HEMOGLOBIN & SERUM ALBUMIN: SALT-MEDIATED HYDROPHOBIC CHROMATOGRAPHY

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SUMMARY: The binding of pure human serum albumin and pure human hemoglobin to L-phenylalinine Sepharose and aniline Sepharose columns, two chromatographic columns of differing hydrophobicity, has been investigated for various concentrations of ammonium sulfate salts.

The binding of hemoglobin at a lower ammonium sulfate concentration than albumin in both hydrophobic support systems parallels the solubility and precipitation characteristics of these two proteins in solution and mirrors the phenomenon of salting out of proteins in solution. Both hemoglobin and albumin bind at lower concentrations on aniline Sepharose than on L-phenylalanine Sepharose, reflecting the greater efficiency of binding by the more hydrophobic support matrix.

INTRODUCTION: In several reports, (1-9), a novel method of protein chromatography has been described. In all cases, the proteins of interest were bound at high concentrations of salt to agarose derivatives containing hydrophobic groups, and were eluted at lower salt concentrations.

The effects of neutral salts , compared to salt-free media,upon intramolecular interactions of macro-molecules (10) and upon the association of enzymes with membranes (11) have been interpreted as due to hydrophobic interaction (10,11). If this same type of interaction is dominant in the precipitation of proteins in ammonium sulfate solutions, then a protein which precipitates at a low ammonium sulfate concentration should likewise be retained on hydrophobic supports at a low ammonium sulfate concentration, and similarly proteins which precipitate at high ammonium sulfate concentrations should require relatively higher concentrations of ammonium sulfate to effect their retention. The experimental test of this prediction is the subject of this communication.

In this connnection, two proteins which are readily available in highly purified form are hemoglobin and serum albumin; their relative solubilities in ammonium sulfate solutions have been investigated (12,13), and it has been found that

hemoglobin precipitates at lower ammonium sulfate concentrations than does albumin. The hydrophobic supports chosen for this study were L-phenylalanine and aniline-Sepharoses whose retention of placental alkaline phosphatase has been studied previously (2).

MATERIALS AND METHODS: Compounds used in this study were: Tris ('ultrapure reagent,' Mann, N.Y., N.Y.), $(NH_4)_2SO_4$ (ultrapure reagent, Mann, N.Y., N.Y.), CNBr (Eastman Organic Chemicals Department, Rochester, N.Y.), L-phenylalanine (Sigma, St. Louis, Mo.), Sepharose 4B (Pharmacia, Uppsala, Sweden), Human Serum Albumin, Fraction V (Sigma, St. Louis, Mo.), Human hemoglobin (2 x crystallized, Pentex, Kankakee, Ill.).

A 20mg/ml (by weight) solution of serum albumin and a 10mg/ml (by weight) solution of hemoglobin were prepared in 0.1M Tris-acetate buffer, pH 8.0. To 0.5ml of these stock solutions were added 0.5 mls of twice the desired $(NH_4)_2SO_4$ concentration in 0.1 ml Tris-acetate, pH 8.0.

Sepharose derivatives were prepared by the procedure of Cuatrecasas (14) by using 20g. of CNBr/100ml (settled volume) of Sepharose 4B in the activation step and 0.2M-ligand with pH 9.8 for the coupling step. The coupling reaction for the preparation of aniline-Sepharose was performed in 80% (v/v) acetone, in which Sepharose has been shown to be stable (15).

RESULTS: Each sample at a known ammonium sulfate concentration was applied to either L-phenylalnine Sepharose or aniline Sepharose columns previously equilibrated with the same concentration of buffered ammonium sulfate, which was then employed for each elution. Before applying the next sample at a different ammonium sulfate concentration, columns were extensively washed with 0.25M Tris base pH 10.5 to ensure complete removal of any protein which might still be bound to the column. All columns were run at a constant pH of 7.4 and temperature of 4^{o} centigrade.

Figure 1 illustrates a typical series of elution profiles obtained for these proteins; in this case, serum albumin is chromatographed on aniline Sepharose at

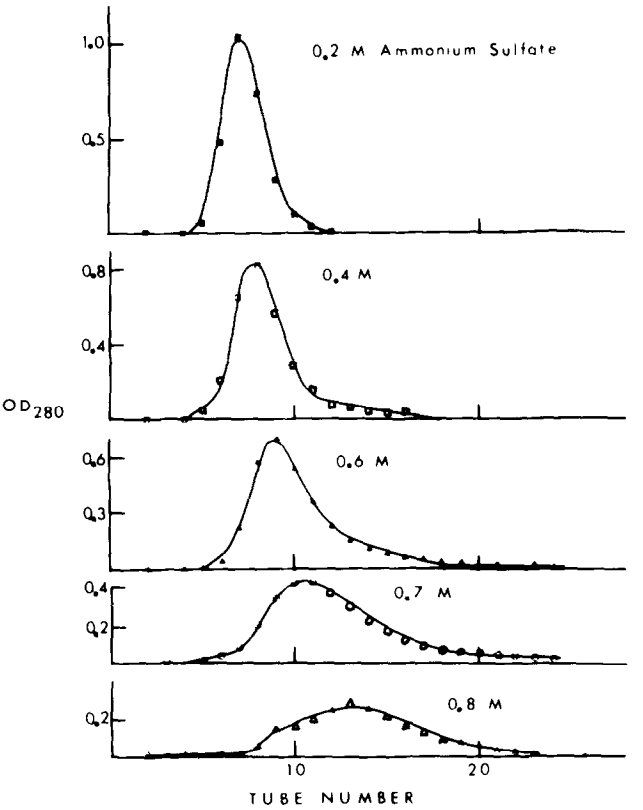

Figure 1. Chromatographic profiles of human serum albumin chromatographed on aniline Sepharose at increasing concentrations of ammonium sulfate in 0.10M Tris-acetate, pH 8.0. Column dimensions 0.9 X 10.3cm, flow rate 0.20ml per minute. 1.30ml per fraction. Temperature 4°C.

stepwise increasing ammonium sulfate concentrations. At a high ammonium sulfate concentration the protein peak occurs at a later position in the elution profile than the peak in the next lower ammonium sulfate concentration indicating an increase in the relative retention of protein on the column at the higher concentra tion. It can also be readily seen that the bulk of protein is also eluted much later from the column and in a greater volume of buffer as the ammonium sulfate concentration used increases. In the case of albumin chromatographed on aniline Sepharose, at 0.2M ammonium sulfate, almost all of the albumin is eluted from the column by tube number 11 in approximately 9.1 ccs of buffer whereas at 0.8M

ammonium sulfate the bulk of protein does not begin to elute until tube number 7, and the protein is completely eluted in a volume of 22.1 ccs of buffer indicative of the increased binding of albumin at the higher ammonium sulfate concentration.

Figure 2 depicts a comparison of the position of the peak elutions of hemoglobin and serum albumin chromatographed on aniline and L-phenylalanine Sepharose as a function of increasing ammonium sulfate concentration. It can be observed that both proteins are retained on aniline Sepharose at lower ammonium sulfate

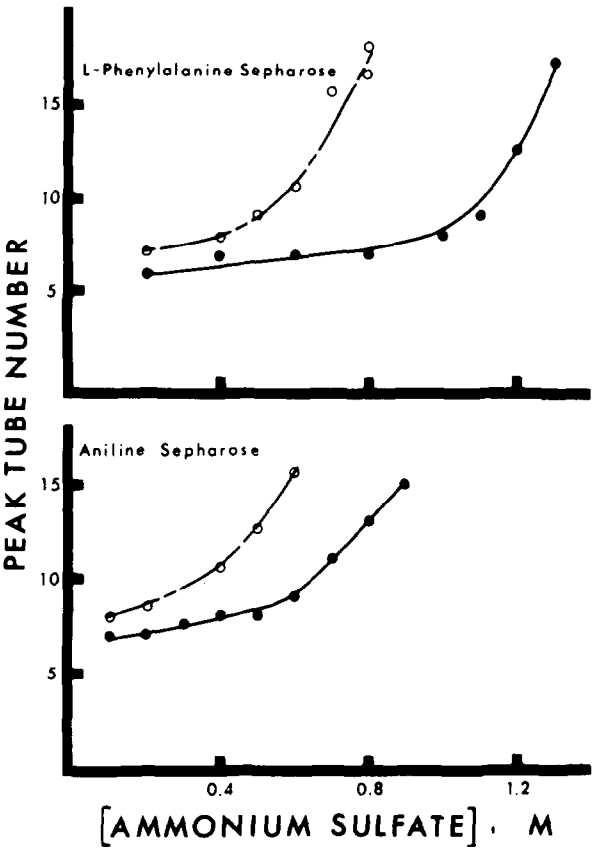

Figure 2. A plot of the peak tube number as a function of ammonium sulfate concentration for the chromatography of human serum albumin and human hemoglobin on columns of L-phenylalanine-Sepharose (top) and aniline-Sepharose (bottom). Column dimensions 0.9 X 10.5cm (L-phenylalanine Sepharose) and 0.9 X 10.3cm (aniline-Sepharose). Flow rate 0.20ml per minute, 1.30ml per fraction. Temperature 4°C. -o-o- is the symbol for hemoglobin and -.-.- represents serum albumin.

concentrations than on L-phenylalanine Sepharose as has been found for placental alkaline phosphatase (2). Binding of either hemoglobin or serum albumin to glycine Sepharose in 1.5M ammonium sulfate could not be accomplished. On both L-phenylalanine Sepharose and aniline Sepharose, hemoglobin was found to be retained at lower ammonium sulfate concentrations than was serum albumin. These results are consistent with the fact that aniline-Sepharose is more hydrophobic than L-phenylalanine-Sepharose. Glycine Sepharose, which lacks a hydrophobic structure, shows no binding of hemoglobin or albumin.

DISCUSSION: On both hydrophobic supports the increased retention of hemoglobin and serum albumin as a function of increasing salt concentration parallels the results obtained in the study of precipitation of these two proteins in solution (12, 13). These results are consistent with the observations of Cuatrecasas (16) concerning the elution properties of nonspecific affinity columns on the chromatographic separation of proteins. Likewise, Rimerman and Hatfield, in comparing the precipitation of bacterial extracts in ammonium sulfate with their chromatography on L-valine Sepharose (4), have also concluded that there is an analogy between salting-out and hydrophobic chromatography mediated by salts.

The generality of the application of this method to protein purification has been considered (2-6,17).In our experience, it has been equally as applicable as salting-out in solution, but has has the added advantage of considerably greater resolution of individual protein components, and of reproducibility of resolution for different samples. This method has been used to separate gamma globulins from other serum proteins including separation of IgA from IgG, IgM and α 2-macroglobulin (17). More detailed descriptions of the purification of IgA (Doellgast, G.J. and Plaut, A.G.) and the purification of IgG (Memoli, V.A. and Doellgast, G.J.) are in preparation. The same principle can be used for the study of ligand-protein interactions, as outlined elsewhere (18).

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