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THE PURIFICATION OF POLYELECTROLYTES BY ION EXCHANGE CHROMATOGRAPHY

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SUMMARY

A homologous series of lysine polypeptides was chromatographed on carboxymethyl-cellulose columns using linear and step gradients of sodium chloride. The effect of changes in flow rates, gradient volumes, salt concentrations, and sample amounts upon the resolution was measured.

The results suggest that in ion exchange chromatography of polyelectrolytes such as polypeptides, proteins, or nucleic acids, convex gradients of about twenty column volumes would give maximum resolution when flow rates and sample amounts are low.

INTRODUCTION

Since the introduction of modified celluloses to column chromatography ion exchange has become one of the most powerful tools for the purification of protein or nucleic acid constituents of complex polyelectrolyte mixtures, such as those found in cellular extracts[1,2]. Several important reviews have been published concerning the application of ion exchange to practical problems, and the correct interpretation of results[3–5]. Experimental data demonstrating general relationships between elution conditions and resolving power are greatly needed on a level that would be meaningful to scientists who are faced with only an occasional purification problem. Frequently such data are buried in "materials and methods" sections of specialized purification procedures, or in mathematical and theoretical relationships and considerations which are too involved and of little interest to many investigators in the biological sciences.

A homologous series of lysine peptides[6], *i.e.*, dilysine, trilysine, tetralysine, etc., has provided a model system for demonstrating relationships between various elution conditions and resolution. Although this system is less complicated than is usually encountered in most ion exchange purification schemes, the data do provide guide

lines for obtaining optimum resolution. Lysine peptides have also been used by the authors in developing theoretical considerations[7–9], but the need for data expressing general relationships between various elution conditions and resolution has become so obvious during the last few years, that it has prompted the publication of these data in a more practical form.

MATERIALS AND METHODS

Lysine peptides

Lysine peptides were prepared by dissolving high mol. wt. poly-L-lysine hydrobromide in 6 *N* HCl and heating for 80 min at 70°. Excess acid was removed by flash evaporation and the resulting lysine peptides titrated to neutrality with NaOH, diluted to 10 mg/ml, and stored at 4°.

Chromatographic elutions

The carboxymethyl-cellulose (Brown Company) used in this work, was sieved upon U.S. standard mesh sieves, and the fraction between 200–325 mesh washed according to a method previously described[10]. Columns, 0.9 × 34 cm, were pump packed[11] with a slurry containing about 4 g of the above cellulose, 0.8 mequiv./g. They were subsequently washed with 25% NaCl and distilled water respectively prior to each elution. The 220 mμ absorbency and the conductivity of column effluents were monitored continuously and recorded on a multichannel potentiometric recorder.

Resolution

As used in this article the resolution is defined as the distance between adjacent peaks on a chromatogram divided by the sum of their half widths, at half maximum concentration.

RESULTS

Flow rate

During routine determination of lysine peptide distributions, flow rates of 1–2 ml/min/cm^2 were commonly used[6–9]. The resolution obtained in these experiments did not seem to be greatly influenced by a twofold change in flow rate. Similar observations have been made for serum proteins in the region of 0.1–0.6 ml/min/cm^2 (ref. 12). In an attempt to obtain a quantitative relationship between flow rate and resolution, lysine peptides were eluted at 0.05–7.0 ml/min/cm^2. When calculated according to the method described above, the resolution between adjacent peaks, *i.e.*, between dilysine and trilysine, trilysine and tetralysine, etc., was represented as a function of the log of the flow rate (Fig. 1). The relatively large change in resolution for the smaller peptides is of little interest because they are well separated from their nearest neighbors even at very high flow rates. Since most proteins and nucleic acids contain a large number of ionic groups they behave more like the larger lysine peptides. As the number of ionic groups per molecule increases at any one flow rate, the resolution appears to approach a minimum value. This relationship is probably characteristic of the hydrolyzate used. Decreasing the flow rate tenfold results in a 50–100% increase in resolution. Such increases are significant among the larger

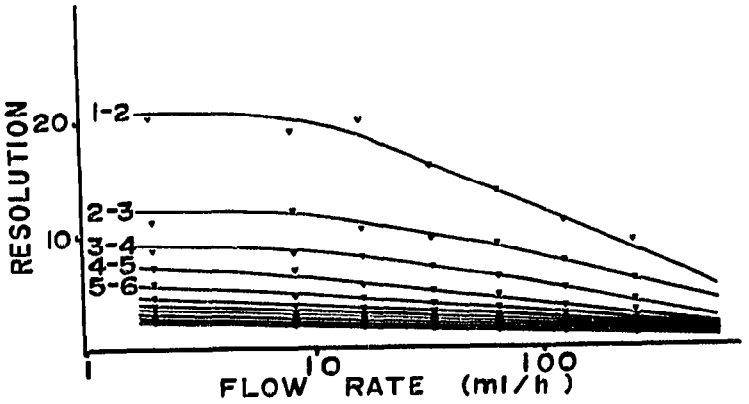

Fig. 1. Resolution between adjacent members of a homologous series of lysine peptides at various flow rates. Numbers on the left refer to the number of lysine residues in the peptides under consideration. Experimental data were calculated from effluent diagrams obtained by eluting 10 mg of a partial acid hydrolyzate of polylysine with a linear 0–1.0 *M* NaCl gradient at various flow rates. See METHODS.

peptides, however, increasing the duration of a given experiment from say 1 to 10 days to obtain this resolution is in most cases not acceptable. It is important to note here that the sodium chloride eluent concentration at the point of emergence of each peak was found not to vary with increasing flow rates. This is in accordance with theoretical predictions[13]. Thus decreasing the flow rate does not change the distances between neighboring peaks on a chromatogram but tends to sharpen such peaks giving an increase in resolution. Broadening of peaks due to diffusion was less than the sharpening brought about by decreasing the flow rate, as previously observed for flow rates of a few ml/h[14].

Gradient volume

Because polyelectrolytes like proteins and nucleic acids have curved adsorption isotherms[15] one frequently observes excessive tailing, unless a salt gradient is used in the eluent. But, this gradient also increases the rate of elution of contaminating molecules still remaining on the column. How steep can the gradient be, *i.e.*, what is the minimum volume in which a certain limiting salt concentration can be attained, without suffering a loss in resolution? It has been inferred in a theoretical treatment that the appropriate gradient volume is a function of column length, and that "symmetrical" peaks are an indication that the length has been correctly adjusted to this volume[15]. Although the data presented in this paper are not described in terms of peak symmetry but rather of the ratio of the distance between neighboring peaks to the sum of their half widths at half maximum concentration (resolution), they tend to substantiate the idea that there exists an appropriate gradient volume for a given column, and an increase beyond this volume results in little if any increase in resolution. In these experiments a linear gradient, 0–1.0 *M* NaCl was used. The gradient volume was decreased in proportion to the flow rate. The resolution of each lysine peptide, as compared with its next higher and lower homolog, was calculated and represented as a function of gradient volume (Fig. 2). As seen before, di-, tri-, and tetrapeptides are well separated even at very small gradient volumes. Very little additional resolution is obtained by increasing the total gradient volume much above

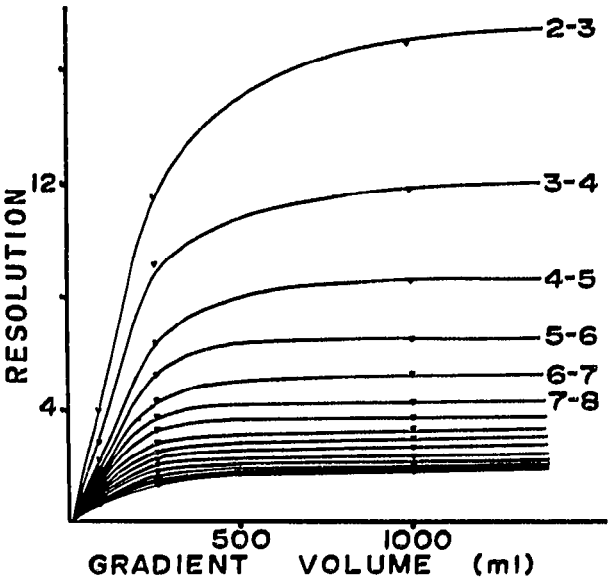

Fig. 2. Resolution between adjacent members of a homologous series of lysine peptides as a function of gradient (linear 0–1.0 *M* NaCl) volume. Elution conditions are similar to those described under Fig. 1. Numbers to the right refer to the number of lysine residues in the peptides being considered.

300 ml. This corresponds to about 15 column volumes. Since the flow rate was increased in proportion with the gradient volume, the tenfold increase in volume, 100–1000 ml, was accompanied with a flow rate increase from 0.6 to 6.0 ml/h. Assuming that the results described above under conditions of varying flow rate can be extended to these experiments, this tenfold increase in flow is accompanied by about a 50% decrease in resolution. Thus at constant flow rates, increased gradient volumes might give significant increases in resolution even at volumes larger than 15 column volumes, but again one is faced with the handicap of prolonged elution times.

Salt concentration

To quantitize the effect of eluent salt concentration on the rate of elution of various members of the homologous series of lysine peptides, a number of one step elutions were carried out at different sodium chloride concentrations, *i.e.*, the peptides were added to the column in water and then eluted by a sudden step change from water to a predetermined fixed salt concentration. The volume of eluent required to elute each peptide from the column, retention volume (Δ), was found to be dependent on the total amount of each peptide and the eluent salt concentration. As mentioned above the retention volume was not altered by changes in flow rate. The sensitivity of lysine peptides toward variations in eluent salt concentration was determined using a constant amount (10 mg) of polylysine partial hydrolyzate. In those cases where various members of the lysine peptide series could not be identified by counting successive peaks emerging after the monomer, co-chromatography of one or more of these peaks with peptides of known identity was necessary[16].

The retention volumes, volume of eluent from the midpoint of the salt front to the apex of each peak, were represented as a function of NaCl concentration (Fig. 3). The data represented here are reminiscent of theoretical predictions in which electro-

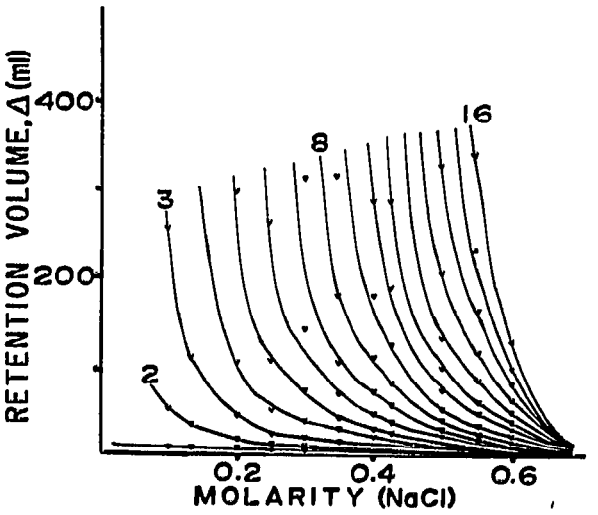

Fig. 3. Relationship between retention volume, Δ(ml), and eluent salt concentration, M (NaCl) for a homologous series of lysine peptides. Numbers refer to the number of lysine residues per molecule. Experimental points were calculated from chromatograms obtained by eluting 10 mg of polylysine partial acid hydrolyzate with various concentrations of NaCl. See text.

static interactions were considered to be the only forces affecting distribution coefficients[17]. The results show an increased sensitivity of larger peptides toward ionic strength. However, it should be noticed that even those peptides containing 16 lysine residues require a small but significant increase in salt to change the retention volume from 300 (about 15 column volumes) to zero, the point where they emerge with the salt front. In similar experiments with cytochrome *c*, β-lactoglobulin, and bovine plasma albumin, we have demonstrated that proteins can be eluted at various retention volumes by proper control of eluent salt concentrations.

Sample amounts

It is well known that polyelectrolytes have curved adsorption isotherms[18], that is, that the volume of eluent required to elute a given polyelectrolyte is dependent upon the amount present. Various amounts of a partial acid hydrolyzate of polylysine were eluted under conditions similar to the one step elutions described above. The retention volumes (Δ) were calculated and represented as a function of the amount of hydrolyzate used (Fig. 4). As expected the larger peptides, those having more ionic groups participating in the exchange, are affected more by the amount of hydrolyzate used than the smaller peptides. The relative values for the retention volumes are of course peculiar to the polylysine hydrolyzate being used, and the conditions of elution. However, it is clear that as the number of ionic groups participating in exchange increases from two in dilysine to five in pentalysine the retention volume becomes more strongly dependent on the amount of sample added to the column. Since proteins and nucleic acids are generally polyvalent, they can be expected to behave in a similar fashion. In practice, curved isotherms become a menace in purification schemes because they induce excessive tailing or peak asymmetry. Decreasing the total amount of polyelectrolyte on a given column produces peaks which are more symmetrical especially when combined with an appropriately chosen salt gradient.

It is realized that phenomena other than ion exchange are involved and may

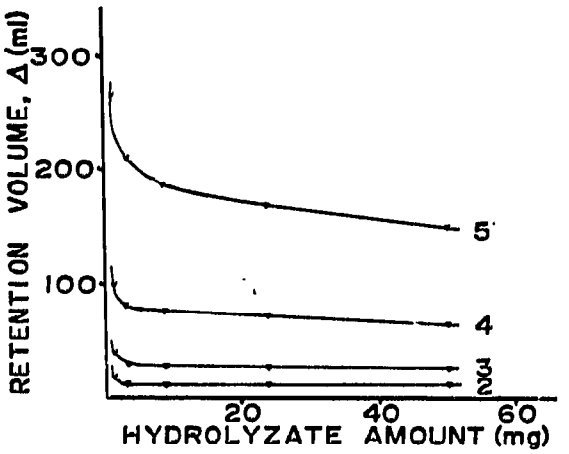

Fig. 4. Relationship between retention volume, Δ (ml), and the quantity of polylysine hydrolyzate added to each column. Numbers to the right of each curve refer to the number of lysine residues per molecule. Experimental points were calculated from a chromatogram obtained by eluting various amounts of hydrolyzate with 0.25 M NaCl.

cause significant deviation from results predictable solely on the basis of electrostatic interactions. But electrostatic interactions generally play an important role in purification by ion exchange. Since the separation of lysine peptides by ion exchange chromatography can be explained rather well by considering electrostatic forces as being the major forces involved in the adsorption of peptides to carboxymethylcellulose[8,13]; these peptides do provide a good system for studying ion exchange chromatography.

DISCUSSION

In conclusion the effect of various elutions conditions on the separation (resolution) of the individual members of a homologous series of lysine peptides can be summarized as follows:

(A) A tenfold decrease in flow rate generally resulted in a 50–100% increase in resolution. At the slower flow rates the resolution of only the smaller peptides reached a plateau below which further decrease in flow rate did not improve their resolution. This is probably a consequence of an approach to equilibrium elution conditions for these peptides, which larger peptides apparently never do attain even at extremely low flow rates.

(B) When salt gradients were employed, near maximum resolution was obtained when the total gradient volume was adjusted to 15–20 column volumes. Volumes smaller than this resulted in a substantial loss in resolution, while larger volumes had no significant effect on the resolution of the larger peptides, when the overall time of such experiments was held constant. It should be pointed out that the use of gradients does not necessarily result in an increase in resolution[19].

(C) An increasing sensitivity of lysine peptides toward increases in eluent salt concentration was found in proceeding from peptides containing only a few ionic groups to those containing many ionic groups. This suggests that a convex salt gradient would produce the most efficient overall separation, *i.e.*, during the early

part of the elution where the peptides containing relatively few ionic groups are eluted, a steep gradient would be flowing through the column; as the larger peptides (which are more sensitive to changes in salt concentration) begin to emerge from the column the gradient becomes progressively less steep. Experimental data supporting this conclusion have already been published[6]. It is important to note that peptides containing at least 16 ionic groups required a 0.1 *M* change in salt concentration from the time migration of these peptides was first detected until they moved as rapidly as the salt front through the column. This is significant because it implies that purification by "true" chromatography is possible as the peptides move down the column.

(D) Lysine peptides were found to have curved adsorption isotherms. Those peptides containing the greatest number of charges were found to be most sensitive toward changes in peptide concentration. Since many proteins and nucleic acids contain multiple ionic groups, and since curved isotherms cause very asymmetric elution profiles, the best resolution can be obtained when the smallest amounts of such polyelectrolytes are eluted from ion exchange columns, *i.e.*, the more symmetrical a peak the less tailing and possible overlapping there will be from one peak to another.

(E) Although, not investigated in this study, various theoretical considerations have shown that resolution is directly proportional to the square root of the column length, *i.e.*, increasing the column length fourfold brings about a twofold increase in resolution[20].

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