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INTERACTIONS OF PROTEINS WITH BONDED-PHASE ION EXCHANGERS

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SUMMARY

The ion-exchange properties of several commercial bonded-phase supports were studied to determine their applicability for protein separations. Because of the complexity and multiple functionality of protein molecules, UV-absorbing ions with unit charges were used initially to probe ion-exchange properties. The anion exchangers studied showed ambiguous properties, but elution of an organic cation from a pellicular cation exchanger was consistent with ion-exchange theory. With proteins, however, contradictions were found. The elution of reference proteins from the cation exchanger showed that mixed mechanisms occurred. For example, bovine serum albumin and ovalbumin were retained at a pH above their isoionic points, and their retention times increased rather than decreased with increasing eluent molarity. The mixed mechanisms and the lower diffusivities of protein led to higher heights equivalent to a theoretical plate. Nevertheless, mixtures of reference proteins and protein isolates were resolved.

INTRODUCTION

Ion-exchange chromatography has been used for many years to separate and isolate proteins. Recently ionogenic groups have been covalently bound to the surface of microparticles of silica or to thin films of porous material which surrounds a solid core. These materials are chemically stable, can withstand large pressure drops and permit faster and more efficient ion-exchange separations by promoting mass transfer. The materials offer great potential for the separation of important biomacromolecules, and some separations of proteins have been reported[1,2]. The purpose of the present research is to observe the behavior of standard proteins and other probes on selected ion-exchange supports in order to obtain information which will lead to development of protein separations on a rational basis.

THEORETICAL

An expression for the chromatographic distribution coefficient, K, has been derived[3] and shown to be applicable for elution of organic cations from silica-based

ion exchangers[4]. The expression for the distribution of an anionic solute between mobile phase (M) and support (S) is

$$K^{A} = \frac{K_{EI}[EI]_S^Z K_{eq}}{[EI]_M^Z (K_{eq} + [H^+]_M^Z)} = \frac{[A^Z]_S}{[A^Z]_M + [C_ZA]} \quad (1)$$

while for a cationic solute, the expression is

$$K^{C} = \frac{K_{EI}[EI]_S^Z}{[EI]_M^Z \left(1 + \frac{K_{eq}}{[H^+]_M^Z}\right)} = \frac{[C^Z]_S}{[C^Z]_M + [CA_Z]_M} \quad (2)$$

where K_{EI} is equilibrium constant for the ion-exchange process, $K_{EI} = [EI]_M^Z [A^Z \text{ or } C^Z]_S / [EI]_S^Z [A^Z \text{ or } C^Z]_M$; $[EI]_M$ is the concentration of univalent counterion in the mobile (eluent) or stationary phase; K_{eq} is the dissociation constant of the solute; and Z is the absolute charge on the dissociated solute species. Under conditions of chromatography, virtually all sites on the support have counterions so that $[EI]_S$ is the capacity of the support. K_{EI} and K_{eq} may also be considered constant under chromatographic conditions. Also $V_R = V_M + KV_S$ where V_R is the retention volume, V_M is the void volume, V_S is the stationary phase volume and K is K^C or K^A. These expressions show that in ion-exchange increasing concentration of counterion in the mobile phase decreases retention, and increasing pH diminishes retention of cationic solutes and increases retention of anionic solutes. For polyvalent solutes, the effects are more pronounced, since $[H^+]_M$ and $[EI]_M$ are raised to the absolute power of the charge on the solute.

K_{eq} cannot be calculated in a straightforward manner for proteins because they contain large numbers of different ionizable species, including carboxyl, amino, imidazole, phenol, sulfhydryl and guanidyl groups. Their dissociation is influenced by the number and location of other groups on the molecule and by the conformation of the protein[5]. K_{eq} in eqns. 1 and 2 is therefore a net value. A typical protein titration is shown schematically in Fig. 1[6]. This protein has over 180 ionizable groups. At the isoinic point (pI) the number of positive charges equals

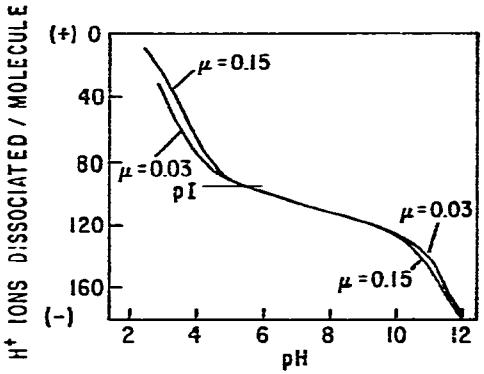

Fig. 1. Schematic of hydrogen ion titration curve of protein.

the number of negative ones. At $[H^+]$ below this, the protein has a net negative charge but still contains a number of positively charged groups except at very high pH values. The concentration of salt alters the charge at a given pH. Complete data for many proteins may be found in the literature[7–9]. These properties of proteins could alter their behavior in ion-exchange systems.

EXPERIMENTAL

Chromatography was performed with a dual Waters* Model 6000-A solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.), a Waters Model 6UK injector and a Perkin-Elmer LC-55 variable wavelength spectrophotometer (Perkin-Elmer, Norwalk, Conn., U.S.A.) operated at 280 nm. The standard 2-ml injector loop was replaced with a 200-μl loop to reduce the lag time between the solvent composition change at the pump and the change at the head of the column during gradient runs. Stainless-steel fittings and tubing were used throughout the system. Connections between columns and detector were of capillary tubing (0.16 cm O.D., 0.02 cm I.D.) to minimize dead volume.

Buffers were prepared with deionized, distilled water and reagent-grade chemicals. Reference proteins were obtained from either Polysciences (Warrington, Pa., U.S.A.) or Sigma (St. Louis, Mo., U.S.A.) and used without further purification. Alfalfa proteins were isolated from leaves as described previously[10]. *p*-Aminobenzoic acid (PABA) was obtained from Matheson, Coleman & Bell (East Rutherford, N.J., U.S.A.) and the *o*-nitrobenzoic acid (ONBA) from Aldrich (Metuchen, N.J., U.S.A.). All samples were dissolved in buffer and passed through a bacteriological filter before use. Samples for chromatography were prepared fresh daily.

Column 1 was assembled from 316 stainless-steel tubing (93 cm × 4.2 mm I.D.) which had been cut to the desired length, thoroughly washed, flushed with acetone, then hexane, and dried under nitrogen. A fritted disk was countersunk into the outlet end of the tubing, and the column was packed by the tap-fill method. The support was Wax (Dupont, Wilmington, Del., U.S.A.), stated to function as a pellicular weak anion exchanger at pH < 4. Particle size was 37–50 μm.

Column 2 consisted of Glycophase DEAE/CPG (Corning, Medfield, Mass., U.S.A.), a research sample obtained from Pierce (Rockford, Ill., U.S.A.), and slurry packed under high pressure into a 25 cm × 4.2 mm I.D. column. The packing surface contained diethylaminoethyl functional groups, with ion-exchange capacity cited to be 40 mg hemoglobin per ml. Particle size of this material ranged from 5 to 10 μm with an average pore diameter of 250 Å. By analogy with DEAE-Sephadex (pK_a 9.7)[11], this material should function as an anion exchanger at the hydrogen ion concentrations used in this study.

Columns 3 and 4 were both commercially packed by their manufacturers. The third column was a Partisil PXS-1025 SAX column obtained from Whatman (Clifton, N.J., U.S.A.). This analytical column was a microparticulate strong anion exchanger in which quaternary nitrogen, $-NR_3$, functional groups are bonded to the

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

surface hydroxyls of Partisil-10 silica gel. Column dimensions are 25 cm × 4.6 mm I.D., and its operable range is from pH 1.5 to pH 7.5. The 10-μm packing material had a high surface area of 400 m^2/g. Column 4 was a strong cation exchanger, a DuPont Zipax SCX column[12]. The support material contained *ca.* 1% by weight of a sulphonated fluorocarbon polymer coated on Zipax pellicular silica support. Its dimensions are 100 cm × 2.1 mm I.D., and its operable range is from pH 2 to 9. This cation-exchange packing is reported to exhibit an exchange capacity of 3.5 μequiv./g.

The presence of protein in chromatographic peaks was determined by modification of the Bradford[13] dye-binding test for proteins. Test reagent was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Approximately 2 ml of dilute reagent (one part concentrate to four parts water) is added to 3 ml of effluent collected at the detector outlet. Presence of protein is indicated by a distinct blue color after the reagent has been added. Precautions should be taken to avoid using eluents which give false positive tests. False positive tests were observed for those eluents containing alcohol or acetone, those with high concentrations of salts such as sulfate, and those which were very basic. For organic solvents, such as methanol, removal of solvent can easily be accomplished by evaporation with nitrogen gas prior to addition of Bradford reagent.

RESULTS AND DISCUSSION

Ion-exchange properties of supports

Because of the complex and multiple functionality of protein molecules, UV-absorbing ions with unit charges were used initially to probe the ion-exchange properties of four commercial high-performance liquid chromatography (HPLC) supports. Elution volume (V_R) of ONBA from the weak anion exchanger (column 1) decreased as salt concentration ($[El]_M$) was increased at constant pH (Fig. 2) as predicted by eqn. 1. However, elution volume decreased as pH was increased over the range of 2.5–4. This likely resulted from the dissociation of positively charged groups on the support so that the capacity ($[El]_S$) term in eqn. 1 was reduced as pH

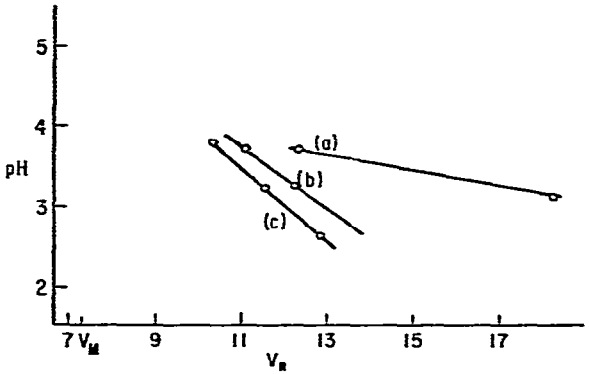

Fig. 2. Effects of pH and salt concentration on elution of *o*-nitrobenzoic acid (pK_a 2.2) from weak anion exchanger (column 1). Mobile phase: 0.01 *M* citrate buffer (0.02% NaN_3) (a); 0.01 *M* $NaNO_3$ added (b); 0.02 *M* $NaNO_3$ added (c). 1 μg injected in 1 μl mobile phase. Flow-rate 2 ml min^{-1}.

was raised, bringing about the concomitant decrease in V_R. Reduction of V_R of ONBA with increasing pH (2–4 range) was also shown by the commercial DEAE-glycophase support (column 2), although numerous applications have been reported on similar materials prepared by the inventor[1]. Surprisingly, the strong anion exchanger (column 3) showed the same trend over a pH 3–5 range and showed no activity at pH 5. None of these anion exchangers seemed appropriate for the further study of protein separations, since they did not demonstrate properties consistent with normal ion-exchange supports and optimization would have to be empirical rather than based on established principles.

The elution of PABA from a pellicular cation support (column 4) followed the patterns predicted by eqn. 2 and the elution equation (Fig. 3). This provided a basis on which to pursue studies of protein behavior.

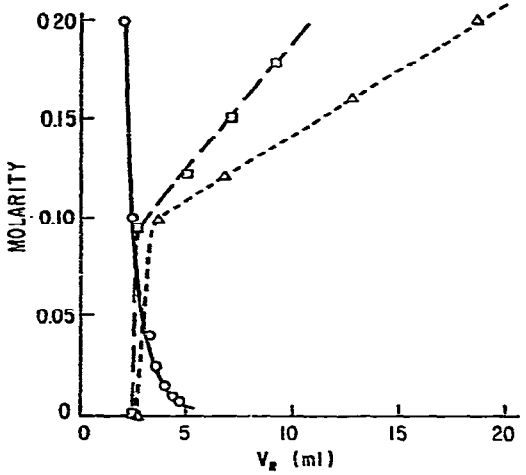

Fig. 3. Elution of *p*-aminobenzoic acid (pK_{a1} 2.5) (○), ovalbumin (□) and bovine serum albumin (△) from pellicular cation exchanger (column 4). Mobile phases: ○, 0.01 *M* citrate buffer (0.02% NaN_3) + $NaNO_3$ (pH 2.5) 20 μg injected in 10 μl mobile phase, flow 1 ml min^{-1}; for proteins, 0.01 *M* citrate (0.02% NaN_3) + $(NH_4)_2SO_4$ (pH 6), 400 μg injected in 200 μl mobile phase. Flow-rate 0.5 ml min^{-1}.

Elution of proteins from cation exchanger

Effect of pH. In general, increasing the pH of the 0.01 *M* citrate buffer decreased retention volume of the model proteins studied. For example, in the absence of NH_4Cl, ribonuclease (RNase, p*I* = 9.4)[7], chymotrypsinogen (CHTG, p*I* 9.3)[8], bovine serum albumin (BSA, p*I* 4.8)[6] and ovalbumin (OVAL, p*I* 4.6)[9] were all retained at pH 4.8 while the latter pair were eluted near the void volume at pH values of 5.3 and 6. At these pH values, the proteins of higher p*I* were not eluted. Data for OVAL and BSA are shown for several salt concentrations in Fig. 4. Large effects due to changes in pH are expected since the $[H^+]$ term in eqn. 2 is to the absolute power of the charge on the solute.

Effect of eluent concentration. Figs. 3 and 4 also demonstrate that above the isoionic point increasing eluent ion concentration increased retention, contrary to what is predicted by eqn. 2. The increase in net positive charges with increasing

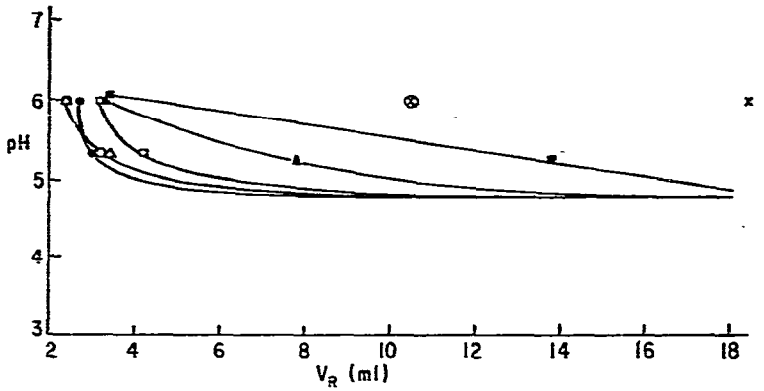

Fig. 4. Effect of pH on elution of ovalbumin (open symbols) and bovine serum albumin (closed symbols) from pellicular cation exchanger (column 4). [NH_4Cl]: ○, no salt added; △, 0.05 *M*; □, 0.01 *M*; X, 0.2 *M*. Proteins were not eluted below pH 4.8 at these concentrations of added salts.

salt concentration (Fig. 1) cannot be considered as an explanation of this retention, since at pH 6, the charge of neither BSA[6] nor OVAL[9] is appreciably affected by salt concentration. A more plausible explanation is that hydrophobic interactions between protein and the support which contains sulfonated alkyl groups are enhanced by increasing salt concentration. The increased retention in reversed phase chromatography due to increased salt concentration has been predicted and observed[14,15]. As pH is lowered, the effect of the charge (*Z*) predominates, although examination of the plots of retention as a function of salt concentration at pH 3.9 indicates that mixed mechanisms occur (Fig. 5). At 0.445 *M* salt RNase eluted near the void volume. A decrease in molarity of NH_4Cl of only 0.005 caused it to be

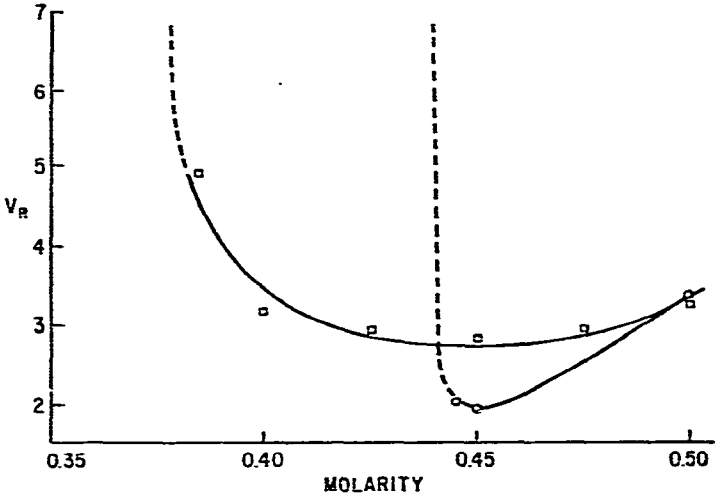

Fig. 5. Elution of ribonuclease (○) and chymotrypsinogen (□) at pH 3.9 (column 4). Mobile phase: 0.01 *M* citrate buffer (0.02% NaN_3) + NH_4Cl. Dashed portions indicate molarities where protein confirmations become indefinite and peak area loss becomes significant. 200 μg injected in 200 μl mobile phase. Flow-rate: 1 ml min^{-1}.

strongly retained. Increase in salt above 0.45 *M* brought about a slight increase in retention. Similar results were obtained for chymotrypsinogen. At lower salt concentrations, the ion exchange effect predominated, *i.e.*, a small change in salt concentration produced a very large effect on retention. Over the range 0.40–0.50 *M*, there was little variation. Experiments were not carried out above 0.5 *M* because of concerns about the reactivity of metal components of the chromatograph with chloride at this pH. BSA was not eluted under these conditions because of the high net positive charge. This protein eluted at the void volume at pH 7.5 with salt concentrations up to 0.5 *M*. At this pH, BSA has a high net negative charge.

The data in Fig. 5 include only those peaks which gave positive protein tests. The presence of protein in the chromatographic peaks was confirmed by a dye-binding test which produced a distinctive blue color. As salt concentration was decreased below a concentration characteristic for each protein, the peak area diminished greatly and the dye-binding tests became inconclusive. The salt concentrations where the decrease became significant are indicated by broken lines in Fig. 5. This suggests a nonlinear sorption isotherm. Eluents containing ammonium sulfate produced similar trends, but they were quantitatively different. The influence of NH_4Cl or $(NH_4)_2SO_4$ in the buffer on retention of two proteins is depicted in Fig. 6. At the same molarity of NH_4^+ ion, the chloride enhanced retention more than the sulfate. The binding of various ions by different proteins has been the subject of wide investigation and may, in part, explain these observations[5].

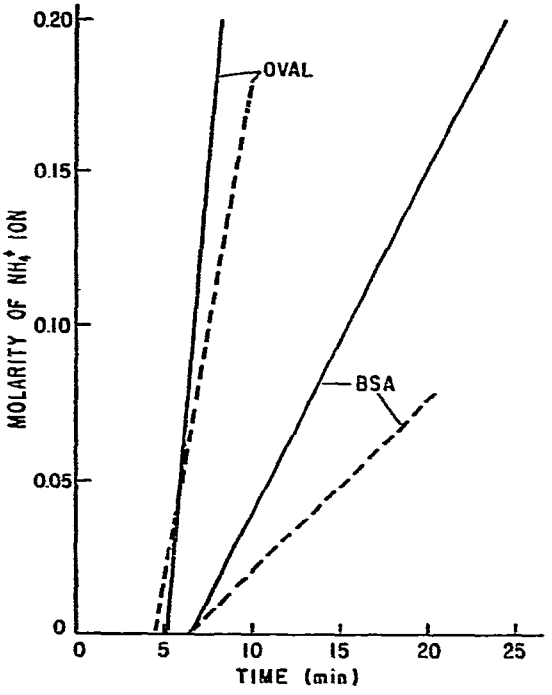

Fig. 6. Effect of salt on elution (column 4): ——, $(NH_4)_2SO_4$; – – –, NH_4Cl. Mobile phase: 0.01 *M* citrate (0.02% NaN_3) + salt (pH 5.3). 200 μg injected in 200 μl mobile phase. Flow-rate: 1 ml min^{-1}.

Relationship to separations

Studies such as those in the previous sections provide information that is required to develop methods for the separation of proteins. Traditional concepts for small molecules do not necessarily apply. Separation of RNase, OVAL and BSA was obtained at pH 5.3 with a stepwise elution starting with 0.5 *M* NH_4Cl and ending with 0.0 *M* (Fig. 7). RNase is eluted first, even though the pH is well below its isoionic point and above that of the other two components. In separate experiments CHTG and hemoglobin were found to coelute with RNase at the initial mobile phase composition, the pH of which is also below their isoionic points. The 0.5 *M* salt brings about elution of RNase by an ion-exchange mechanism but causes retention of the other proteins, most probably by hydrophobic mechanisms. Therefore, reduced salt levels are needed for their elution. Obviously, under these conditions, order is not related to isoionic point. OVAL and BSA elute in the order of hydrophobicity found by Albertsson[16] and Hofstee and Otillio[17] (BSA > OVAL).

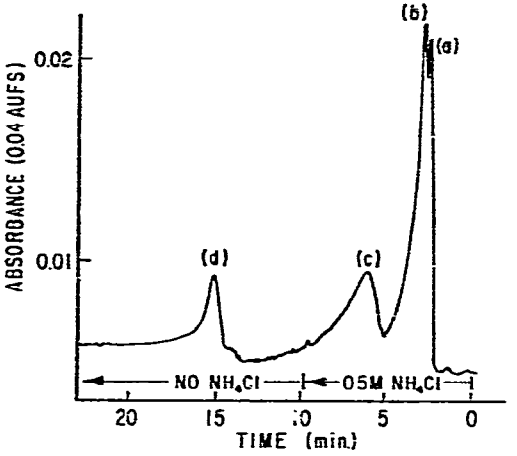

Fig. 7. Separations of proteins on pellicular cation exchanger (column 4): a, non-protein; b, RNase (66 μg); c, OVAL (66 μg); d, BSA (134 μg). Mobile phase: step elution, 0.01 *M* citrate (0.02% NaN_3) + 0.5 *M* NH_4Cl (pH 5.3) for 10 min, then 0.01 *M* citrate (0.02% NaN_3) without NH_4Cl (pH 5.3). 200 μl of mix injected. Flow-rate: 1 ml min^{-1}.

Chromatography of RNase and CHTG at pH 3.9 produces some interesting results. For example, examination of Fig. 5 shows that a constant salt concentration of 0.45 *M* RNase would elute first; however, it would follow CHTG if a gradient from 0.40 *M* to 0.45 *M* were used.

Practical aspects of column preservation are influenced by the behavior of proteins revealed in these studies. No single pH or ionic strength condition would purge the column. Combinations of both high and low concentrations of salt-containing buffers are needed.

The heights equivalent to a theoretical plate (HETPs) calculated from the chromatograms of proteins were an order of magnitude or more higher than those calculated for PABA at the same flow, and there was more skew to the protein peaks.

Several factors may influence this difference. The diffusivities of proteins are about one-tenth that of PABA, so increased band spreading is predicted from mass transfer considerations[18]. As discussed earlier, proteins contain a large number of different types of groups which can interact with the support. For ion exchange alone, K_{eq} in eqn. 2 is a composite value, each type of positively charged group having its own energy of binding to the negatively charged support. When mixed mechanisms occur, the energetics of interaction are more complex. Giddings[19] described how non-homogeneous binding leads to increased band spreading.

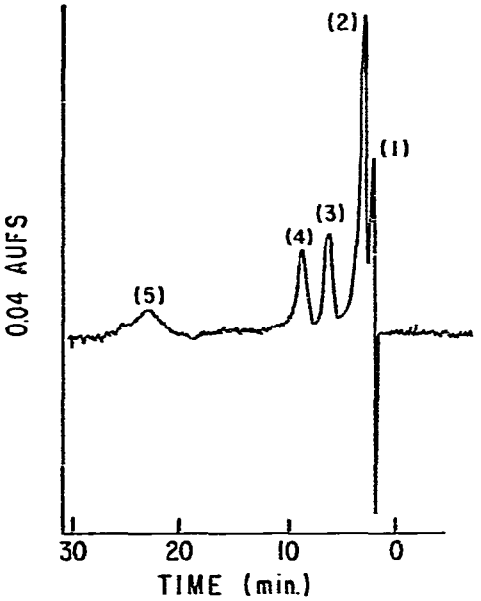

Fig. 8. Separation of an alfalfa leaf protein isolate on pellicular cation exchanger (column 4). Mobile phase: 0.01 *M* citrate buffer + 0.05 *M* $NaNO_3$ (pH 2.9). Peaks 2, 4, 5 gave definite positive protein test; peak 3 slightly positive; peak 1 negative. 200 μg injected in 200 μl mobile phase. Flow-rate: 1 ml min^{-1}.

In spite of the difficulties, separations of natural mixtures of proteins may be achieved by use of the pellicular support. An example is the chromatogram obtained from the injection of a soluble protein fraction isolated from alfalfa leaves (Fig. 8). Four peaks were eluted which gave protein-positive tests. To optimize such separations and to be able to glean information about protein molecules from chromatographic data, more studies such as those in this report are needed.

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