AN APPROACH TO THE ION EXCHANGE CHROMATOGRAPHY OF POLYELECTROLYTES

II. EXPERIMENTAL EVALUATION OF THE MODEL

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INTRODUCTION

In the preceding paper[1] one of us has suggested a model for the chromatography of polyelectrolytes by ion exchange. The model is based essentially on the law of mass action, as applied by LANGMUIR to surface phenomena. Although the validity of the treatment is certainly limited by the assumptions made, it did bring out most of the peculiarities observed in the chromatography of polyvalent ions when compared with monovalent ions. Reviews have appeared which summarize some of the most important concepts of ion exchange as applied to separations of complex polyelectrolyte mixtures[2-6]. An ideal system for reducing the above model to experimental verification would be the chromatography of a family of polyelectrolytes of increasing charge and size. Such a family or homologous series can be obtained from partial hydrolysis of macro ions such as polylysine[7,8], polyglutamic acid[9], polyadenylic acid[10], and polythymidylic acid[11]. As was initially shown by STEWART AND STAHMANN[12], the first 20 members of the lysine homologous series can be fractionated on carboxymethyl-cellulose[13]. The chromatographic properties of the members of this series, under various elution conditions, have been used to test certain relationships predicted by the theory. Exchange isotherms of lysine polypeptides of various concentrations have been determined, and they give reasonable supporting evidence to the model used.

EXPERIMENTAL

Column packing

All of the experiments described in this manuscript were carried out at room temperature on carboxymethyl-cellulose[13], CM-C, with a capacity of 0.8 mequiv./g**, which had been sieved on U.S. standard mesh sieves, and the fraction between 200–325 mesh washed and prepared according to the method of PETERSON AND SOBER[4]. The sodium form of the exchanger was used. Columns were packed by pumping a dilute

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slurry into Pyrex cylinders, initially filled with the slurry[14]. These cylinders were stoppered at either end with special adapters, designed to keep mixing of effluents at a minimum.

Column elutions

Three types of elution were used depending on the experiment being performed. The first made use of an exponential gradient, the second of a linear gradient, and the third was an elution at constant eluent concentration. The exponential gradient mixing device consisted of a one liter mixer[12], and the linear gradient was produced by using two of the three 500 ml chambers of a modified multichambered gradient mixing device[14].

All of the chromatograms were monitored at 220 mμ, which is a convenient wavelength for peptide bond detection[15,16]. Except for the batchwise determinations, effluents were continuously monitored in 1 cm quartz flow-cells in either the Zeiss PM Q II or Beckman DB spectrophotometer. The Cary 11 was used for determining concentrations from the batchwise experiments. A peristaltic finger pump was convenient for pumping eluents at about 1.5 ml/h and faster, however, some difficulties were experienced using this pump at slower flow rates.

Preparation of hydrolyzates

Forty milligrams of high molecular weight polylysine* (mol. wt. 110,000) was suspended in 6 ml of 6 *N* HCl at 70°, and 2 ml aliquots removed at 20, 40 and 80 min. After excess HCl was removed by rotary evaporation, each aliquot was analyzed by column chromatography as previously described[12]. It was found that the 80 min hydrolyzate gave a size distribution which was suitable for the large scale isolation and purification of peptides containing from 1–15 lysine residues. Material prepared in this manner was also used in experiments which were designated to test the chromatographic relationships predicted by the theory.

Preparation of lysine polypeptides

Five hundred milligrams of the polylysine was hydrolyzed as above and suspended in 50 ml of distilled water and chromatographed in 40 mg portions on a 0.9 × 34 cm column containing about 3.5 g of CM-C at 2 ml/min with an exponential gradient[12]. Peptides of equal size were pooled, diluted about 10 fold, added directly to the same column at the same flow rate, and rechromatographed. All peptides probably did not require this 10-fold dilution, but the lower limit for quantitative recovery was not determined. Volatile buffers were not used because of their absorption in the region used for monitoring column effluents.

Determination of exchange isotherms

Concentration isotherms of lysine peptides containing from 3–13 residues were determined batchwise in 10 ml polyethylene disposable syringes, containing a filter disc and 0.1 g of CM-C. These syringes provided a convenient method for adsorbing the chromatographically purified peptides from high dilutions, for determining peptide concentrations at various levels of the experiment, and for mixing the slurry during each equilibration period.

* Pilot Chemicals, Inc., Watertown, Mass., Lot No. L-22.

The high dilution of the peptides was a consequence of diluting out the NaCl to such a level that each peptide could be quantitatively adsorbed by percolating the same through the CM-C bed, which was allowed to settle in each syringe. Following this adsorption each bed was flushed with distilled water, which was allowed to drain freely from the syringes. An additional 0.5 ml was removed by compressing the bed with the plunger. At this point 3.0 ml of 0.43 *M* NaCl was added through the filter disc of each syringe. Buffer was not used because it did not prove to be necessary during the column chromatography of lysine peptides by gradient elution[7].

The concentration of NaCl used during the isotherm determinations was chosen because at this concentration a representative family of peptides could be obtained by constant salt elution of a partial acid hydrolyzate of polylysine. A control syringe was used to determine non-peptide absorbing material which washed from the CM-C during the experiment. This absorption was subtracted from that obtained during all syringe experiments.

The exchange isotherms were calculated from the effluent histories of each batch experiment. The amount of peptide in the stationary phase at any one mobile phase concentration was calculated by subtracting the total amount of peptide in the latter phase from that remaining in both phases. The volume of the mobile phase was about 3.2 ml. Concentrations were determined by using a molar extinction coefficient of 600 for the peptide bonds. Absorbancy measurements provided the information required for determining the individual peptide concentrations.

Symbols

The symbols used in this paper have been taken from the previous publication where the theoretical considerations are reported[1]. DP is the abbreviation for degree of polymerisation.

DISCUSSION AND RESULTS

As mentioned earlier, this publication is directed toward the experimental verification of certain relationships predicted by the mass action law for polyelectrolyte exchange in chromatography. The model used has been discussed in some detail in the preceding publication[1], and it is the purpose of this paper to check the theory with a homologous series of lysine polypeptides.

Establishment of equilibrium

One of the basic assumptions is that local equilibrium be established between the solid and mobile phases in the ion exchange column at every stage of the chromatographic development. Under ordinary elution conditions equilibrium is seldom established[17], however, it can certainly be approached. One of the effects of non-equilibrium is the broadening of the solute bands as they move down the column[17]. The criteria used for determining the flow rate at which equilibrium was approached was the height and sharpness of peptide zones as they emerged from the column. Such zones seemed to reach a maximum height and sharpness about 10 ml/h/cm^2.

Retention volume predictions

If for a first approximation the assumptions made in the theoretical treatment

are reasonably good[1], then during constant salt elutions a plot of the log Δ versus n-1 should give a straight line, the slope being log $K_M/(E)$. Because n is unknown, Δ was plotted as a function of the degree of polymerization, DP, as in Fig. 1. The degree of polymerization was obtained from the order of emergence of each peptide from the chromatographic column[12], and it is probably proportional to n. The value of K_M as calculated from the slope was 705.

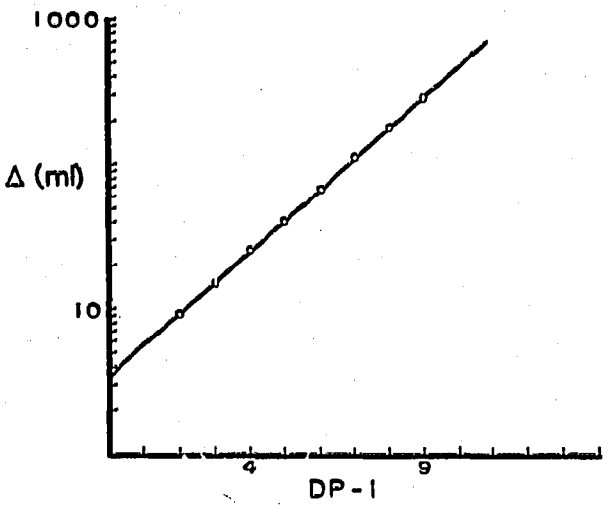

Fig. 1. Relationship between the log Δ and DP. Δ = retention volume. DP = degree of polymerization. The experimental points were calculated from the chromatogram shown in Fig. 2.

The experimental points used in calculating the above retention volumes were obtained from a chromatogram in which 10 mg of hydrolyzate was eluted with 0.43 M NaCl, at 6 ml/h as shown in Fig. 2. A similar experiment performed at one-third this flow rate gave comparable retention volumes. The fact that the zones emerging from the column became increasingly more asymmetric as DP increased is in good agreement with the theory[1].

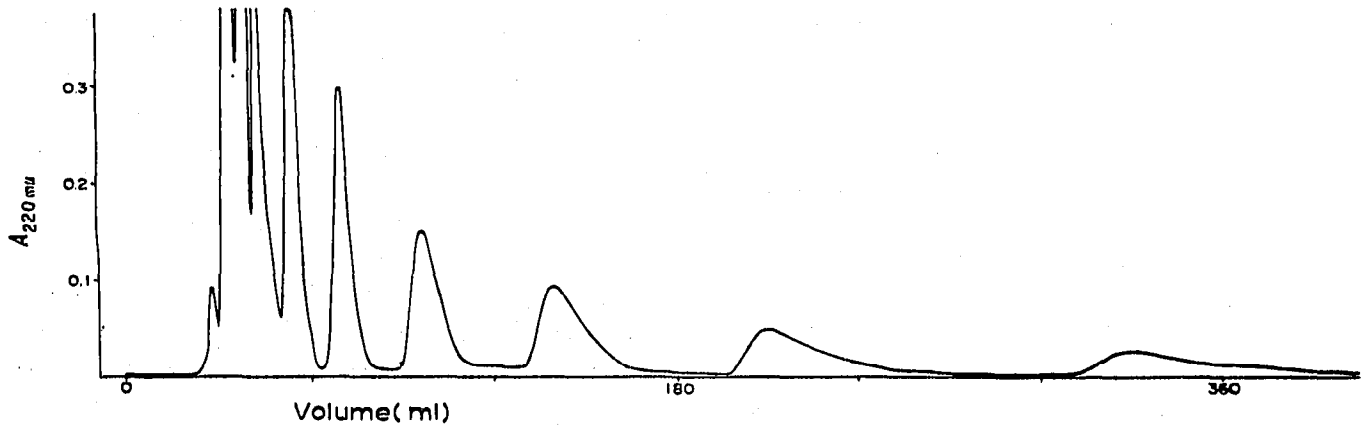

Fig. 2. Elution at constant eluent concentration of a partial acid hydrolyzate of poly-L-lysine hydrobromide. Ten milligrams of polylysine was added to a 0.9 × 34 cm column packed with about 3.5 g of carboxymethyl-cellulose 0.8 mequiv./g and eluted with 0.43 M NaCl at 6 ml/h. The salt front emerged with the front shoulder of the first peak, lysine. Hydrolyzate was prepared by reacting high molecular weight polylysine with 6 N HCl for 80 min at 70°.

The derivation of the above and subsequent relationships required that $\theta_E \gg \theta_P$. This assumption is probably valid in that the total peptide in any one zone on the column was always considerably less than 1.0% that present during isotherm determinations at high peptide concentrations.

Gradient elution predictions

If one accepts the approximations mentioned in the previous section, and operates the column under gradient conditions such that all of the peptides reach an R_F of 1, or nearly 1, before they emerge from the column, a plot of log (E) versus 1/DP should give a straight line. The intercept at 1/DP = 0 is equal to log K_M. To obtain such a system, a series of chromatograms were run such that the eluent volume was decreased proportionally with the flow rate, the maximum concentration of the eluent and overall time of the experiment remaining constant. A plot representing the data can be seen in Fig. 3, in which the concentration of the effluent at the point of emergence of each peptide was plotted as a function of 1/DP. If the linear portion of each curve is extrapolated to 1/DP = 0, values for K_M between 690 and 800 are obtained. The point to which all of the curves have been extended represents high molecular weight polylysine (110,000), 860 residues per molecule.

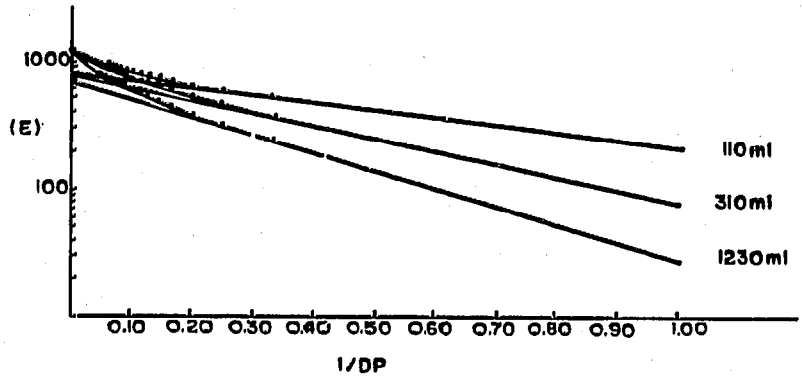

Fig. 3. Relationship between log (E) and 1/DP. (E) = elution concentration of various lysine peptides, DP = degree of polymerization. The experimental points used for plotting the curves were taken from chromatograms in which 10 mg of polylysine hydrolyzate were eluted with a constant gradient, 0–1.0 M NaCl, of 1230, 310 and 110 ml, at 6, 1.6 and 0.6 ml/h, respectively. The point to which the curves are extended is the elution concentration of unhydrolyzed high molecular weight poly-L-lysine hydrobromide, containing an average of 860 residues per molecule.

The fact that one obtains a curve and not a straight line can probably be attributed to the effect of increasing NaCl concentrations on the activity coefficients. As DP increases, the effect of this is felt more than for low DP. The change in activity coefficients would turn up essentially as a "salting out" effect which is known to appear much before the substance actually precipitates[18]. This effect, by increasing the affinity of the polylysines for the adsorbent, would make them emerge at a later point of the gradient, *i.e.*, at higher salt concentrations. Such an effect should be less apparent in constant salt experiments.

Determination of exchange isotherms

The isotherms were determined batchwise according to the method described above (Fig. 4). The fact that some of the curves do not fit well in the family of curves

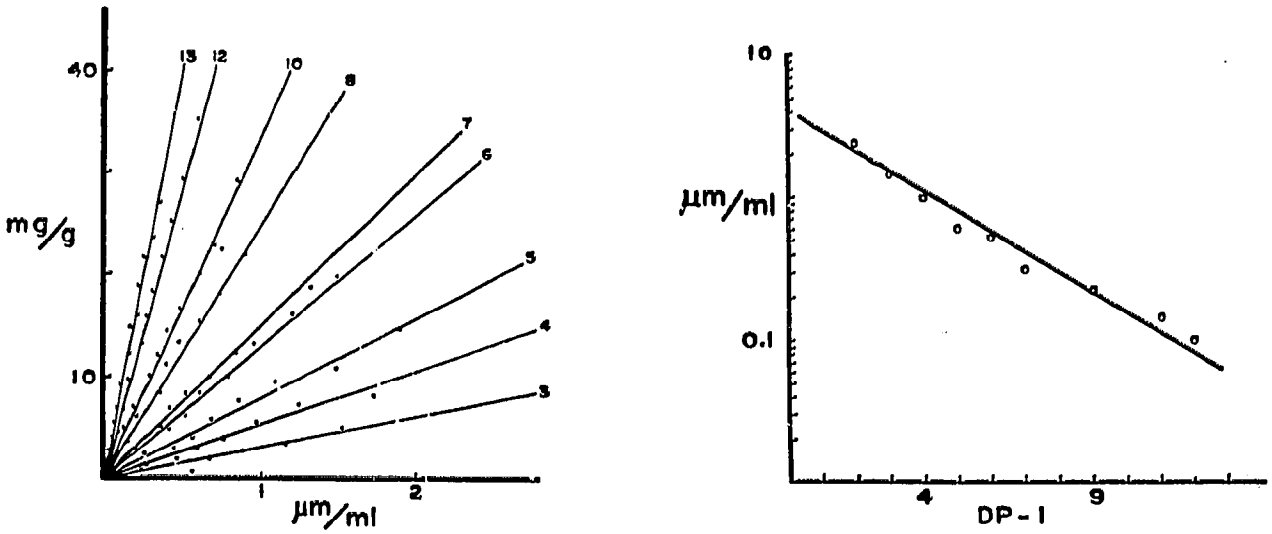

Fig. 4. Isotherms of a series of lysine peptides as determined from batch experiments. Each isotherm was determined on 0.1 g of carboxymethyl-cellulose, 0.8 mequiv./g, at 0.43 *M* NaCl. The ordinate represents mg of peptide per g of exchanger, and the abscissa the concentration of peptide in the mobile phase, 3.2 ml. The degree of polymerization is given above each curve.

Fig. 5. Concentration relationship between isotherms of a series of lysine peptides. Ordinate gives peptide concentration in mobile phase at a constant stationary phase concentration, 7.7 mg/g (Fig. 4). DP = degree of polymerization.

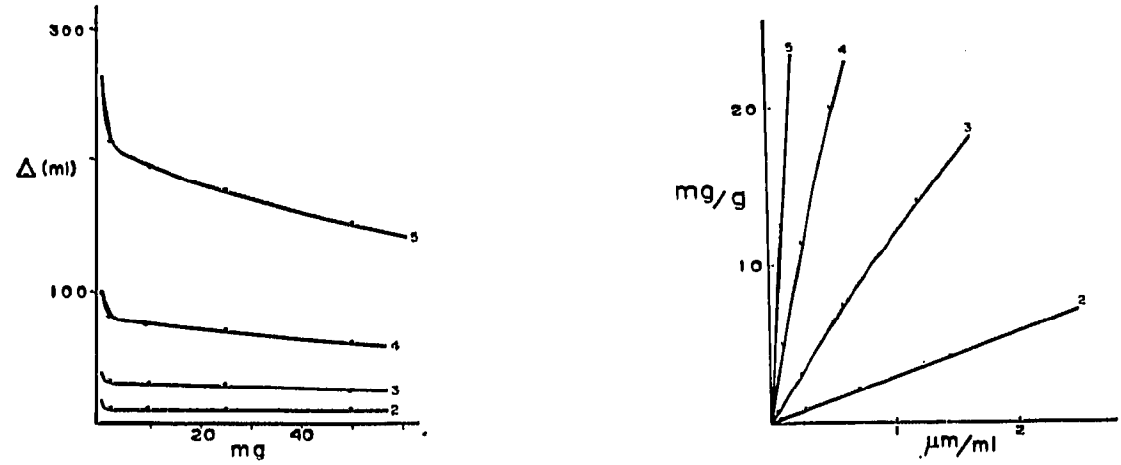

Fig. 6. Relationship between the retention volume and mg of poly-L-lysine hydrolyzate used during one step elutions. Experimental points were taken from chromatograms similar to Fig. 2, the difference being that they were determined at 0.25 *M* NaCl at 60 ml/h with 1–50 mg hydrolyzate. The degree of polymerization is given to the right of each curve.

Fig. 7. Isotherms of a series of lysine peptides as calculated from retention volumes, Fig. 6, using 0.72, a value calculated from Δ intercept at DP = 0 (Fig. 1), as the proportionality constant. Peptide concentrations corresponding to particular retention volumes were taken as a fraction, mg hydrolyzate added to column/50 mg, of the maximum concentration of each peptide obtained during the 50 mg elution.

set by the others may be explained by differences in the hold up volumes of the various syringes. Variations of 0.1 ml were shown to have a significant effect on the slope of the isotherms eventually obtained. The release of 220 mμ absorbing material from columns to which no peptide had been adsorbed also added some uncertainty.

Equation (11) of the preceding paper[1] states that a plot of log (P) against (n — 1) should give, at constant q_P, a straight line with a negative slope (— log [K_M/(E)]). The experimental points obtained do, in fact, lie on a line (Fig. 5). The value of K_M calculated from this plot, 586, is of the same order of magnitude as that obtained from constant salt elutions at the same NaCl concentration.

During the experiments reported above and carried out under conditions of gradient or constant salt elution, it was observed that the relative position of each peptide was not a function of flow rate. In another set of experiments we have determined the relative distribution coefficients, and therefore the isotherms, under non-equilibrium conditions. Chromatograms were made at 60 ml/h, with increasing amounts of hydrolyzate (1–50 mg) in 5 ml of distilled water, and eluted at 0.25 M NaCl. The data are represented in terms of retention volumes (Fig. 6). In this figure, Δ, and therefore the distribution coefficient, is indeed a curved function of polyelectrolyte concentration and DP.

If, however, the data are represented in terms of mobile and stationary phase peptide concentrations, comparable with Fig. 4, such non-linearity is difficult to detect (Fig. 7).

CONCLUSIONS

The mass action theory of ion exchange chromatography, as presented in an earlier paper[1], has been tested with a homologous series of lysine peptides. These experimental tests were based on the elution characteristics of this polylysine series under various chromatographic conditions. There was good correlation between the theoretical predictions and the experimental data. Isotherms of these peptides were shown to be related in a manner predicted by the theory. Moreover, data were also obtained on the isotherms of polylysines under non-equilibrium conditions.

Although the relationships used in testing the theory were derived by making a series of assumptions and approximations which are only rarely approached in real systems, they do seem to describe adequately the ion exchange process involved in the chromatography of lysine peptides, which presumably is dominated by electrostatic type forces.

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SUMMARY

This publication is directed toward the experimental verification of certain relationships predicted by a mass action theory for polyelectrolyte exchange in chromatography. These relationships describe the ion exchange process as determined with a family of lysine polypeptides of increasing size and charge. It is concluded that the theory, in addition to explaining certain peculiarities observed in protein chromatography, provides a mathematical treatment which, as a first approximation, was experimentally verified.

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