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UNUSUAL FLOW BEHAVIOR AND LOSSES OF PROTEINS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY CAPILLARY TUBING

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SUMMARY

Several different types of experimental result point to a surface adsorption process when proteins are passed through narrow (0.25 mm I.D.) capillary tubing of the type used as connecting tubing in high-performance liquid chromatography. Injection-type experiments show highly erratic behavior in terms of peak shapes and heights in both high and low ionic strength aqueous mobile phases, with the most erratic behavior at low ionic strengths and relatively low flow-rates (*ca.* 100 μl/min). Flow-rate changes affect polymer loss both in injection experiments and in frontal analysis experiments. The polymer lost to the capillary surface is difficult to remove and, because of changes in hydrodynamic patterns, small amounts of deposited polymer apparently have profound effects upon the amount of polymer loss in subsequent experiments. We believe losses of the type described are responsible for a significant portion of polymer losses in protein and other macromolecules in liquid chromatography.

INTRODUCTION

Only after careful optimization through alteration of the mobile phase composition can one hope to obtain greater than 90–95% recovery of many biological macromolecule samples in most forms of liquid chromatography (LC). The reason for losses of these macromolecules usually has been attributed to adsorption of column packing material[1]. For most biological macromolecules, relatively high ionic strengths (0.1–0.5) are necessary for maximum recovery during chromatographic runs. However, for some proteins higher salt concentration causes larger losses[1]. Apparently each macromolecule requires a different mobile phase composition to minimize losses during LC. However, at least in one instance yields of *ca.* 100% protein recovery have been reported[2].

We have conducted experiments in which samples of various proteins and other macromolecules have been passed through stainless steel capillary tubing of the type used in high-performance liquid chromatography (HPLC) as connecting tubing between the sample injection valve and HPLC column or between the column and the detector. With a number of different mobile phase compositions significant polymer

losses occur and unusual chromatographic behavior is encountered in terms of peak shapes and reproducibility.

EXPERIMENTAL

The typical arrangement for our experiments is a conventional HPLC experimental arrangement except for the substitution of: (1) a 50–100 cm × 0.25 mm I.D. 316 stainless steel capillary in place of the packed HPLC column and (2) a Sage Model 220 syringe pump in place of the HPLC pump with a plastic 10-ml syringe holding the mobile phase.

Enzymes and proteins obtained from Worthington, Sigma, Pharmacia and Boeringer-Mannheim were of the highest purity available and were used without purification. Water was singly or triply distilled. Buffers were made from analytical reagent grade chemicals.

Scanning electron micrographs were taken on milled and gently water-rinsed 0.25 mm I.D. stainless steel capillary tubing in a Philips PSEM 501 scanning electron microscope. Specimens were mounted on an aluminum stub with conducting cement.

RESULTS AND DISCUSSION

Aqueous solutions of a number of proteins have been injected in plug flow into capillary tubing which is monitored at its outlet with a conventional HPLC detector for concentration of the injected protein as a function of time. The observed shapes of elution curves for low-molecular-weight species are in good agreement with theoretical predictions based upon the work of Taylor[3] and of Golay and Atwood[4]. However, for proteins the experimental elution curve shapes are often in sharp contrast with those predicted. From preliminary studies of such elution curve shapes, electron micrographs of capillary tubing, spectrophotometric studies of effluent, and frontal analysis curve shapes, we have come to the following tentative conclusions:

(1) Protein is being lost to the capillary wall in the form of a coating which tends to build up with successive injections and alters, sometimes markedly, the hydrodynamic flow properties of the capillary with each subsequent injection.

(2) The degree of protein loss is dependent upon the previous injection history and the nature of the inside surface of the capillary.

(3) The protein coating on the wall is a metastable substance which may dissolve back into solution very slowly, if at all, and is quite often very dificult to remove even by fairly drastic measures.

(4) Under many flow and solvent conditions aggregation of the protein occurs, often resulting in the formation of higher-molecular-weight dissolved species or of a suspended precipitate or colloid in the mobile phase. These species may dissolve or disaggregate after passage through the capillary and detector because of low shear forces after passage through the capillary.

(5) Protein losses from the mobile phase are dependent on the ionic strength and flow-rate of the mobile phase as well as the chemical nature of protein.

Some of our evidence for the above tentative conclusions follows.

Figs. 1 and 2 demonstrate the differences between the inside surfaces of capillary tubing which have and have not been exposed to multiple injections of protein so-

lutions. There are clearly visible macro (Fig. 1) and micro (Fig. 2) deposits in the tubing exposed to protein solutions. The pits in Fig. 2a are due to salt crystals formed when capillaries containing 1 M salt solution were allowed to dry. However, the unusual behavior reported below was only slightly changed by the presence or absence of these pits. The point of interest in Fig. 2b is the manner in which the icing-like polymer
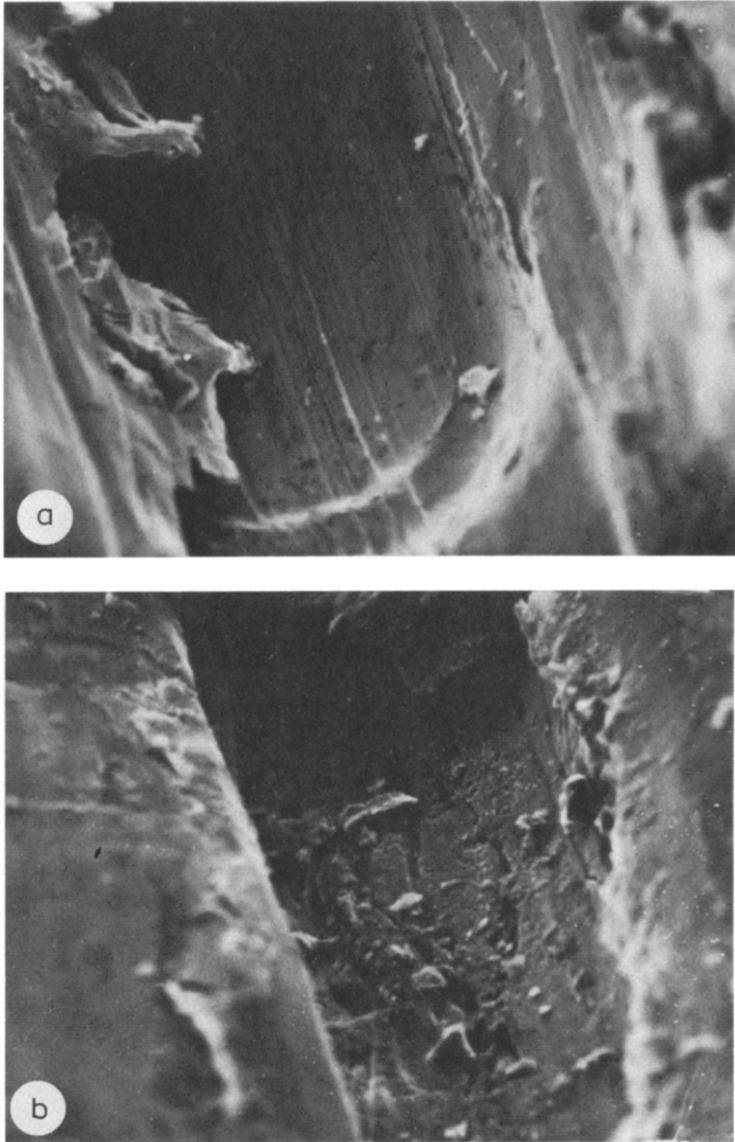

Fig. 1. Scanning electron micrographs (SEM) of inside surface of two stainless steel capillaries, one of which (a) was not exposed to protein solutions and the other (b) which was exposed to multiple injections of many different proteins in a variety of mobile phase compositions. Both capillaries were milled to expose the inside surface of the tube and were gently rinsed with distilled water in the same manner. Magnifications are: (a) ×387 and (b) ×319.5.
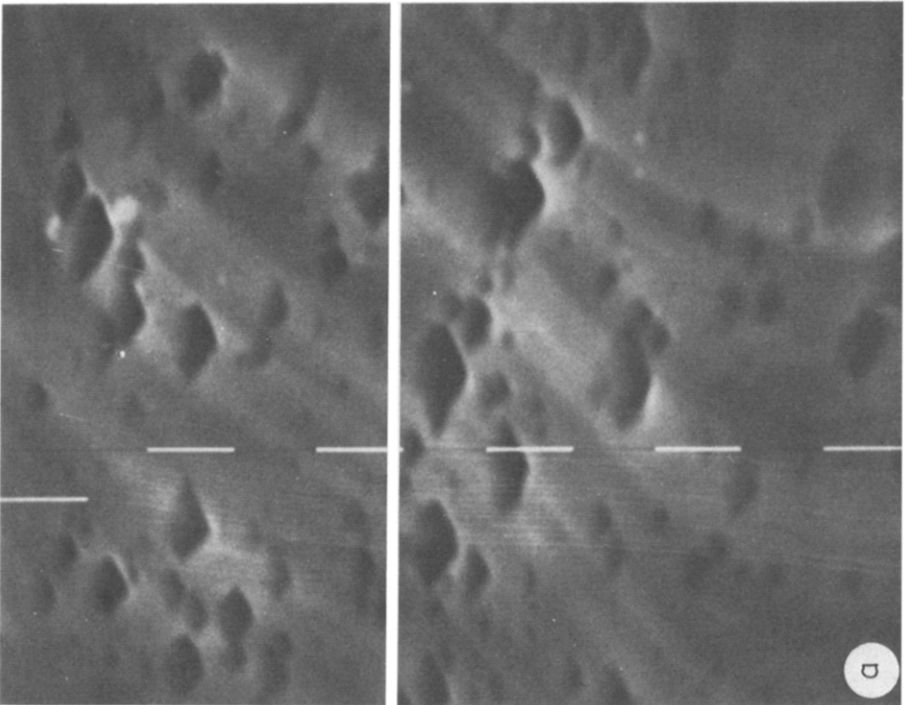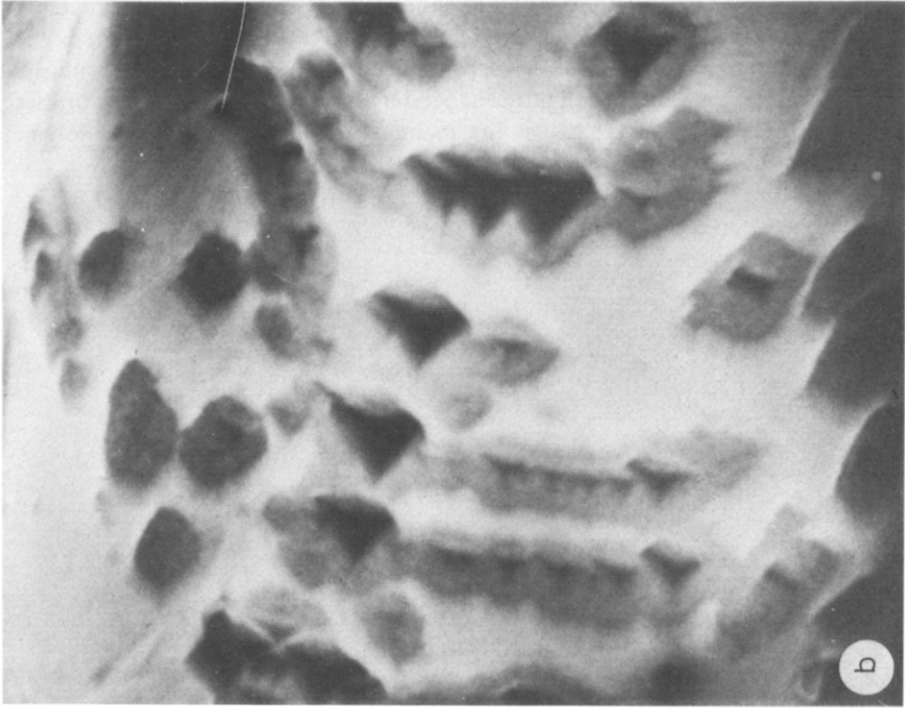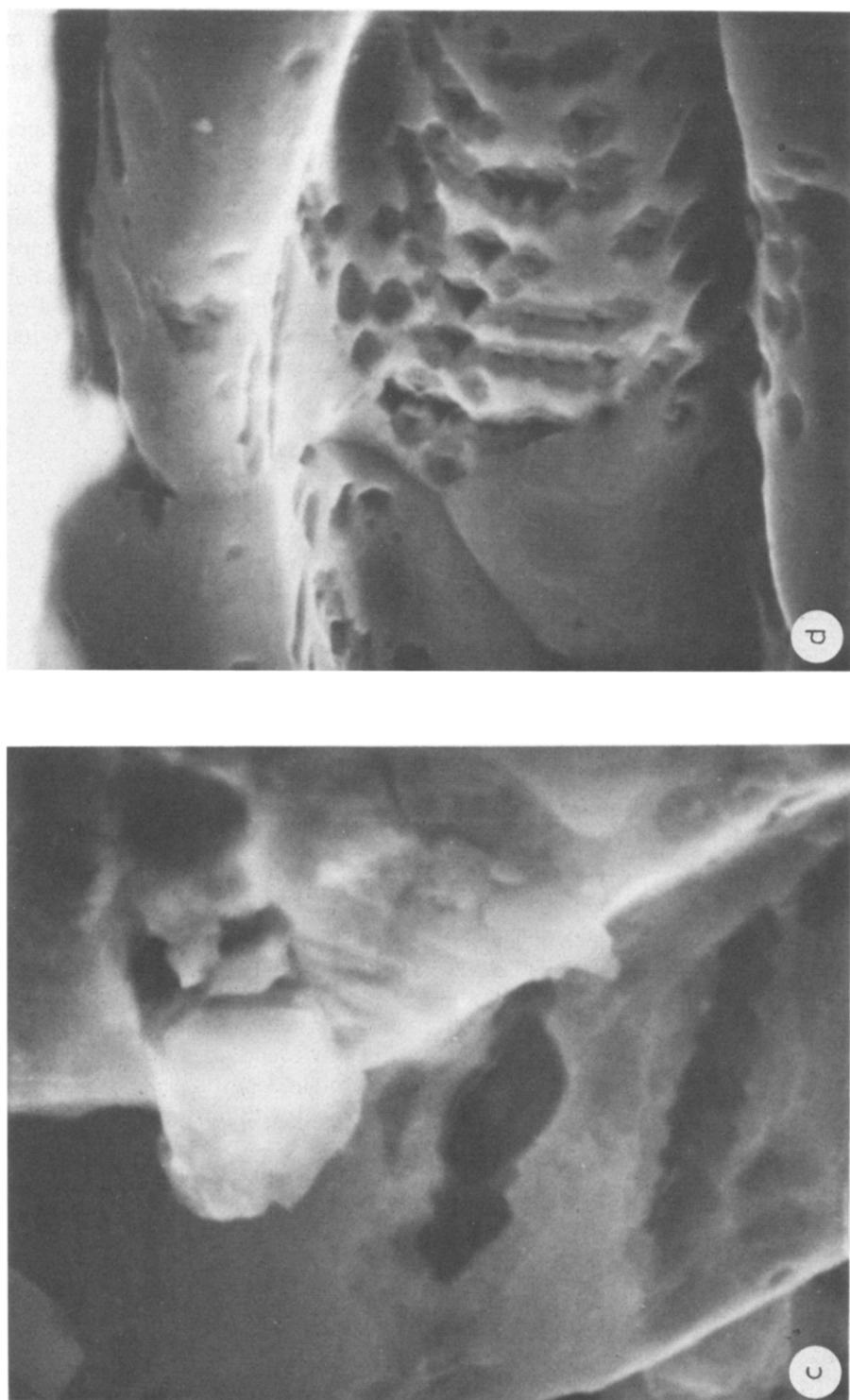
Fig. 2.

Fig. 2. High magnification SEMs of the inside surfaces of stainless steel capillary tubes which contained pits induced by evaporation of salt solutions: (a) tube exposed only to salt solution (×9000); (b) and (c) tube exposed to multiple injections of proteins (same as in Fig. 1b, ×9000); (d) same area as in (b) at lower magnification (×4500).

precipitate is absent from the region immediately surrounding the pits. We interpret this as an indication that a shear-induced aggregation phenomenon is minimized in regions of low shear near these pits. Aubert and Tirrell[5] and Metzner et al.[6] have demonstrated that polymers will selectively migrate toward zones of low shear.

Further evidence for a protein wall deposit is seen in the chromatogram shapes in Fig. 3. The mobile phase and sample buffer system was that used by Schmidt et al.[2] in their studies of protein retention on HPLC columns. Each successive injection of identical volume of a gamma globulin sample caused a different peak height and/or peak shape. After a series of rinses of the column with Triton X-100 the peak shape returned to approximately that observed at the beginning of the series of injections but again showed signs of degradation upon further injections, implying the removal or alteration of the polymer buildup pattern on the capillary wall by the Triton X-100 rinses.
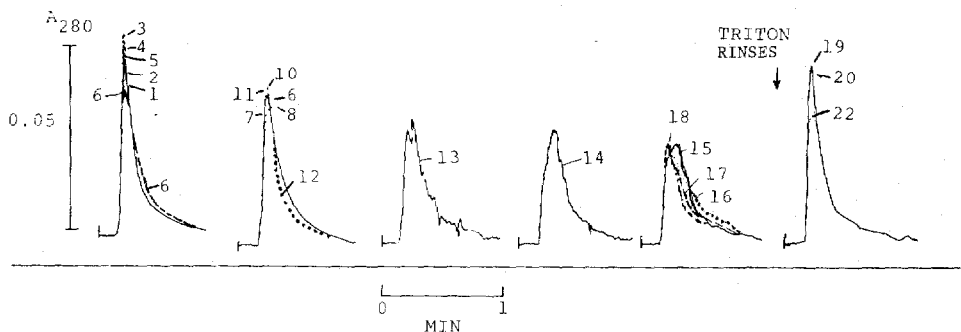

Fig. 3. Repeated injections of gamma globulin into capillary at high ionic strength. Column, direct injection into the ca. 15 cm × 0.25 mm I.D. lead in capillary tubing attached to the HPLC detector cell of the LKB Uvicord S UV monitor; mobile phase, 0.1 M sodium acetate, 0.1 M Na_2SO_4 (pH 5) buffer; flow-rate, 170 μl/min; samples, 22 successive 3-μl injections of γ-globulin (0.5 mg/ml) dissolved in mobile phase. Numbers (e.g. 1–6) indicate peak heights of peaks with similar shape as others in the same group.

Further evidence for buildup of a capillary surface coating is presented in Figs. 4 and 5. Fig. 4 illustrates a general buildup of a second peak in a series of ferritin solution injections into a capillary column. Given the flow-rate and column length employed, we predict from the work of Golay and Atwood[4] and Atwood and Golay[7] there should be double-peaked chromatograms observed under the experimental conditions for the data in Fig. 4. Apparently, such behavior is only found after the wall surface was altered by repeated injections and the resulting protein deposits. Note also in Fig. 4 the upward drift of the baseline. In another experiment with ferritin under slightly different conditions (Fig. 5) much more drastic behavior is observed. In a similar series of repeated injections of identical volume under identical flow-rate, hydrodynamic flow patterns are so markedly changed with each injection that the nature of the polymer buildup apparently alternately destroys and re-establishes the parabolic flow profile necessary for establishing the double peak. For another high-molecular-weight polymer, gamma globulin, a different pattern of peak shapes is observed in Fig. 6 again illustrating chromatograms resulting from a series of protein injections under identical conditions. Under the experimental conditions in Fig. 6, gamma globulin should give a double peak according to the Atwood and Golay theoretical plate analysis[7]. However,
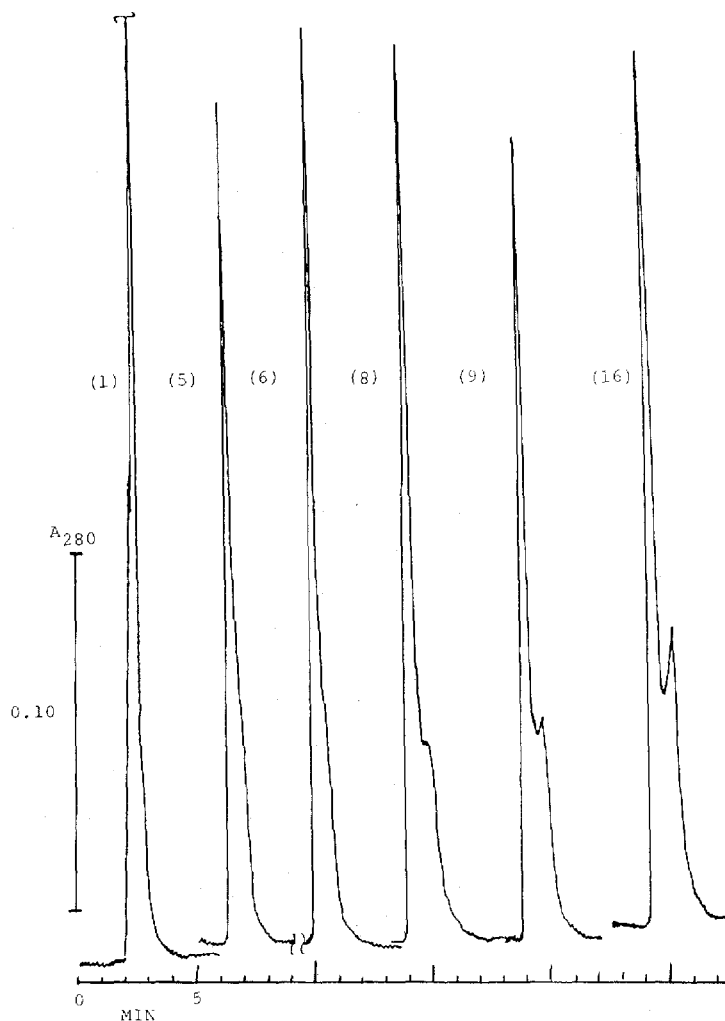
Fig. 4. Gradual transformation of peak shape upon repeated injection of an aqueous solution of ferritin. Column, 73 cm × 0.25 mm I.D., 316 stainless steel capillary corroded by drying after exposure to 1 M NaCl, resulting in a pitted surface; mobile phase, water; flow-rate, 68 μl/min; detector, UV (280 nm); samples, 16 successive 2-μl injections of a 0.25 mg/ml solution of ferritin (horse spleen) in water.

only a single peak of diminishing intensity is observed followed by an off-scale peak and then a continuation of the pattern of diminished peak heights.

Further evidence for protein losses from the mobile phase is implied by frontal analysis experiments, observations in which an interfacial zone between pure solvent (leading) and solution containing protein (following) passes through a chromatographic detector. The detector response would be a step function were it not for the parabolic flow profile of the interface. Instead, a rather sudden rise followed by an asymptotic approach to a plateau value of higher absorbance is expected for a sample absorbing at the detector wavelength. Figs. 7 and 8 illustrate the nature of successive frontal analy-
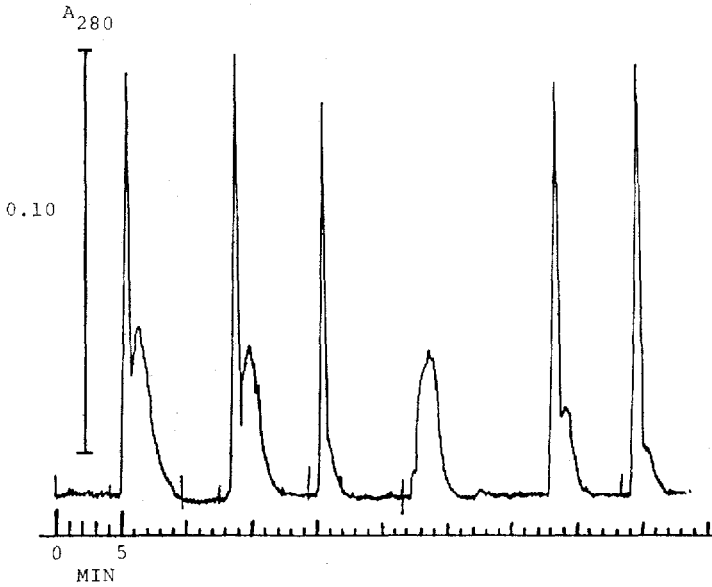
Fig. 5. Highly erratic and irreproducible chromatographic behavior resulting from six successive ferritin injections under identical conditions. Column, 72 cm × 0.25 mm I.D., 316 stainless steel capillary treated with 1 M NaCl and air dried (not the same column shown in Fig. 2); mobile phase, water; flow-rate, 68 μl/min; detector, UV (280 nm); samples, six successive 5-μl injections of a 0.13 mg/ml solution of ferritin in water.
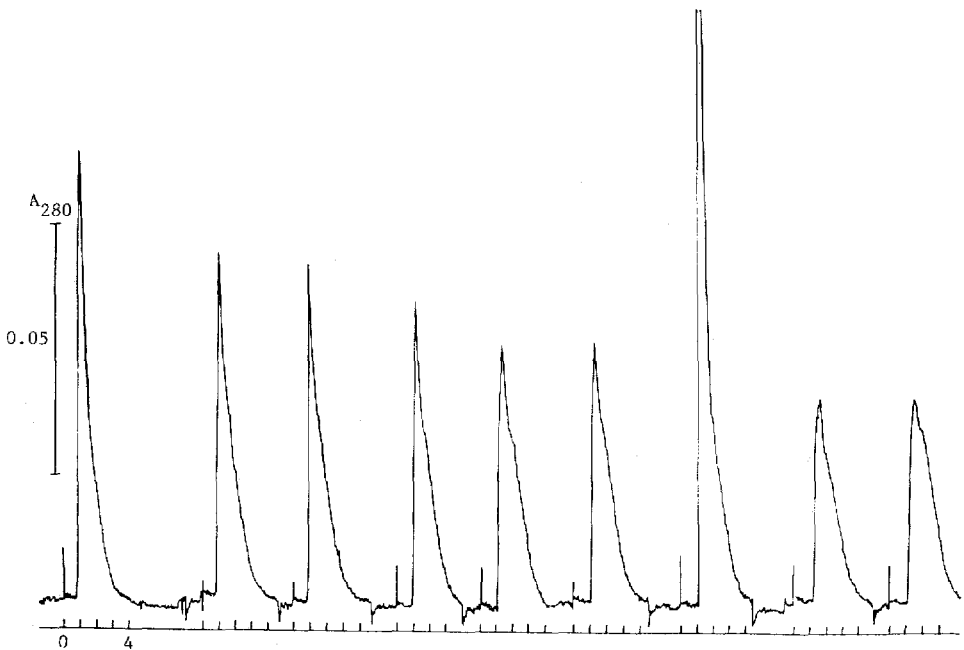

Fig. 6. Erratic chromatographic behavior during repeated injections of gamma globulin into a stainless steel capillary column. Column, same as used in Fig. 5; mobile phase, water; flow-rate, 68 μl/min; detector, UV (280 nm); samples, nine successive 1-μl injections of a solution of gamma globulin (6 mg/ml) in water.

sis experiments with the same solution and capillary column. Fig. 7a shows two unusual features for a ferritin sample, namely a peak and a subsequent drastic change in initial slope in a repeat of the initial experiment. Fig. 7b demonstrates the same type of experiment with a lower-molecular-weight protein exhibiting less irreproducible frontal chromatograms. Control experiments on lower-molecular-weight species showed both reproducibility and anticipated frontal analysis curves. Attempts to clean the capillary result in unanticipated frontal analysis patterns such as that shown in Fig. 8. Again it would appear from Figs. 7 and 8 that initial polymer loss processes determine subsequent polymer losses to the wall.

Some injections yielded chromatograms in which the tail of the peak was asymptotic to a flat plateau higher than the original baseline. We believe this is caused by a coating of protein film on the detector cell wall similar to that coating the capillary wall.
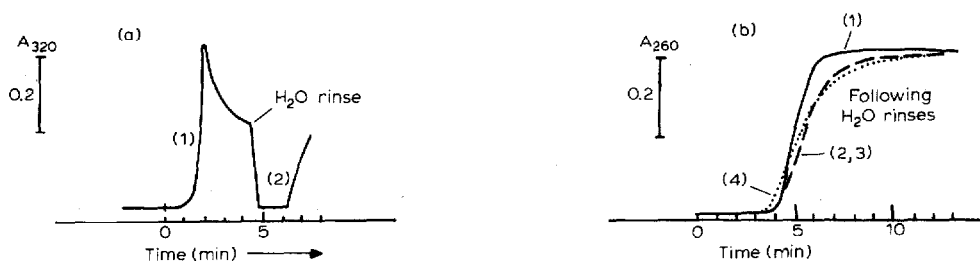

Fig. 7. (a) Frontal analysis of (1) ferritin solution showing an unexpected peak and subsequent altered frontal analysis pattern in second run (2). Column, 73 cm × 0.25 mm I.D., stainless steel capillary column, corroded with concentrated NaCl resulting in surface pitting on capillary wall; mobile phase, water; flow-rate, 34 μl/min; detector, UV (320 nm); sample, 10-ml syringe containing aqueous ferritin (0.025 mg/ml) preceded by pure water. (b) Frontal analyses of RNAase samples illustrating irreproducibility of repeated frontal analysis experiments. Column, 78 cm × 0.25 mm I.D., stainless steel capillary; mobile phase, water; flow-rate, 27 μl/min; sample, 0.9 mg/ml ribonuclease in water in 3-ml syringe run in same system as in (a) above.

When homogeneous solutions of certain solutions flowed through a capillary, a noisy detector baseline (Fig. 9) was observed in comparison with that observed with only the pure solvent or low-molecular-weight, UV-absorbing species flowing through the detector. The absorbance of the flowing solution was a function of flow-rate, the absorbance decreasing with increasing flow-rate. In one instance (Fig. 9d) the noise level *increased* significantly at higher flow-rates. Thus, it would appear that particles of protein are formed in a flow-rate-dependent manner in the mobile phase or on the wall and flaking off during the passage of the mobile phase. In Fig. 9d the numbers below the recorder tracings are separately measured absorbances of the effluent collected from the output of the detector during the times marked with arrows. These absorbance readings do not bear a direct relationship with the changes in absorbance of the HPLC detector, although the changes are relatively small. No particles were observable in the effluent during this experiment.

In another experiment in which the total sample effluent was collected from single injections (Table I) of a ferritin solution, the percent recovery as measured by spectrophotometry was found to increase somewhat with flow-rate. However, this flow
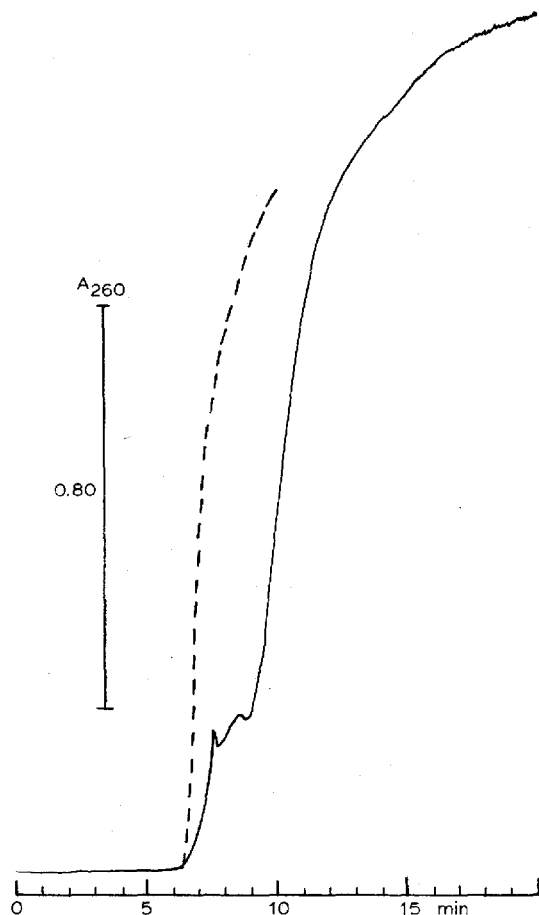
Fig. 8. Effect of multiple cleanings of capillary before frontal analysis experiment with gamma globulin. Column, 78 cm × 0.25 mm I.D., 316 stainless steel capillary. Column soaked overnight in 1 M HNO$_3$, rinsed with distilled water and then flushed repeatedly with methanol. Mobile phase, water; flow-rate, 27 μl/min; sample, gamma globulin (2.0 mg/ml in water) in 3-ml syringe run interfaced with pure water. (---) Calculation based upon Taylor's equation[2] for frontal analysis with no diffusion of sample (case A2) using x = 78 cm and U_o = 108 cm/min. x = Length of column; U_o = maximum velocity of fluid along axis of capillary.

dependence can be understood by calculations which show that molecules deposited in the center of the capillary did not have time (assuming laminar flow) to diffuse to the wall during the sample front transit time. Thus, the faster the flow-rate, the fewer molecules are able to collide with the wall or experience the higher shear rates near the capillary wall. However, those that are close to the wall are subject to shear-induced aggregation and wall deposition.

    The response to changes in flow-rate is also dependent upon the chemical nature of the protein as demonstrated in Fig. 10. In this experiment identical volume injections were made at two different flow-rates, one twice as fast as the other; chart speed was also increased by a factor of two at the faster flow-rate. Control injections of non-protein, low-molecular-weight UV-absorbing molecules gave identically shaped peaks
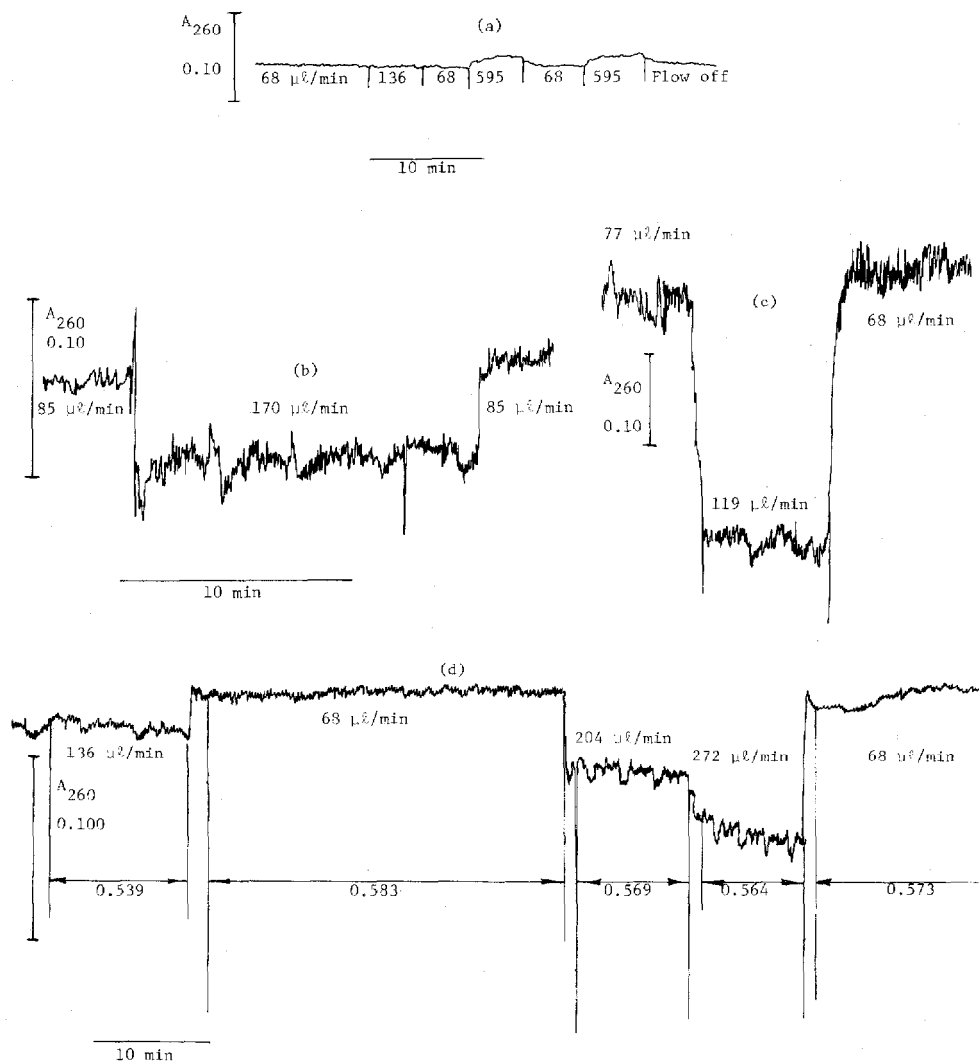
Fig. 9. Effect of flow-rate change on steady-state UV detector readings on polymer solutions flowing through a capillary column preceding the detector. Column, 79 cm × 0.25 mm I.D., 316 stainless steel capillary; mobile phase, water; flow-rates, as indicated in μl/min; detector, UV (260 nm); samples: (a) 0.1 % (v/v) aqueous acetone in 10-cc syringe run in system shown in Fig. 7; (b) aqueous Blue Dextran, 0.5 mg/ml; (c) aqueous Blue Dextran, 1.0 mg/ml; (d) aqueous lysozyme, 0.7 mg/ml. Five fractions collected (←   →): A_{260} readings on fractions are given in lower portion.

within experimental error. However, in Fig. 10 it is seen that exactly opposite changes in peak shape are recorded in response to increasing flow-rate, implying that the chemical nature of the polymer determines the flow-rate dependent losses and unusual chromatographic behavior.

Although higher-molecular-weight molecules tend to give more irreproducible and unusual results, lower-molecular-weight species such as lysozyme (a basic protein

TABLE I

FERRITIN RETENTION EXPERIMENTS

Samples (10 μl) of aqueous ferritin (0.83 mg/ml) injected into a stainless-steel column 79 cm × 0.25 mm I.D.

Flow-rate (μl/min)	Recovery (%)
68	84
136	84
340	95
680	90

with a molecular weight of 14,000) give similar non-conventional behavior. In general, less problems with protein loss were encountered at higher ionic strengths. However, unconventional behavior was a complex function of the chemical nature of the protein or other high-molecular-weight compound, the ionic strength, the flow-rate and the previous injection history of the column.

In our work we have used mobile phases which consist primarily of pure water
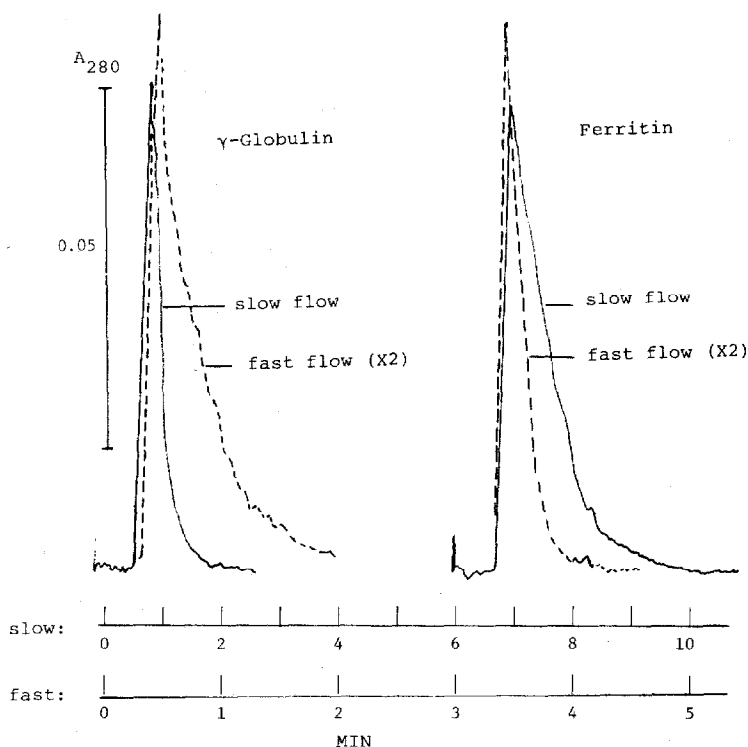

Fig. 10. Effect of flow-rate on peak shape of chromatograms of individual components with both flow-rates and chart speeds increased by a factor of two. Column, 73 cm × 0.25 mm I.D., stainless steel capillary corroded with 1 *M* NaCl; mobile phase, water; flow-rate, slow (68 μl/min); detector, UV (280 nm); chart speed, 0.5 in./min at 68 μl/min, 1.0 in./min at 136 μl/min; samples, 0.5 μl (γ-)globulin (6 mg/ml), 0.25 μl ferritin (0.25 mg/ml).

or aqueous inorganic buffers. However, we also have carried out one series of experiments using 2-propanol as the mobile phase with a sample consisting of histone proteins. Chromatograms from this experiment show sharp spikes on the trailing edge of the complex protein peak in the chromatogram. These sharp spikes were correlated with visible precipitates in the effluent from the detector (the histone sample did not precipitate when diluted with 2-propanol). Thus the precipitates would appear to be formed near the capillary wall and to be the result of a shear-related process.

Glass and Teflon tubing gave results which were similar to or worse than stainless steel tubing in terms of protein loss or unusual concentration profiles. Often clogging of the columns would occur.

We can postulate no single mechanism which explains all of the above results at this time from the limited amount of reproducible data available. However, we believe some mechanistic speculations may be in order at this time.

The flow-rate dependence of deviations from anticipated behavior may be a consequence of the higher shear rates close to the capillary wall at higher flow-rates. There are some indications in the literature that other polymer precipitates may have formed because of similar shear forces[6]. These precipitates were also very difficult to dissolve once formed[8]. If the conformations of proteins are altered by shear forces near the wall, it is likely that more hydrophobic regions will be exposed on the polymer surface. Intermolecular hydrogen bonding between intramolecular hydrogen bonding partners created from intramolecular hydrogen bonds broken by shear forces are also possible candidates for forces stabilizing mobile phase aggregates, precipitates, or protein wall coatings. Initial capillary wall coatings may be created through ionic attractions. Once the shear-induced precipitate is deposited on the wall surface, it may act to attract similarly shear-deformed protein molecules from the mobile phase and a metastable precipitate may form in multiple layers.

Often large absorbances are seen in the detector when the mobile phase flow is commenced after a period without mobile phase flow through the capillary. This result may be due to relaxation of the shear stress on the metastable precipitate on the wall surface —causing an increased solubilization of the surface molecules. While cleaning agents are quite often able to remove some UV-absorbing material from capillaries showing evidence of polymer buildup, the process is very slow and there is usually a non-asymptotic approach to a "clean" surface with repeated cleanings.

We believe the above results imply that similar processes may take place in a packed LC column itself. We have not explored the effects of mobile phase composition on polymer loss to the wall in a systematic fashion but this may be a fruitful avenue for investigation. We report elsewhere[9] that lowering the flow-rate causes anticipated diffusion behavior of proteins. Cohen[10] postulates that water-soluble polymers diffuse away from the wall because of an entropy gradient due to the shear force near the wall. However, the condition of the wall surface is reported to be critical in terms of the surface contours[11]. Therefore, it would seem to be worthwhile investigating the relationship between polymer loss processes and the nature of the topology of chromatographic packing material.

The investigations reported here are the result of using an apparently common practice of substituting a capillary "blank" for a packed column in order to determine the percent loss due to "adsorption" by the column[2]. Our results imply that, since losses in the capillary column are of the same order as those reported for losses on columns,

that there may be *greater* losses in the capillary than on the column. This might account for "percent recoveries" of proteins greater than $100\%^2$. Since similar size capillary tubing and flow-rates to those we have used are employed in conventional LC, we believe our results should be of concern to workers engaged in non-HPLC chromatography.

CONCLUSIONS

Because of the above results, we believe the following warnings seem appropriate to those engaged in *both HPLC* and *conventional* LC of proteins and other polymers:

(1) Capillary tubing should not be employed as a blank in testing the percent loss of protein since it is possible that *more* protein will be lost in the capillary than on the column, thus giving misleading "percent recoveries".

(2) Capillary length should be minimized to minimize high shear regions.

(3) Capillary tubing *diameter* used in protein LC should be investigated for its role in helping reduce protein losses by reducing shear rate, since these losses appear to be related to the shear forces in the capillary. Dead volume considerations may be less important than possible protein losses due to narrow diameter shear-induced losses.

(4) Consideration should also be given to shear-induced losses in syringes, sample loops, injection valves and detector tubing and cells.

(5) The chemical nature of the mobile phases should be considered in terms of its ability to discourage aggregation and wall coating behavior of shear-stressed proteins. Special attention should be paid to addition of hydrophobic mobile phase additives which will discourage aggregation and wall adsorption. However, suspended precipitates or colloids may replace wall adsorption and care should be taken to examine the nature of the effluent.

ACKNOWLEDGEMENTS

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