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Purification of complex protein mixtures by ion-exchange displacement chromatography using spacer displacers

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ABSTRACT

The anion-exchange separation of complex protein mixtures by displacement chromatography using spacer displacers driven by high-affinity final displacers is demonstrated. Guinea pig serum was separated on a medium-resolution adsorbent using a single heterogenous mixture of carboxymethyldextran displacers to space the protein components. Mouse liver cytosol was separated on a low-resolution adsorbent using six carboxymethyldextran spacer displacers of increasing column affinity. The demonstration of the purification of alkaline phosphatase from *E. coli* periplasm by displacement chromatography on a high-performance liquid chromatography column is reviewed. The benefits of spacer displacers for separating minor components from complex biological protein mixtures is discussed. A simplified method for preparing carboxymethyldextran displacers is presented.

INTRODUCTION

Although there have been many advances made in the chromatographic separation of proteins, large-scale purification still presents challenges. The purification of a single protein produced by procaryotic or eucaryotic cells is a formidable task because of the complexity of mixtures of proteins produced by organisms. The biotechnology industry has become acutely aware of these problems, as is it estimated that 50–80% of the total production costs of genetically engineered proteins are related to purification [1]. Many purification procedures developed in research laboratories for protein characterization and amino acid sequencing are not suitable for production because of low recoveries from multiple chromatographic and electrophoretic procedures [2]. To reduce the costs of therapeutic proteins, purification methods of high resolution, selectivity, capacity, and recovery must be used [3].

It was recognized over 40 years ago that displacement chromatography offered several advantages for preparative separation. Tiselius and associates published several manuscripts describing the theory of displacement chromatography, using simple mixtures of sugars on charcoal columns as models [4]. Porath and Li [5,6] pioneered the application of displacement chromatography for the separation of polypeptides. They showed that bacitracin and insulin could be highly purified on charcoal columns using various alcohols as carrier displacers. This research showed that very high sample loads of simple mixtures could be separated with high resolution and high recovery [5]. However, it was obvious that the separation of complex mixtures of typical proteins from biological samples was much more difficult. This work was largely abandoned because of the lack of adequate protein adsorbents and carrier displacers. Displacement chromatography for proteins has been revived in recent years, using

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modern column technology and synthesized carrier or spacing displacers [3,7–9].

It was shown in the late 1970s that unfractionated preparations of carboxymethyldextran (CM-D) establish multicomponent displacement trains on anion-exchange columns, the affinity of each CM-D molecule being a function of the number of negative charges it can present to the adsorbent. Proteins of human serum were separated within the displacement trains according to their column affinities [10]. These CM-Ds behaved like classic displacers [11] as described by Tiselius [4]. Tailing, noted by Porath with single displacers, was eliminated because of the gradual, progressively increasing affinities of the multiplicity of CM-D species within the displacement train. These displacers have been used to purify proteins from complex biological mixtures, including serum [12] and *E. coli* periplasm [13]. High recovery, resolution, and capacities have been accomplished on various anion-exchange adsorbents, including high-performance liquid chromatography (HPLC) columns.

Chondroitin sulfate and Nalcolyte 7105 have been used as displacers to separate simple protein mixtures on anion- and cation-exchange columns [14,15]. High-affinity displacers like chondroitin sulfate and Nalcolyte establish displacement trains based upon protein–protein displacement since they have higher affinities than any of the proteins. Subramanian and Cramer [15] have shown that simple mixtures of proteins can be separated by protein–protein displacement when conditions were adjusted to separate crossing isotherms. Although protein–protein displacement can be useful when the target proteins are substantial components of the feed stock, it has not been shown to offer high recovery and high resolution for the separation of a single minor protein component from a complex biological mixture such as serum or a cell culture fluid.

Research in the last 15 years has shown that proteins can be separated by displacement chromatography on many types of protein adsorbents [7,8,10]. However, to be useful in separating therapeutic proteins from complex protein mixtures, effective spacer displacers must generally be used. These displacers must be relatively inexpensive, produced by controlled conditions, cover a wide column affinity range, the nontoxic, and be removeable from the final product. The CM-D displacers were designed to fulfill these properties.

Our recent work has largely involved simplifying the synthesis and purification of CM-Ds. The present manuscript demonstrates the separating power of displacement chromatography employing carrier or spacer carboxymethyldextrans as displacers in resolving the complex mixtures of proteins present in mouse liver cytosol, guinea pig serum, and alkaline phosphatase from *E. coli* periplasm on low resolution, medium resolution, and HPLC adsorbents, respectively.

EXPERIMENTAL

Materials

Fractogel DEAE 650S was purchased from Pierce (Rockford, IL, USA). DEAE-cellulose (DE-52) was obtained from Whatman (Hillsboro, OR, USA). The silver staining gel kit, AG1-X8 (OH), and Dowex 50W-X8 (H) were products of Bio-Rad (Richmond, CA, USA). Industrial grade XL dextran of 9000 average molecular weight was a product of Pharmachem (Bethlehem, PA, USA). Chloroacetic acid, lithium hydroxide monohydrate, and sodium borohydride were Fisher Scientific (Pittsburg, PA, USA) products. Phenylmethylsulfonyl fluoride (PMSF), DNase I, RNase A, and molecular weight standards (Dalton Mark VII-L) for gel electrophoresis were obtained from Sigma (St. Louis, MO, USA).

Preparation of spacer carboxymethyldextran

In a 125-ml glass flask, 7.0 g of chloroacetic acid were dissolved in 56 g of water, using a $1\frac{1}{2} \times \frac{5}{16}$ in. magnetic stir bar. Then 47 g of dextran was added in many small portions, reducing the rate of addition as the increasing viscosity slowed dispersion. The resulting solution was cooled to 7°C in a small ice bath; then 6.2 g of $LiOH \cdot H_2O$ were added in a trickle to the rapidly stirred solution at a rate adjusted to keep the temperature at 20°C or below as the heat of neutralization was absorbed by the ice bath. When slightly more than half of the $LiOH \cdot H_2O$ had been added, a rapid color change to deep yellow indicated that the solution had become strongly alkaline. After this was confirmed with a pH test paper, the ice bath was removed, about 300 mg of sodium borohydride was added, and the re-

maining LiOH · H_2O was dropped in. Stirring was continued to complete solution.

The flask was sealed and placed in a weighted screw-cap plastic jar, which was then supported in a 37.5°C water bath for 48 h. The pale yellow product was diluted with partially deaerated deionized water to 450 ml.

CM-D was separated from low-molecular-weight reaction products by passing the diluted reaction mixture at 90 ml/h through a series of three ion-exchange columns; 120 ml of AG1-X8 (OH), 120 ml of Dowex 50W-X8 (H), and 30 ml of AG1-X8 (OH), all 20–50 mesh. In order to provide density stability, the columns were connected to each other in such a way as to produce upward flow through the resin beds. When all the diluted reaction mixture had entered the system, partially deaerated deionized water was pumped after it until all of the purified product had been collected. The CM-D emerged in the free acid form, so collection was begun when the effluent turned a strip of Congo Red paper blue. Soon after this point, the first column was inverted, without disconnecting it from the others, in order to preserve density stability when, much later, the water entered the column. The second and third columns were similarly inverted soon after pumping water into the first column was begun. Collection of the CM-D was ended when the effluent was no longer acid or was very weakly acid to Congo Red paper.

The entire product was adjusted from pH 2.46 to 7.85 with 4.4 ml of 10.0 *M* NaOH. The final product was 467 ml of 11% CM-D having a reciprocal of pellet volume (RPV) of 7.

Preparation of carboxymethyldextran final displacer

In a 100-ml glass beaker, 8.6 g of chloroacetic acid were dissolved in 17.0 g of water, and 17.0 g of dextran were added in many small portions as in the procedure described above. The resulting solution was cooled in an ice bath to 6°C before starting the addition of 7.6 g of LiOH · H_2O. The very high viscosity slowed the addition of the alkali markedly, since mixing and solution were strongly affected. When the mixture became permanently alkaline, the bath was removed, about 110 mg of sodium borohydride was added, and the remaining LiOH · H_2O was dropped in. Stirring was continued to complete solution. This mixture was heated at 37.5°C for 48 h in the manner described above for the preparation of low-affinity CM-D. The light yellow product was diluted to 180 ml with partially deaerated deionized water and purified by the ion-exchange procedure described above. The purified acidic fraction was adjusted from pH 2.10 to 7.8 with 5.5 ml of 10.0 *M* NaOH. The final product was 211 ml of 11% CM-D having an RPV of 26.

Sample preparations

Normal guinea pig serum was dialyzed against 10 m*M* sodium phosphate, pH 7.0.

Mouse liver cytosol was prepared from livers perfused with 10 ml of phosphate-buffered saline, each. Ten livers were homogenized with a Potter-Elvehjem homogenizer in an equal volume of Littlefield's medium (50 m*M* Tris · HCl, pH 7.2, 250 m*M* sucrose, 25 m*M* potassium chloride and 5 m*M* magnesium dichloride). The homogenate was filtered through a single layer of silk. The filtrate was centrifuged at 100 000 *g* for three hours in a Spinco Type 40 rotor. PMSF was added to 0.1 m*M*, and magnesium acetate was added to 200 m*M*. DNase I and RNase A were added to 20 μg/ml ad 50 μg/ml, respectively, and the pH adjusted to 7.3 with 0.5 *M* NaOH. The nucleic acid was digested overnight at room temperature. The protein-rich cytosol was dialyzed against the respective column buffer before use. A Lowry protein determination showed a protein concentration of 13 mg/ml.

Column chromatography

The anion-exchange columns were equilibrated in the appropriate buffers. The flow-rate was 10 ml/h for the serum separation and 5 ml/h for the cytosol chromatography and 2.5-ml fractions were collected in both experiments. The temperature was 5°C.

Gel electrophoresis

The non-denaturing buffering system of Davis [16] was used in a 35-place vertical slab (90 × 244 × 2 mm) of 7.5% acrylamide for evaluation of samples taken directly from the column fractions in the serum and liver cytosol experiments (40 and 70 μl, respectively). The proteins were stained with Coomassie Blue G-250 [17].

Analysis of *E. coli* lysate and purified *E. coli* alkaline phosphatase (see Fig. 3) was performed by so-

dium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide running gel with a 6% stacking gel [18].

RESULTS AND DISCUSSION

The first two thirds of the chromatographic separation illustrated in Fig. 1 (up to fraction 23) is a displacement of the most lightly bound proteins of the guinea pig serum sample within a frontal analysis of the very heterogeneous low-affinity CM-D preparation. Application of the final displacer resulted in the emergence in fraction 24 of a brief spike of both CM-D and protein having somewhat higher affinities, with loss of potential resolving power within that range. Adsorption of the very high affinity CM-D used as final displacer resulted in a displacement of counter ion such that the background buffer rose from 10 m*M* phosphate to 21 m*M* (not plotted), sufficient to eliminate the affinities of the components of fraction 24. The pH rose only slightly, to 7.27, and therefore did not contrib-

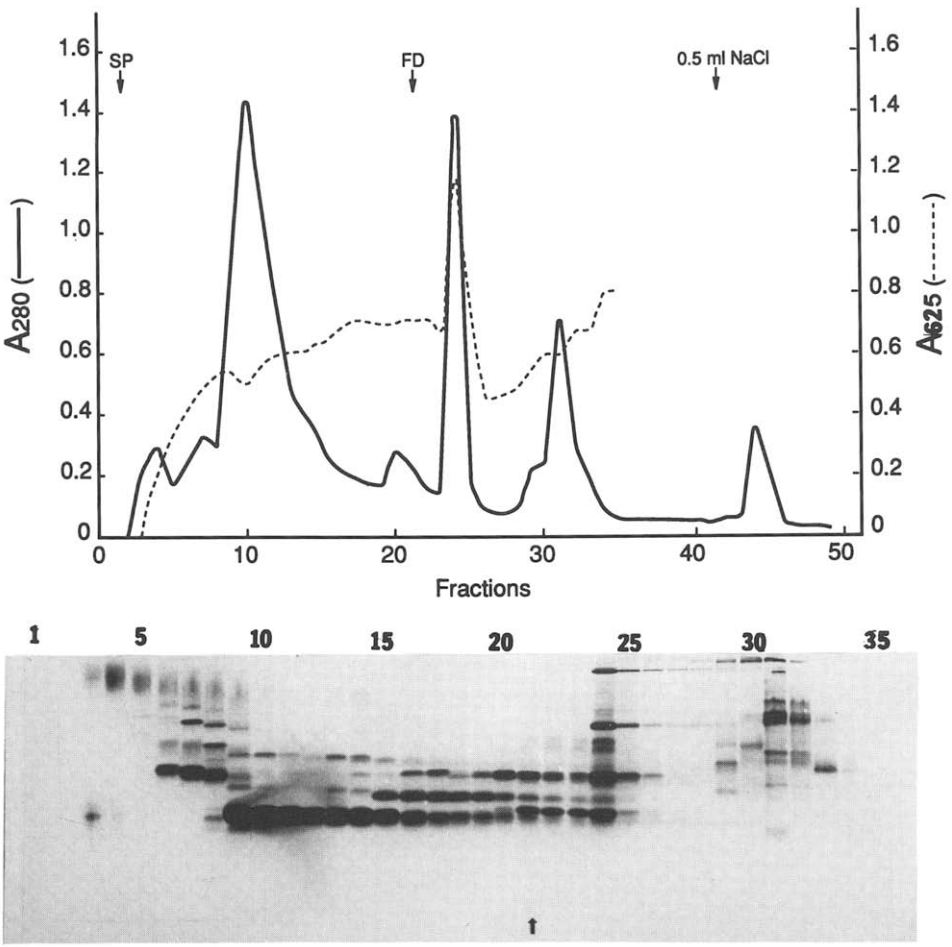

Fig. 1. Displacement chromatography of guinea pig serum on Fractogel DEAE 650S and electrophoretic evaluation of fractions. Sample, 1.1 ml, dialyzed against 10 m*M* sodium phosphate, pH 7.0, was pumped into the bottom of a 7-ml column equilibrated with the same buffer. Displacers: 51 ml of 0.6% solution of spacer CM-D (RPV = 7) and 50 ml of a 1% solution of the final displacer (FD) (RPV = 26), both in the starting buffer. Heavy line represents absorbance at 280 nm, light line the absorbance at 625 nm after reaction of 10-μl samples with anthrone [23] as a measure of CM-D. For electrophoresis, 40-μl samples were taken directly from the fractions. The point at which FD was applied is marked by the arrow below the electrophoretic pattern of fraction 21.

ute to the desorption of the proteins in fraction 24. Thereafter, the final displacer, much more homogeneous with respect to affinity than the spacer CM-D, drove a classical displacement train comprising the higher affinity components of the serum sample and the spacer CM-D. Finally, the adsorbent was stripped of CM-D by passing 20 ml of 0.5 *M* NaCl through the column. This CM-D emerged with the salt front in fractions 45 and 46.

Examination of the bands obtained by electrophoresis shows that individual protein species generally emerged in one or two fractions. Some protein classifications were too heterogeneous to permit such clear resolution: γ-globulins (upper left), in which a progressive increase in electrophoretic mobility revealed their well known extreme heterogeneity; the transferrins (starting in fraction 6), which appear to be more easily separated chromatographically than electrophoretically under these conditions; albumin (starting at fraction 8), which has long been known to be chromatographically heterogeneous in its untreated form because of its ability to bind many low-molecular-weight substances such as fatty acids and other lipids; and albumin

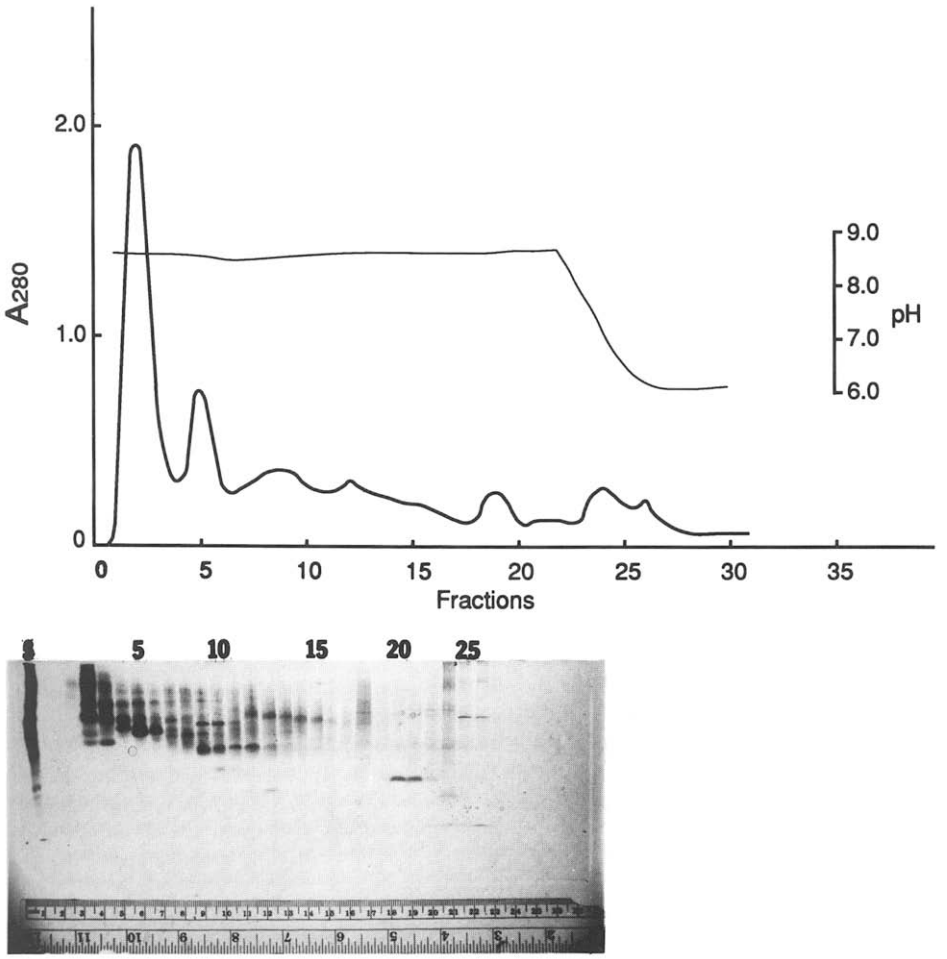

Fig. 2. Displacement chromatography of mouse liver cytosol on DEAE-cellulose and electrophoretic evaluation of fractions. Sample, 2 ml, dialyzed against 40 m*M* Tris–5 m*M* phosphate, pH 8.6, was applied to a 3.6-ml column of DE-52 equilibrated with the same buffer. Displacers: six 6-ml portions of 1% solutions of six narrow range CM-Ds [19] increasing progressively in RPV from 5 to 15 and decreasing progressively in pH from 7.5 to 6.5, all containing 5 m*M* phosphate, were applied successively and followed with 35 ml of an 0.8% solution of CM-D having an RPV of 26 in 5 m*M* Tris phosphate pH 5.9.

dimer (starting at fraction 15). The extreme heterogeneity of serum protein is evident, and most fractions contain several protein species that have affinities, under these conditions, that are too close for their resolution to be achieved in this system. However, rechromatography of appropriate fractions with the same highly heterogenous displacers at another pH is likely to improve the separation. Group specific globulin, a vitamin D binding protein, for example, was highly purified from human serum by two-step displacement chromatography at pH 7.1 and 8.1 [12].

In work to be published elsewhere, samples of human serum as large as 10 ml (about 600 mg of protein) have been applied to the 7-ml column used here, resulting in the rapid emergence of gammaglobulin, transferrin, and most of the albumin in an unresolved fore peak, leaving proportionately greater amounts of the higher affinity proteins on the column to be separated by displacement within a frontal analysis of a single spacer CM-D solution in a manner analogous to that employed here for the low-affinity serum proteins.

The experiment shown in Fig. 2 provides another example of the resolution by displacement chromatography of a complex natural mixture of proteins, intracellular in this case. Here the small size of the column (3.6 ml) was partly compensated for by the use of six narrow-range CM-Ds [19] applied in succession so as to approximate a partially developed displacement train before they entered the column. This situation is very different from the frontal analysis of heterogeneous CM-D in Fig. 1, where all the constituents of the spacer CM-D entered the column simultaneously until the final displacer was applied. Although dominating heterogeneous classifications such as the γ-globulins, transferrin and albumin of serum are less evident in mouse liver cytosol, the array of proteins presents a formidable problem in resolution. Examination of the gel shows that high- and low-molecular-weight proteins are highly resolved, often in a couple of fractions. In this experiment, a progressive decrease in pH incorporated into the CM-D schedule was buffered by the adsorbent bed (equilibrated at pH 8.6) and did not begin to emerge until fraction 22. Under other conditions of pH and salt a different picture can be expected, and when the objective is the isolation of a single species, poor resolution among other proteins can be ignored.

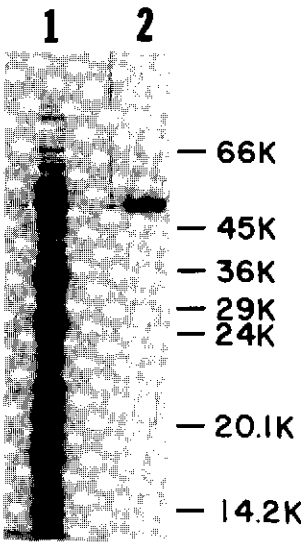

Fig. 3. Silver-stained SDS-PAGE analysis of *E. coli* periplasma sample containing alkaline phosphatase (lane 1) and purified alkaline phosphatase monomer (lane 2). The protein was purified as a dimer of molecular weight 94 000, but examined here in the reduced state. Numbers at the right represent relative positions of molecular weight markers in kilodaltons (K).

The analysis of the *E. coli* periplasma sample and purified alkaline phosphatase is shown in lanes 1 and 2, respectively of Fig. 3. This purification of alkaline phosphatase was previously reported by Dunn *et al.* [13] and is not described here except to demonstrate briefly the purification of a single protein from a complex mixture. The resolution of most of the periplasm mixture was ignored in this case in order to achieve the purification of alkaline phosphatase. Only two peaks were displaced from the column: the first containing alkaline phosphatase with two minor components, and the second containing numerous proteins. The minor contaminants were removed by anion-exchange elution chromatography, using a shallow gradient.

The fullest realization of the high capacity inherent in displacement chromatography would be achieved if protein–protein displacement could be made the basis of the separation. However, most of the proteins in biological mixtures are minor constituents surrounded by other minor proteins, and in a displacement train comprised only of proteins, each of these would occupy a volume so small that neighboring and otherwise separable proteins

would be remixed in the column and appear in the effluent with very little separation. Greatly reducing the concentration of the final displacer would reduce the mixing by increasing the volumes of all the bands, but they would remain contiguous. Moreover, one cannot yet assume that all proteins can participate effectively in protein–protein displacement. The model mixtures used for protein–protein displacement studies have been limited to proteins of relatively low molecular weight. From the earliest experiments involving the chromatography of proteins on cellulosic ion-exchangers [20], it has generally been observed that when biological mixtures containing colored adsorbable proteins are applied to adsorbents (*e.g.* fibrous DEAE-cellulose) that offer more or less uniform access to proteins of essentially all molecular sizes, the adsorbed proteins immediately form a series of colored bands that widen and migrate the accommodate the increasing widths of the bands behind them as additional sample enters the column. However, an attempt to drive such a train of human serum proteins through a column by applying a high-affinity displacer without spacers resulted in very poor resolution [10], in part because the fractions collected were too large to preserve whatever separation survived remixing in the column.

The application of suitable intermediate-affinity displacers subsequent to the application of the sample does not reduce the amount of sample that can be applied to the column, but it does greatly improve the spacing of the numerous minor species. Also, the passage of low concentrations of such displacers through the developing train on their way to appropriate positions ahead can be expected to increase the rate at which the higher-affinity proteins attain their appropriate positions in the later portions of the train. When the adsorbent employed does not provide uniform access for all the proteins to potential adsorption sites (*i.e.*, some degree of molecular sieving occurs), the presence of displacing molecules of intermediate affinity and relatively low molecular weight prevents the retention of low-molecular-weight proteins within the inner recesses of the adsorbent, away from competition with higher-affinity proteins of higher molecular weight.

The use of displacers having intermediate affinities for spacing does, of course, increase the volume of the effluent. However, much more can be achieved in a single pass, and when only one protein species is being isolated, the added spacer can be limited to the region of the train containing that protein, with reliance on protein–protein displacement in the rest of the train and even sharp elution with salt after the desired protein has been collected. Generally, displacement chromatography would be the first chromatographic step in the isolation of the product, so subsequent fractionation to attain final purity provides opportunity for the removal of spacing displacers, whether by ion exchange [12], hydrophobic interaction [22], molecular sieving, affinity chromatography or electrophoresis.

The very fine, uniform beads and compact packing of HPLC columns are very helpful in small-scale displacement separations for maintaining the sharp boundaries that are developed in the trains [21]. For example, *E. coli* alkaline phosphatase was purified on an HPLC DEAE-5PW column with excellent results. However, the self sharpening effect of a displacement train allows high resolution on a relatively low-resolution adsorbents. Molecular forms of ovalbumin which differ by 0.1 pH units in their isoelectric points have been separated on DE-52 [19]. In this manuscript we have demonstrated the separation of guinea pig serum on a medium-resolution beaded adsorbent (DEAE-Fractogel), and the separation of mouse liver cytosol on a low-resolution microgranular cellulose (DE-52). All three of these separations showed high resolution as demonstrated by the electrophoretic evaluation of fractions. It has been our experience that once the chromatographic conditions are established, the columns are interchangeable. For example, the β-lactoglobulins A and B were separated by the same spacer displacer on low-pressure anion-exchange columns [19] and HPLC anion-exchangers [21]. General conditions for preparative columns can thus be worked out rapidly on HPLC columns. In large-scale separations relatively inexpensive beaded adsorbents such as those now available for chromatography at moderate pressure should be very effective because the much larger volumes of the developed protein bands will make any effects of the increase in the size of the beads less significant. The close packing that is possible with beaded adsorbents remains important in large-scale work since it minimizes the interstitial volume, thus reducing

mixing at the boundaries. On the other hand, separation problems vary so widely in the challenges they impose that it is likely that in some cases non-beaded adsorbents will be effectively employed and in others the full power of HPLC will be required.

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