected to alter the chromatographic behavior of a protein. To explore this possibility a search was made for proteins with such characteristics. Perhaps the best indication of asymmetric charge distribution is the measurement of the dielectric increment. Data on the dielectric increment of proteins are rare, but values for a few proteins, including ovalbumin, serum albumin, hemoglobin, myoglobin, and β-lactoglobulin, have been cited by Oncley[17,18]. All proteins for which values have been obtained have

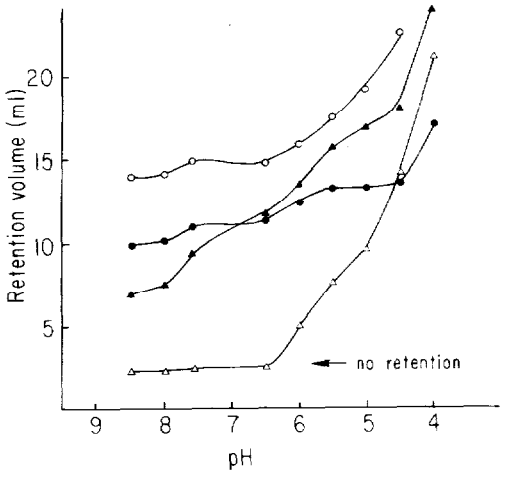

Fig. 7. Retention map of various proteins chromatographed on the cation exchanger, Mono S, at different pH values. Proteins were chromatographed as described under Materials and methods, with a linear gradient of sodium chloride from 0 to 0.43 *M*, starting at 4 ml and terminating at 24 ml. Buffers: 50 m*M* sodium formate, pH 4.0; 50 m*M* sodium succinate, pH 4.5; 50 m*M* sodium acetate, pH 5.0; 50 m*M* methyl ethanesulfate, pH 5.5, 6.0 and 6.5; 50 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.6 and 8.0; 50 m*M* N,N-bis(2-hydroxyethyl)glycine (BICINE), pH 8.5. ○ = Cytochrome *c*; ● = chymotrypsinogen A; ▲ = ribonuclease A; △ = lentil lectin.

dielectric increments between 0.10–0.04 $\Delta D_0/g$*, with the exception of β-lactoglobulin, which has an unusually high dielectric increment of 3.0 $\Delta d_0/g$ (ref. 17). Otherwise, β-lactoglobulin A does not appear to be an unusual protein. It contains two apparently identical subunits of 17,500 daltons, and its intrinsic viscosity of 3.4 ml/g is well within the range of other proteins regarded as globular[19]. While the molecule appears to be globular and symmetrical, the high dielectric increment indicates that the distribution of charge on the molecule must be high asymmetrical.

Polyacrylamide electrophoretic titration curves were determined for β-lactoglobulin and a number of other proteins of similar size, shape and isoelectric pH as β-lactoglobulin. The titration curves appeared unexceptional. For example, α-lactalbumin exhibited a slightly lower mobility than β-lactoglobulin at both above and below pH 5.2, the isoelectric point of both proteins (Fig. 8a). Ovalbumin and soy bean trypsin inhibitor formed curves nearly parallel but always below (more negative charge) than the curve of β-lactoglobulin (Fig. 8b). Bovine serum albumin and amyloglucosidase exhibited a much higher anionic charge density than β-lactoglobulin (Fig. 8c).

Based on these data, it appears that ovalbumin, soy bean trypsin inhibitor, bovine serum albumin and amyloglucosidase have higher anionic charge densities than β-lactoglobulin at most pH values. They should be eluted from an anion exchanger at higher salt concentration than β-lactoglobulin. On the other hand, α-lactalbumin should be eluted at a salt concentration equal to or slightly below that at which β-lactoglobulin is eluted.

* $\Delta D_0 = D_0 - D^0$, where D^0 is the dielectric constant of the solvent and g is the concentration of solute in g/l.

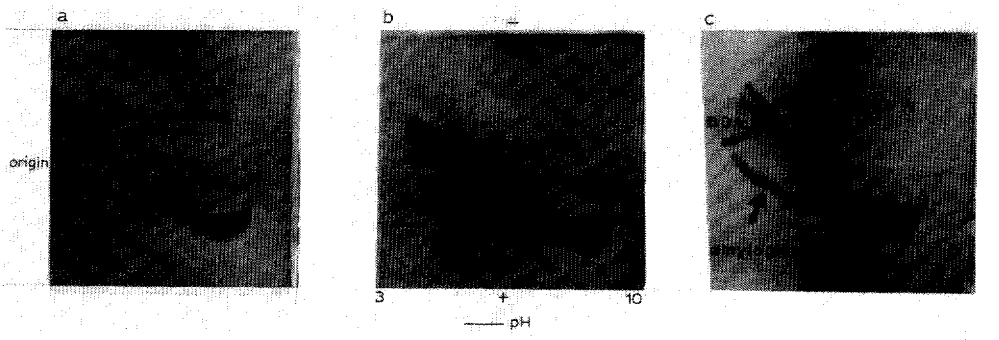

Fig. 8. Electrophoretic titrations of β-lactoglobulin A. a, α-Lactalbumin and β-lactoglobulin; b, ovalbumin, soy bean trypsin inhibitor and β-lactoglobulin; c, bovine serum albumin (BSA), amyloglucosidase and β-lactoglobulin.

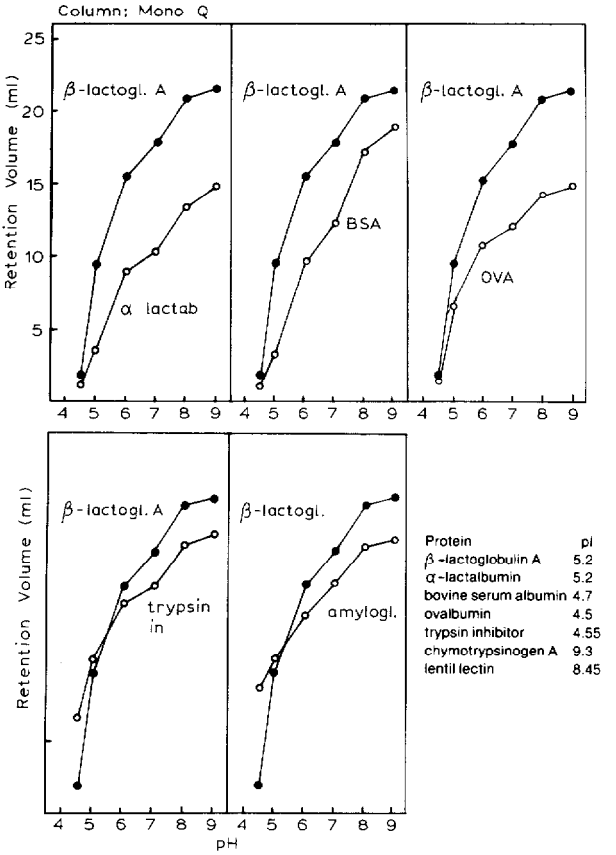

Fig. 9. Retention maps of β-lactoglobulin A and some acidic proteins on the anion exchanger, Mono Q. Proteins were chromatographed with a 0–0.35 *M* sodium chloride gradient starting at 2 ml and terminating at 22 ml. Buffers: 20 m*M* N-methylpiperazine, pH 4.5; 20 m*M* piperazine, pH 5.0 and 6.0; 20 m*M* Bis-TRIS-propane, pH 7.0; 20 m*M* tris(hydroxymethyl)aminomethane (Tris), pH 8.0; 20 m*M* ethanolamine, pH 9.0.

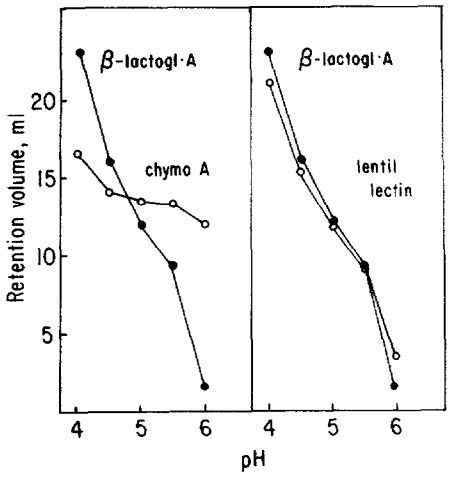

Fig. 10. Retention maps of β-lactoglobulin A, and some basic proteins, on a cation exchanger, Mono S. Proteins were chromatographed with a linear 0–0.43 *M* sodium chloride gradient, starting at 2 ml and terminating at 22 ml. Buffers: 50 m*M* sodium formate, pH 4.0; 50 m*M* sodium succinate, pH 4.5; 50 m*M* sodium acetate, pH 5.0; and 50 m*M* 2-N-morpholinoethanesulfonic acid (MES), pH 5.0 and 6.0.

In practice, the chromatographic behavior of β-lactoglobulin was not as predicted. On the anion exchanger, Mono Q (Fig. 9), β-lactoglobulin behaved as a much more highly charged protein than indicated electrophoretically. Throughout the entire pH range between 5 and 9, β-lactoglobulin was eluted at a much higher salt concentration than α-lactalbumin, bovine serum albumin and ovalbumin. Trypsin inhibitor and amyloglucosidase, which apparently had substantially higher electrophoretic mobilities than β-lactoglobulin at most pH values, were more weakly retained by the anion exchanger than β-lactoglobulin. β-Lactoglobulin was weakly retained by the anion exchanger at pH 5.0, 0.2 units below the p*I* of the protein, a pH at which it was not expected to be retained.

The behavior of β-lactoglobulin on a cation exchanger, Mono S, also was not as expected (Fig. 10). A retention map of β-lactoglobulin on Mono S between pH 4 and 6 was prepared, and its chromatographic behavior was compared with that of two relatively basic proteins, chymotrypsinogen A (p*I* 9.6) and lentil lectin (p*I* 8.4). As expected, these two basic proteins exhibited very high electrophoretic mobilities in titration curves on the cationic side (results not shown). However, despite the fact the β-lactoglobulin was a much more acidic protein, it was retained more tighly by Mono S than chymotrypsinogen A between pH 4–4.5, and its retention behavior was nearly identical with lentil lectin (Fig. 10). β-Lactoglobulin was weakly retained by the matrix at pH 5.4, 0.2 pH units above its p*I*, the expected point of non-retention.

DISCUSSION

Prediction of the ion-exchange characteristics of proteins is obviously a much more difficult problem than prediction of the behavior of smaller molecules or of molecules with less variable secondary and tertiary structures, such as nucleic acids. It is indeed pleasantly surprising that electrophoretic experiments carried out in an environment very different from that inside an ion-exchange matrix can serve to predict chromatographic performance fairly accurately[8,9].

A conclusion supported by this study and others[8,9] is that retention of proteins in ion-exchange chromatography was linearly proportional to charge density of the proteins, which is in turn closely related to electrophoretic mobility in low percentage gels. On the other hand, it is a fairly common experience that polymers such as oligonucleotides are typically eluted at salt concentrations that are linearly proportional to their total charge, and not their charge density. By extrapolation, shape must also lay a role in determining the ion-exchange characteristics of a protein, since highly fibrous proteins would probably be bound in a manner more dependent on their total charge. In this study we did not work with any fibrous proteins, such as myosin. In globular proteins, a major factor must be the limited surface area of the globular molecule that can contact the absorbent surface. In either case, electrophoretic methods possess the advantage of measuring only the external, exposed charged groups which are presumably the same groups responsible for the ion-exchange characteristics of the protein.

We postulate that most of the discrepancies between electrophoretic and chromatographic behavior of globular proteins can be explained on the basis of inhomogeneous charge distribution. Electrophoresis measures a time average of a protein's net surface charge and is not expected to reveal charge inhomogeneity. However, charge inhomogeneities exist, as can be shown by physical measurements such as dielectric increment.

The exact mechanism of ion exchange of proteins is still unknown, since many details such as the kinetics of absorption and desorption are largely unknown. It is likely that proteins are bound to ion exchangers at many different sites on the exchanger, although any localized site with relatively higher charge density should have a higher probability of binding. Since many electrostatic bonds are probably formed with the sorbent, a protein may tend to be nearly irreversibly bound during the initial binding step. During the course of an ion-exchange experiment in which the salt concentration rises, proteins bound through weakly charged areas should be desorbed at relatively low ionic strengths, and then bound again by the matrix, but usually to sites of higher charge density. By extension, the salt concentration at which a protein is finally eluted should be determined by the site of highest surface charge density on the protein. In actuality, of course, the situation will be complicated by other factors. Not the least of these is the fact that over the course of time a single protein will be in equilibrium with nearly countless different ionic forms[20], and the kinetics of this process are not well understood. Also, the ion exchanger itself may alter the conformation of a bound protein. Such phenomena could be reasonably expected to cause peak broadening or formation of multiple peaks in chromatography. Proteins containing subunits can be expected, in some cases, to exchange subunits or to exist in multiple conformations.

In this work, we studied the chromatographic behavior of β-lactoglobulin A, a protein known to have an unusually high degree of separation between positive and negative charge centers. This protein behaved as though it were much more highly charged than predicted by electrophoretic titration, on both anion and cation exchangers. In fact, β-lactoglobulin was retained by both cation and anion exchangers at its pI, although nearly all the other proteins tested failed to be bound unless chromatographed 0.5–1.5 pH units off their isoelectric points. The effect cannot be attributed to low solubility of β-lactoglobulin at its pI, nor can it be attributed to the

Donnan effect within the ion exchanger because this effect would tend to be constant for all proteins, leading to increased retention on both cation and anion exchangers.

Charge asymmetry could also be expected to lead to delayed retention in chromatofocusing[21]. A recent study[22] on the chromatofocusing of whey proteins on the chromatofocusing (anion-exchange) gel PBE 94 indicates that, in this mode, β-lactoglobulins A and B are also eluted somewhat uncharacteristically. While most of the proteins in this study were eluted at pH values within 0.3 units of their isoelectric points, the β-lactoglobulins were eluted about 0.8 pH units below their isoelectric points. While it is appealing to speculate that these phenomena are solely due to asymmetric charge density, further studies need to be conducted with other proteins which also have unusual dielectric properties.

Charge heterogeneity may explain why examples were found in which two proteins with different electrophoretic mobilities at a given pH cannot be separated chromatographically at that pH (*i.e.*, points of intersection in Fig. 7). It may also predict that two proteins with apparently identical electrophoretic mobilities could easily be resolved by ion exchange, because the interaction with the ion exchanger would actually involve critical sites containing a high charge. A particularly interesting example of the second type of phenomenon is the separation of the two M_2H_2 isoenzymes. These two isoenzymes have identical primary structure and apparently represent the two possible arrangements of two subunits of two different types in a tetrahedral structure. The polyacrylamide titration curves indicate a barely discernable split, indicating that the conformers possess slightly different charge densities. However, these conformers can be easily and nearly totally resolved by ion-exchange chromatography (Fig. 4). To obtain this degree of resolution, the ion exchanger must be sensitive to a parameter different from the net ionic characteristics of the molecule. It is most likely sensitive to a region of the M_2H_2 isoenzyme sterically altered by the differences in arrangements of the two isoenzyme types.

In summary, these experiments indicate that electrophoretic titrations are highly useful in predicting the ion-exchange characteristics of protein mixtures. The results must be interpreted with caution, because an as yet unknown proportion of proteins may contain highly asymmetric charge distributions. We believe that a useful method for exploring the charge heterogeneity theory would be the construction of hybrid molecules by covalent crosslinking of basic and acidic proteins. Such molecules would probably contain more charge heterogeneity than normally found in nature, and their chromatographic behaviors should be unusual. It is becoming clear, we believe that in many cases the resolving power of high-performance ion exchangers exceeds that of electrophoresis. Retention mapping may in many cases be more predictive than electrophoretic titration, and the use of high-performance ion-exchange media allows the construction of such maps in a reasonable time.

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REFERENCES

1 J. T. Edsall, in E. J. Cohn and J. T. Edsall (Editors), *Proteins, Amino Acids and Peptides*, Hafner, New York, 1965, p. 447.
2 R. A. Alberty, in H. Neurath and K. Bailey (Editors), *The Proteins*, Academic Press, 1953, p. 505.
3 A. Rosengren, B. Bjellqvist and V. Gasparic, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, p. 165.
4 A. Bianchi Bosisio, C. Loeherlein, R. S. Snyder and P. G. Righetti, *J. Chromatogr.*, 189 (1980) 317.
5 K. Ek and P. G. Righetti, *Electrophoresis*, 1 (1980) 137.
6 R. Krishnamoorthy, A. B. Bosisio, D. Labie and P. G. Righetti, *FEBS Lett.*, 94 (1978) 319.
7 K. Ek, E. Gianazza and P. G. Righetti, *Biochim. Biophys. Acta*, 626 (1980) 356.
8 L. Fägerstam, L. Söderberg, L. Wahlström, U.-B. Fredriksson, K. Plith and E. Walldén, *Protides Biol. Fluids*, 30 (1982) 621.
9 J. Richey, *Amer. Lab.*, 14 (1982) 104.
10 J. Bergström, L. Söderberg, L. Wahlström, R.-M. Müller, A. Domicelj, G. Hagström, R. Stalberg, I. Källman and K.-A. Hansson, *Protides Biol. Fluids*, 30 (1982) 641.
11 J. Richey and L. Beading, *Amer. Lab.*, 13 (1981) 100.
12 L. Fägerstam, L. Wahlström, U.-B. Fredriksson, R.-M. Müller and V. Nylund, *Protides Biol. Fluids*, 30 (1982) 657.
13 L. Söderberg, R.-M. Müller and L. Fägerstam, *Protides Biol. Fluids*, 30 (1982) 661.
14 F. Stolzenbach, *Methods Enzymol.*, 9 (1966) 287.
15 A. Manrique and M. Lasky, *Electrophoresis*, 2 (1981) 315.
16 A. Pesce, T. P. Fondy, F. Stolzenbach, F. Castillo and N. Kaplan, *J. Biol. Chem.*, 242 (1967) 2151.
17 J. L. Oncley, in E. H. Grant, R. J. Sheppard and G. P. South (Editors), *Dielectric Behavior of Biological Molecules in Solution*, Clarendon Press, Oxford, 1978.
18 J. L. Oncley, in E. J. Cohn and J. T. Edsall (Editors), *Proteins Amino Acids and Peptides*, Hafner, New York, 1965, p. 543.
19 H. A. Sober (Editor), *Handbook of Biochemistry*, The Chemical Rubber Company, Cleveland, OH, 2nd ed., 1970.
20 J. T. Edsall, in E. J. Cohn and J. T. Edsall (Editors), *Proteins, Amino Acids and Peptides*, Hafner, New York, 1965, p. 461.
21 L. A. A. E. Sluyterman and J. Wiudenes, in B. J. Radola and D. Graesslin (Editors), *Proc. Int. Symp. Electrofocusing and Isotachophoresis, Hamburg, August 2–4, 1976*, De Gruyter, Berlin, 1977, p. 463.
22 R. J. Pearce and R. M. Shanley, *Aust. J. Dairy Tech.*, Sept. (1981) 110.