THE UNIVERSITY OF MANITOBA

SURFACE HYDROPHOBICITY MANIPULATION THROUGH AMMONIUM SULFATE CONCENTRATION

BY
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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFULLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE
WINNIPEG, MANITOBA
OCTOBER, 1988

ISBN 0-315-47977-9

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGMENTS

I would like to express my deep appreciation and gratitude to my advisor Dr. M.A.H. Ismond for her invaluable advice, her constructive criticism and her patience. Also the efforts of Dr. E.D. Murray and Dr. R. Marquardt were greatly appreciated, especially considering the small amount of time allotted to them. I am extremely grateful to the Department of Food Science for allowing me to complete this study on a part-time basis while working as a member of the technical staff. I would like to thank A. Bernatsky for her valuable technical support and S. Arntfield for sharing her considerable knowledge in the field of differential scanning calorimetry.

I would like to take this opportunity to show my appreciation to the late Dr. P.E. Cansfield. It was through his concern and encouragement that I first contemplated attempting a Master's program.

Finally, I would like thank my wife Carole for her encouragement and her patience during all those times when we couldn't.

ABSTRACT

Five standard proteins (lysozyme, ovalbumin, ribonuclease A, aldolase and legumin) were analyzed by high-performance hydrophobic interaction chromatography to determine their retention characteristics as a function of initial ammonium sulfate concentration. Lysozyme was retained most strongly, followed by ovalbumin and ribonuclease. Aldolase and legumin were not retained. At a characteristic initial salt concentration the retained proteins were bound to the column at an increased rate. It was also found that for each protein a characteristic initial salt concentration existed where the elution salt concentration of that protein reached a maximum. These two characteristic concentrations correlated well and occurred at a point were the capacity factor attained a value of 1.

Thermal analysis of the three retained proteins revealed that stability increased in direct proportion to the amount of ammonium sulfate in the environment until a characteristic salt concentration was reached. Beyond this point the rate of change in stability as a function of salt concentration decreased. This change was attributed to a steric limitation to the number of intra-molecular hydrophobic interactions that could occur. The salt

concentration at which this effect occurred correlated well with the characteristic salt concentrations noted in the chromatographic analysis.

Surface hydrophobicity was determined fluorometrically using 1-analino-8-naphthalenesulfonate (ANS) as a probe. It was shown that the probe began to bind to the protein more rapidly at the same ammonium sulfate concentration that marked the change in stability for each protein. It was concluded that the increase in binding of the probe to the protein and of the protein to the column was a result of the decrease in the number of intra-molecular hydrophobic interactions resulting in a proportionally larger number of inter-molecular interactions.

It was shown that the ratio (B) of ANS surface hydrophobicity to molecular weight was ten times greater for proteins which were retained to the column as opposed to those that were unretained. It was suggested that both B and the characteristic ammonium sulfate concentration at which the rate of hydrophobic interaction formation increases show promise as indices of retention for hydrophobic interaction chromatography.

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1. INTRODUCTION

Hydrophobic interaction high-performance liquid chromatography (HIC) is a relatively new technique for the separation, quantitation and evaluation of proteins. Like reversed-phase high-performance liquid chromatography (RPC), HIC utilizes differences in the strength of hydrophobic interactions between the proteins and the column to effect separation. The conditions under which the separation takes place are far less harsh than is the case during RPC. The reasons for this are that the density of hydrophobic ligands on the column is much less and that an aqueous salt mobile phase is used instead of organic solvents. Even under these milder conditions some proteins undergo conformational changes or denaturation.

In terms of chromatographic behavior, changes in the protein structure may result in broader peaks, changes in retention time, or even multiple peaks. These effects make it difficult to analyze protein mixtures accurately. Protein functionality in both biological and food systems is affected if these changes are of a permanent nature. Conformational changes may be brought about by the hydrophobic character of the chromatographic column or by the influence of the salt used in the mobile phase of the

HIC system. The former has been investigated by Ingaham et al. (1985). It has been shown by Ismond et al. (1986a) that increased amounts of lyotropic salts such as ammonium sulfate increase the stability of proteins by means of electrostatic and hydrophobic interactions.

It is the purpose of this investigation to study how ammonium sulfate, in the concentrations used during HIC, affects the stability and conformation of a number of standard proteins. An attempt will also be made to relate changes in surface properties produced by different salt conditions to the retention mechanism.

2 LITERATURE REVIEW

2.1 Hydrophobicity

The model of globular protein structure proposed by Kauzmann (1959) postulated that nonpolar amino acids would tend to be buried in the center of the molecule whereas the exterior surface of the molecule would be surrounded by an outer layer of polar residues in contact with an aqueous solvent. He suggested that hydrophobic interactions played an important part in the stabilization of the globular structure. A hydrophobic interaction is the adherence of two hydrophobic entities in aqueous solution. When an amino acid of hydrophobic character enters a solution the structure of the water in the immediate vicinity becomes more ordered and hydrogen bonding between water molecules increases. This requires an increase in energy to maintain the system. When two such amino acids come into contact the water between them is excluded from the contact area resulting in a corresponding reduction in water structure and a lower energy requirement. For this reason hydrophobic interactions may occur spontaneously.

Hydrophobic character in amino acids is associated with their side chains. Based on Kauzmann's model, Tanford (1962), plus Nozaki and Tanford (1970) developed a scale of

values for the hydrophobicity of the amino acid side chains in terms of the change in free energy of transfer between ethanol and water. These values approximated the change found when a protein unfolds and the side chains are exposed to water. Bigelow (1967) calculated the average hydrophobicity of several proteins by summing the transfer free energies for all residues present and dividing by the number of residues. His values were intended to be an estimation of the hydrophobic contribution to the stability of the protein.

Neutral salts, can effect the degree to which hydrophobic interactions take place. Hofstee (1975) has shown that salting-out ions enhances intramolecular, as well as intermolecular, hydrophobic interactions as indicated by the stabilization of the protein's core. Tanford (1968) indicated, however, that while high concentrations of these salts enhanced hydrophobic associations, the solubility of the protein could decrease since the availability of water molecules is reduced. Conversely, salting-in ions do not favor hydrophobic interactions. They have been termed chaotropic by Hamaguchi and Geiduschek (1962) because they provoke unfolding, extension and dissociation on the macromolecules.

Klotz (1970) plus Lee and Richards (1971) have shown through x-ray diffraction data that a significant number of

the nonpolar amino acid residues in proteins are exposed to the water interface, as opposed to the model set forth earlier by Kauzmann (1959). While Klotz (1970) classified residues as either exposed to the solvent or buried within the molecule he realized that some amino acids were only partially buried and a more comprehensive measurement was required. In 1977 Rackovsky and Scheraga attempted to define hydrophobicity and hydrophilicity in terms of the distance of the residues from the center of mass and the orientation of the side chains. This study was extended by Meirovitch et al. (1980) when they developed two scales of hydrophobicity based on the average reduced distance from the center of mass and the average orientation angle of the side chains. Chothia (1976) calculated the accessible and buried surfaces of a number of proteins and discussed the implications this had on the theory of protein structure. Rose et al. (1985) used proteins of known structure to measure the average area each residue buries upon folding and found a strong correlation with residue hydrophobicity as defined by Nozaki and Tanford (1971).

There are a number of problems that arise when calculations are based on x-ray diffraction measurements. They are not completely accurate since parts of the interior are blocked from measurement by the exterior atoms. Furthermore, those data are of a rigid structure while in actual fact, a protein is quite dynamic. This would affect the calculation of solvent accessible surfaces. Finally,

although several scales of hydrophobicity have been devised, there still exists some disagreement with regard to residues such as proline as to their relative hydrophobicity. This disagreement results in some uncertainty as to the true contribution of hydrophobicity to protein stability.

2.2 Hydrophobic Interaction Chromatography

Affinity chromatography has been described by Cutrecasas et al. (1968) as having near-ideal properties for selective column chromatography. In this mode of chromatography a biospecific entity such as an inhibitor is bonded to a nonsoluble hydrophilic support such as agarose by means of a spacer-arm or ligand. Enzymes and other proteins without a significant affinity for the bound entity pass through the column whereas those which recognize the entity are retarded in proportion to their affinity constant. Elution is achieved by changing the ionic strength, the pH or by adding a competitive inhibitor. However, in 1974a O'Carra et al. pointed out some serious disadvantages to this technique due to non-specific adsorption. O'Carra et al. (1974b) reported that while some of these problems may have been due to ionic effects, the most serious interferences stemmed from hydrophobic interactions between the spacer-arms and the enzymes. They showed that a hydrophilic spacer-arm eliminated most of the non-specific adsorption.

Er-el et al. (1972) coated Sepharose with hydrocarbons having sidechains which varied in length and found that some proteins were bound more tightly as the length of the sidechains increased. Shaltiel (1974) showed that the increased affinity was due to the increased hydrophobicity rather than an increased ability of the arm to bind inhibitors or to reach deeper into the active site. He suggested that a homologous series of hydrocarbons coated onto agaroses could be used to separate proteins. Each member offered hydrophobic "arms" or "yardsticks" of different sizes which would interact with accessible hydrophobic pockets in various proteins, retaining only some proteins out of the mixture. Further separation was achieved by altering the nature of the eluting solvent. This study formed the basis of what is now known as hydrophobic interaction chromatography (HIC).

In affinity chromatography, proteins which had been adsorbed to the column were eluted by passing a mobile phase through the column which contained a competing inhibitor, by changing the pH of the solution or by changing the ionic strength of the mobile phase. The salt used in the latter mode often deformed the active site causing the protein to be released. An increasing linear salt gradient was used for this purpose. As HIC developed, the same methodology was carried over into this technique. Rimerman and Hatfield (1973) plus Hjerten (1973) demonstrated that hydrophobic

interactions could be induced between proteins and a hydrophobic adsorbent by concentrations of salt greater than 1 M. The order of effectiveness followed the Hofmeister series. Proteins were then eluted by means of a decreasing salt gradient. Pahlman et al. (1977) studied the effect of high salt concentrations on the conformation of proteins. Through the use of circular dichroism at constant ionic strength, they showed that neutral salts can cause conformational and structural changes. However, salting-out ions caused only minor conformational changes while salting-in ions could cause severe conformational and structural changes. If salts such as ammonium sulfate, sodium sulfate, and sodium chloride are used most proteins would not be irreversibly denatured.

2.3 High-performance Hydrophobic Interaction Chromatography

Until the mid-1970's HIC had been performed mainly on derivatized agarose supports which are not suitable for use on high-performance liquid chromatography (HPLC) since these supports are unable to withstand the necessary pressures. In 1976 Chang et al. showed the feasibility of combining these two techniques when they were able to derivatize silica with heptanol and polyethylene oxide. It was also shown by Pfannkoch et al. (1980) that silica based size-exclusion columns for HPLC showed a weakly hydrophobic character at high salt concentrations.

2.3.1 Column development

Kato et al. (1983) reported that two high-performance (HP) HIC stationary phases had been developed by bonding butyl and phenyl groups to TSK-GEL G3000SW, a wide-pore silica used in size-exclusion chromatography (SEC). The starting material contained a hydrophilic layer to which the active groups were bonded by means of ether linkages. The degree of substitution was 0.12 and 0.14 mmole/gram of support.

Fausnaugh et al. (1984) used polyamine-bonded silica as a hydrophilic matrix from which to produce seven different stationary phases of increasing hydrophobicity. These materials were synthesized through acylation with anhydrides and acid chlorides. The columns were characterized with regard to protein retention, resolution and loading capacity.

A series of six HIC columns was reported by Gooding et al. (1984). These supports were prepared by covalently attaching alkyl and phenyl moieties to a hydrophilic polymeric matrix bound to macroporous silica. The effect of chain length on resolution and selectivity was studied.

The use of wide-pore silica-based hydrophilic ether-bonded phases for the HIC separation of proteins was also studied by Miller et al. (1985). The hydrophilic matrix was

varied as well as the ligand chain length. The columns were found to have no ionic interactions at low salt concentrations and were thus suitable for SEC as well as HIC.

Two resin-based columns were recently released by Toya Soda Manufacturing Company Limited. Both columns were developed by bonding hydrophobic groups to TSKgel G5000PW, which is a hydrophilic-polymer-based material of large pore size used for high-performance gel filtration. The first of these, reported by Kato et al. (1984a), incorporated phenyl groups having a density of about 0.1 mmole/mL by means of ether linkages. This column is sold as TSKgel Phenyl-5PW. The second column known by the name TSKgel Ether-5PW contains oligoethylene glycol bonded to the matrix. The performance characteristics of this column was also reviewed by Kato et al. (1986).

2.3.2 Influence of Ligand Length and Density

Er-el et al. (1972) and Shaltiel (1974), working with agarose open columns, have shown that hydrophobicity increases as the chain length of the "spacer-arm" increases. Tanford (1972) reported that the hydrophobicity of a linear aliphatic carbon chain increased linearly with increasing numbers of CH_2 groups. Similarly, Shaltiel and Er-el (1973) and Hofstee (1974) have observed that since there was often

a sharp increase in affinity for a given protein as the number of methylene groups increased it should be possible to select pairs of adsorbents that show dramatic differences in affinity towards one or a few proteins and only small differences toward others.

Kauzmann (1959) pointed out that the introduction of double bonds or branched chains reduced hydrophobicity as compared to straight chains. This was confirmed by Tanford (1972) who also stated that aromatic agarose derivatives may possess intermediate hydrophobicity between two consecutive members of the alkyl-agarose series. Benzene for example was equivalent to that of a 3-4 straight chain hydrocarbon.

The density of the hydrophobic groups on the agarose was also found to make an important contribution. Jennissen and Heilmeyer (1975) presented evidence that adsorption of proteins to alkyl-agarose derivatives takes place at a critical group density and is a function of its hydrophobicity. They also showed that the degree of substitution determines the capacity of the support. Pahlmann et al. (1977) stated that the critical hydrophobicity could be attained by increasing the density or the chain length. However, he cautioned that higher binding affinities required harsher eluting conditions which might in turn lead to denaturation of the sample.

Investigators in HP-HIC have confirmed that the

relationships between the length and density of the hydrophobic groups bound to the stationary phase and the hydrophobicity of the column hold true for this new technique. Fausnaugh et al. (1984) report that as the alkyl group was changed from methyl through pentyl and phenyl the retention of a given protein increased. Weakly hydrophobic columns such as the methyl column produced sharp peaks but resolution between groups was poor. The stronger pentyl and phenyl groups retained proteins but the peaks were broad and therefore showed limited resolution. Binding butyl groups to the matrix gave the best results. Ligand density for these columns was determined to be 1.4 to 1.6 umoles per square meter. Gooding et al. (1984) found that retention generally increased in the order: hydroxypropyl, propyl, benzyl, isopropyl, phenyl and pentyl. It was found that a small change in ligand length, from propyl to isopropyl for example, could result in a large increase in retention time for some proteins. They pointed out that differences in retention can also be caused by changes in ligand density but that the differences in selectivity that they found had to be attributed to the nature of the hydrophobic group. Miller et al. (1985) developed columns that not only varied in the length of the alkyl group but also in the length of the ethylene oxide groups in the hydrophilic matrix covering the silica. Their results confirmed the effect of increased ligand length but also showed that increasing the length of the ethylene oxide groups decreased the retention of a given

protein. This would increase the ability of the chromatographer to modify the column hydrophobicity. Kato et al. (1986) studied the differences between the resin based columns TSKgel Phenyl-5PW and TSKgel Ether-5PW. Over all it was found that the Phenyl column gave better resolution but that the hydrophobicity of the ether column was less and gave better results when separating proteins that were subject to denaturation.

2.3.3 Mobile Phase Effects

Since HIC was developed from affinity chromatography it is not surprising that the early workers in this new form of chromatography used methods similar to those used in affinity chromatography to bind proteins to the column and then elute them. A stationary phase was selected which would bind all or some of the proteins in the sample and then a mobile phase was selected which was strong enough to elute the proteins in order of increasing hydrophobicity. The solvents contained salting-in salts, organic solvents and detergents. Rimmerman and Hatfield (1973) were the first to report the use of mobile phases of high ionic strength, greater than 1 M., to induce hydrophobic interactions between the proteins and the hydrophobic groups on the column. The proteins were then eluted from the column with a decreasing linear salt gradient. The order of effectiveness of the salts used to induce binding followed the Hofmeister series which is based on the effectiveness of salts in

causing the precipitation or "salting-out" of proteins in aqueous solution (Nisikawa and Bailon, 1975; Pahlmann et al., 1977).

Gooding et al. (1984) reported a comparison of sodium chloride, sodium sulfate and ammonium sulfate in the mobile phase. Ammonium sulfate was used most extensively since halide ions corrode stainless steel in the equipment and sodium sulfate gave solubility problems. Kato et al. (1984b) tested sodium sulfate, ammonium sulfate and potassium phosphate. It was found that sodium sulfate retained weakly hydrophobic proteins best but had a maximum solubility of 1.5 M at 25°C. Salt concentrations used in HP-HIC frequently exceed this value. Ammonium sulfate and potassium phosphate were similar in their ability to retain proteins. However, it was found that at pH 8 or greater ammonium sulfate would produce ammonia and was therefore not stable. This effect was also found to be temperature dependent. Nevertheless, ammonium sulfate has proven to be quite popular with researchers. Schmuck et al. (1986) compared 2 M ammonium sulfate with 4 M ammonium acetate and found that the former retained proteins more strongly under almost all conditions.

Initial salt concentrations were also shown to have a significant effect. Kato et al. (1983) showed the dependence of capacity factors on ammonium sulfate concentrations in chromatography on Butyl-G3000SW and Phenyl-G3000SW columns.

The capacity factors increased from near zero to large values in non-linear fashion as the salt concentration was increased from 0 to 2 M. Fausnaugh (1984) found that by reducing the initial concentration of sodium sulfate a given protein could be made to elute early, midway or late in the gradient. In the first and last cases the peak width was narrow but when the peak occurred in the middle of the gradient it was wider. He concluded that this may be due to greater resolution at intermediate ionic strengths resulting in subfractionation of the protein or to a greater number of adsorption-desorption steps occurring with a concomitant increase in band-spreading. His results also showed that selectivity was affected by changes in initial ionic strength since the most tightly bound proteins were affected the least. It was also felt by this group that since, for most proteins, a critical ionic strength is reached where the protein begins to be strongly retained by the column, this point could be used as an estimate of the native or surface hydrophobicity of the various proteins.

Studies have shown that pH, mobile phase additives, flow-rate, gradient duration, column length and column temperature affect retention and selectivity. Most of the HP-HIC studies cited here have been carried out in the pH range 6 - 7 since most proteins are stable in this region. Kato et al. (1984b) have studied the effect of pH on resin columns and Schmuck et al. (1984) have studied the influence of pH changes on silica-based columns. Both report that some

proteins exhibit slight variations in retention under different pH values. These changes were not as great as when ionic strength and ligand length were varied but it was felt that differences in selectivity thus produced could be used to improve resolution, keeping in mind the sensitivity of silica to extremes in pH.

Kato et al. (1984b) explored the effect of column length (1 or 2 -75 mm columns), flow-rate (0.25 - 1.5 ml/min), and the length of the gradient (30 or 60 min) on protein separation by HIC. They found that longer columns gave better resolution due to narrower peaks. A slight improvement was found in resolution with slower flow rates and this was especially true when longer gradients were used. Longer gradients produced better separation especially as flow rates increased. These improvements came at the expense of longer analysis time and, in the case of higher flow rates and longer gradient times, greater sample dilution.

The effect of adding organic solvents and chaotropes to the final buffers on the elution time and recovery of protein has also been considered by Kato et al. (1984b). Of the solvents tested, isopropanol proved the most effective in decreasing the retention of late eluting compounds. When added to the second buffer, an increase in concentration produced a decrease in retention until a level of 7% was

attained at which point the trend was reversed. Different proteins were affected to different extents by this technique; as a result this can therefore be used to enhance selectivity. Urea (2 M) and guanidine hydrochloride (4 M) were also examined for this purpose. The results attained were similar to 5% isopropanol but the elution patterns were different. This study did not discuss the denaturing effects of these substances since the authors were mainly concerned with the elution properties of the column.

Goheen and Engelhorn (1984) looked at the effect of temperature changes on the elution of myoglobin, bovine serum albumin (BSA), cytochrome c and lysozyme. Myoglobin proved to be the most ideal of the compounds studied. It never eluted at the solvent front and increased in retention as the temperature was increased. On the other hand lysozyme was relatively unaffected by temperature changes in the 0 - 45^{o} C range studied. Between 0^{o} C and 30^{o} C cytochrome c eluted at the solvent front. Between 30^{o} C and 35^{o} C a wide broad peak appeared and above this temperature retention increased and the peak became narrow and sharp. BSA eluted as a single broad peak above 30^{o} C but as the temperature dropped below 25^{o} C a second earlier eluting peak appeared which was not completely resolved from the broad peak. As the temperature was reduced to 0^{o} C the sharp peak grew at the expense of the wide peak until at 0^{o} C the wide peak was not discernible from the baseline. It was also noticed that the presence of methanol in the mobile phase caused BSA to

be eluted as a sharp peak at all temperatures studied.

Kato et al. have studied protein recovery on silica based HIC columns (1983) and on resin based columns (1984a) as well as the effects of flow rate, gradient duration, pH, initial salt concentration and mobile phase additives (1984b). They reported, as did Gooding et al. (1984), Miller et al. and Fausnaugh et al. (1984), that protein mass recovery on HP-HIC was generally between eighty and one hundred per cent and that the recovery of enzymatic activity was almost complete. Exceptions did occur, however. Gooding et al. (1984) found that a pentyl column bound most proteins irreversibly with 1 - 2 M salt. The n-butyl column tested by Miller et al. (1984) also eluted no proteins. Kato et al. (1984b) found that α-lactoalbumin was eluted as a broad peak, γ-lactoalbumin was not eluted and α-amylase was eluted with a low yield on a TSKgel Phenyl-5PW column. However the less hydrophobic TSKgel Ether-5PW column studied by the same group (1986) eluted these compounds with sharp peaks and good recovery. It was concluded that when the hydrophobicity of the stationary phase exceeded a certain level proteins can be retained irreversibly. This level was not the same for all proteins.

Both reversed-phase liquid chromatography (RPLC) and HIC separate proteins based on hydrophobic interactions. In both techniques hydrophobic groups are bound to the

substrate and used to effect the separation. The greatest physical difference between the two matrices is in the density of the hydrophobic groups. Reversed-phase packings commonly have ten to one hundred times the density of hydrophobic groups found on hydrophobic interaction packings (Goheen, 1984). Geng and Regnier (1984) stated that it is generally accepted that the mobile phases used in RPLC alter the 3 dimensional structure of most proteins during the chromatographic process. Strongly hydrophobic surfaces are considered by Cohen et al. (1984) to be capable of denaturing proteins upon adsorption. Because the length and density of ligands used in HIC were less and because HIC elutants were much milder it would be expected that the possibility of denaturation would be much less using this new method of separation.

2.3.4 Denaturation on HPLC columns

Upon studying the effects of solvents and matrices on protein structure, Lau et al. (1984) concluded that they had demonstrated that the hydrophobicity of reversed-phase columns resulted in the denaturation of proteins by disrupting the hydrophobic interactions stabilizing the native conformation. Denaturation occurred on binding to the matrix. Even ultra-short (C_3), 300 A pore matrix with relatively low carbon binding (2.9%) did not prevent denaturation of extremely stable synthetic 2 stranded α-helical coils.

Ingraham et al. (1985) investigated the possibility of denaturation on Bio-Gel TSK-Phenyl-5-PW HIC columns. It was felt that some of the enzymes tested by Goheen et al. (1984) had undergone reversible denaturation on the column and that multi-subunit proteins might not always be recovered in their native state. By comparing the results obtained from reversed-phase columns and the previously mentioned phenyl column in the reversed-phase mode with those from the phenyl column in the HIC mode it was possible to judge the effect of temperature and the phenyl matrix on the conformation of the proteins. Their data indicated that temperature increases between 0 and 50 ^{o}C generally enhanced protein binding to the HIC matrix without conformational change.

Chromatographic evidence of conformational change was shown by the increase in peak width and the growth of a later eluting peak at the expense of the earlier native peak with a temperature increase (Ingraham et al). For example, myoglobin developed a very broad peak as temperature rose, evidence that it could exist in many partially unfolded forms which were in rapid equilibrium. Cytochrome c on the other hand moved from an early eluting sharp peak to a broad peak to a second later eluting sharp peak with increasing temperature. This later behavior was interpreted as an indication that this protein had undergone a localized conformational change.

Ingraham et al. (1985) also studied the behavior of two

synthetic peptides, TM-22 and Tm-36. They showed by means of circular dichroism that both peptides were 100% α-helical and both were dimers, although TM-36 was more stable as a dimer than TM-22. Chromatographic evidence suggested that TM-36 was eluted as a dimer at 0°C while TM-22 was eluted as a monomer. It was felt that the quaternary structure of TM-22 had been denatured by the phenyl matrix. As the temperature was increased the quaternary structure of TM-36 and the tertiary structure of TM-22 were both changed - characteristic of denaturation. This was evidenced in the chromatograms of the two compounds.

Wu et al.(1986) studied the thermal behavior of proteins in HIC on the methyl column described by Miller et al. (1984). They used on-line spectroscopic data obtained with a photodiode array detector to support their interpretation of the chromatograhic information. In addition they have developed a general index (Z value) characterizing protein retention as a function of salt concentration. The Z value is equal to the slope of the plot of log k' as a function of log %B where k' is the capacity factor of the column and %B is the fraction of the mobile phase that is represented by the B buffer. They demonstrated that this value was shown to be equivalent to the number of moles of water displaced per mole of protein adsorbed to the stationary phase and as such was related to the contact area of the adsorbed protein on the surface. As the protein

denatured the value of Z would increase. They found that this new index was a sensitive measure of conformational change.

This study was extended by Wu et al. (1986). In this paper the authors further examined the value of Z as an indicator of conformational change. It was concluded that Z can be viewed as a measure of the free energy change of adsorption under the condition of constant salt concentration as a function of temperature. This value could also be influenced by ionization, association, conformation, etc. Thus a change in Z did not in itself mean a conformational change but with a variation in temperature, a knowledge of thermal unfolding in solution, and on-line spectral analysis, a body of evidence could be assembled which was consistent with conformational change.

Using the techniques described previously, Wu et al. have examined the behavior of α-lactalbumin under HP-HIC. Their results indicated that the stationary phase acts as a catalyst for the thermal denaturation of proteins and that the extent of the catalytic effect was dependent on the hydrophobicity of the stationary phase. They suggested that operating at subambient temperatures would reduce denaturation of labile proteins. They also demonstrated that metal binding proteins such as α-lactalbumin could be rendered more stable, or more unstable, depending on which metal ions were present in the mobile phase.

2.4 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) has been used by many researchers to follow the thermodynamic properties of denaturation. The temperature and heat of transition between states (ordered/disordered, solid/liquid, etc.) provided the most important information yielded by DSC analysis, since the more useful and interesting applications of the technique stemmed from an appreciation of how these parameters varied with experimental or technological conditions (Wright, 1984). In the area of food research the observed DSC transitions relate mainly to processes such as protein denaturation, starch gelatinization, and fat crystal melting. These processes appeared as a peak on the thermal curves because of their high order of co-operation, ie. the rupture of inter- and intra-molecular bonding was almost simultaneous. The narrower the peak the more intramolecular co-operativity that exsisted. Given the proper conditions, denaturation can be followed by aggregation which would be exothermic in nature. Should aggregation occur in the same temperature range as denaturation the determination of the denaturation enthalpy would be hindered. Furthermore, renaturation would be prevented by aggregation preventing a rigrous thermodynamic analysis since it would not be possible to follow the thermodynamic changes that would occur during renaturation.

Pfiel and Privalov (1976a,b,and c) overcame the

problems created by aggregation through the careful choice of experimental conditions such as concentration and extremes of pH. Privlov and Khechinashvili (1974), in their study of the thermal denaturation of ribonuclease, lysozyme, chymotrypsin, cytochrome c and myoglobin, showed that the area of the thermal adsorption peak represented the effective enthalpy of denaturation (ΔH) and the temperature at which the peak maximum occurred was termed the temperature of denaturation (T_d). They concluded from their study that the enthalpy of denaturation was largely attributable to the heat of rupture of intra-chain hydrogen bonds and that its observed temperature dependence was a result of an exothermic contribution from the cluster formation of water molecules around newly exposed non-polar groups of the proteins. This ordering effect decreased with increasing temperature with the result that the apparent enthalpy of denaturation increased with increasing temperature.

DSC has been used to study the interaction of proteins with a range of other substances, including polysaccharides (Imeson et al., 1977), sugars (Donovan et al., 1975; Back et al. 1979), polyhydric alcohols (Back et al., 1979), and monohydric alcohols (Velicelebi and Sturtevant, 1979). Sugars and polyols were found to stabilize proteins against thermal denaturation. This was believed to be due to their ability to alter the structure of water and thus the

strength of the hydrophobic interactions. Alcohols caused a destabilizing effect which was believed to be once again due to its effect on water structure. In this case hydrophobic interactions were hindered.

Delben and Crescenzi (1969) investigated the thermal denaturation of lysozyme in concentrated aqueous solutions of urea, guanidine hydrochloride, and hexmethylenetetramine. Wright and Boulter (1980) have shown through the use of DSC analysis that the legumin globulin from various pulse crops undergoes single step denaturation. Donovan and Mapes (1975) followed the conversion of ovalbumin to S-ovalbumin and Sochava et al. (1985) utilized the DSC to study the reversible and irreversible thermal denaturation of concentrated globular proteins. The capability of DSC to measure changes in protein stability brought about by the presence of different salts has been demonstrated by Ismond et al. (1986a), Arntfield et al. (1986) and Harwalkar and Ma (1987). DSC can be used to follow protein conformational change with environmental variations. In particular, it has been shown that differences in protein stability and conformation under various salt environments can be assed by monitoring the temperature of denaturation and enthalpy of denaturation levels. Therefore, this technique would be of great value in evaluating changes in protein structure under the salt conditions used in HIC.

2.5 Fluorescence

The study of proteins by fluorescence using cis-parinaric acid (CPA) as an extrinsic probe was first developed by Sklar et al. (1977). CPA, a natural polyene fatty acid, fluoresed under a hydrophobic environment and readily simulated natural lipid-protein interactions. This method was adapted by Kato and Nakai (1980) to determine the effective hydrophobicity of proteins. They used the initial slope (S_o) of the plot of relative fluorescence intensity as a function of protein concentration as an index of protein hydrophobicity. The S_o values obtained for native proteins, denatured proteins and surfactant-bound proteins correlated well with the effective hydrophobicities obtained by other methods. They showed that as the heat denaturation of ovalbumin and lysozyme proceeded, S_o values increased in direct relation to the increase of hydrophobic sites exposed. This work was extended by Kato et al. in 1981 when the partial denaturation of ovalbumin and lysozyme was correlated to their foaming and emulsifying properties.

Several other workers have used CPA to study the effects of changes in surface hydrophobicity brought about by protein conformational change. Townsend and Nakai (1983) employed CPA in the investigation of how surface hydrophobicity affected foaming properties. They concluded that while S_o played an important roll in foaming, there was no direct correlation. Voutsinas, et al. (1983) also used

CPA to study the effect of heat denaturation on the emulsifying properties of a wide range of proteins. Ismond et al. (1985) used DSC and surface hydrophobicity to follow the changes in vicilin conformation due to electrostatic modifications brought about by derivatization with maleic anhydride. In 1986a, Ismond et al. studied the effects of stabilizing and destabilizing anions on the ability of vicilin to form micelles. The effect of pH on the same phenomenon was also reported by Ismond et al. (1986b). Results from these studies were correlated with surface hydrophobicity data assessed using CPA.

Hayakawa and Nakai (1985) have provided evidence that hydrophobicity may be subclassified into aliphatic hydrophobicity as determined by CPA fluorescence and aromatic hydrophobicity as determined by using 1-anilino-8-napthalene sulfonate (ANS) as the probe. They found that aromatic hydrophobicity was closely correlated with protein insolubility.

Conformational change can be expected to alter the number of hydrophobic sites accessible to the environment of the protein. Fluoresence studies using CPA and ANS have been shown useful in assessing the hydrophobic character of the protein surface. When used under conditions of changing environment where quenching does not occur these techniques should prove useful in monitoring changes in surface

hydrophobicity which may occur under HIC. The combination of DSC, fluoresence and HIC should provide strong evidence of any changes in hydrophobic interaction and protein structure which take place.

3 MATERIALS AND METHODS

3.1 Source of Proteins

Ribonuclease A (Type XII-A) from bovine pancreas, Grade I lysozyme from chicken egg white, Type IV rabbit muscle aldolase, and Grade V ovalbumin from chicken egg were purchased from Sigma Chemical Company. Legumin was prepared from faba bean (*Vicia faba*) meal according to the method of Georgiou, (1987). Aldolase was supplied as a crystaline suspension in 2.5 M ammonium sulfate, 0.01 M Tris, 0.001 M EDTA, and pH 7.5 from Sigma Chemical Company. Extensive dialysis against distilled water was used to remove the salts from the aldolase preparation; the protein was then lyophilized and stored at 4^{o} C.

3.2 Hydrophobic-Interaction Chromatography

Hydrophobic-interaction chromatography (HIC) was carried out on a Waters High-Performance Liquid Chromatography (HPLC) System consisting of one M-45 pump, one Model 510 pump, a Model 680 automatic gradient controller, a U6K injector, and a Model 441 absorbance detector set at 280 nm. The column used was a 75 x 7.5 mm

Bio-Gel TSK-Phenyl-5-PW column from Bio-Rad Laboratories. Retention times were determined using a Shimadzu CR34 Integrator.

Solutions having a concentration of 2 mg/mL of protein in 0.1 M phospate buffer, pH 7, were prepared for each of the selected proteins and injected individually onto the HIC column. Initially, elutions for each protein were carried out using a flow rate of 1 mL/min and a 15 min linear gradient from 1.7 M ammonium sulfate in 0.1 M phospate buffer, pH 7, to 0.1 M phosphate buffer. These were the conditions recommended by the manufacturer (Bio-Rad Laboratories). A peak was produced at approximately 2.4 min when only the phosphate buffer was injected. It was assumed that this represented the void volume of the column. This value was used to calculate the salt concentration in the mobile phase at which each protein was eluted. The calculations were carried out according to the following relationship:

$$C_E = \left[1 - \frac{(t_R - t_m)}{t_g}\right] \times C_A$$

where C_E = salt concentration at elution

C_A = initial salt concentration

t_R = peak retention time

t_m = void volume

t_g = time of gradient

If the C_E were greater than 0 M, further elutions were carried out in which the starting concentration was set equal to C_E and then to values between 1.7 M and C_E. In all cases the 15 min linear gradient was maintained and the C_E was calculated as before. The initial concentration of the ammonium sulfate was adjusted by means of the gradient controller rather than preparing new solutions for the initial gradient concentration. Also calculated for each chromatogram were the adjusted retention time (t'_R) and the capacity factor (k'). The first value is the difference between the retention time and the time needed for the void volume to elute. The capacity factor represents the amount of time a solute remains in the stationary phase of the chromatographic column. The latter is the ratio of time spent in the stationary phase (t_R) to the time spent in the mobile phase (t_m). The capacity factor is a commonly used measure of the ability of a column to retain a solute.

3.3 Differential Scanning Calorimetry

The thermal analysis of the proteins was carried out according to the method of Arntfield and Murray (1981) using a Model 9900 Dupont Thermal Analysis System. The cell base was a Dupont Differential Scanning Calorimeter, Model 910, operated under 300 psi of nitrogen. Samples were prepared by mixing 10 mg of protein in 100 uL of 0.1 M phospate buffer, pH 7.0, to which had been added the desired concentration of

ammonium sulfate. In addition to the initial 1.7 M ammonium sulfate concentration, salt concentrations equivalent to the concentration calulated to exsist at the time of elution, 0.0 M, and other selected intermediate salt concentrations were used. These are shown in Table 1. Approximately 10 mg of each sample were weighed to an accuracy of 0.01 mg using a Mettler AE 163 analytical balance, placed into a preweighed DSC pan and hermetically sealed. Samples were scanned as they were heated from ambient temperature to 120^{o}C at a rate of 10 ^{o}C/min. The only exception to this was in the case of aldolase where it was necessary to increase the upper temperature limit to 150 ^{o}C. The reference was an empty, hermetically sealed DSC pan. The computerized data analysis system which is a part of the Model 9900 recorded the temperature of denaturation and calculated the enthalpy of the reaction. The changes in heat flow as a function of temperature were plotted and the results were printed on a Hewlett Packard Model 7470A two pen plotter.

3.4 Fluorescence

The proteins were prepared as 2 mg/mL solutions in 0.1 M phosphate buffer, pH 7.0, containing the concentrations of ammonium sulfate given in Table 1. These solutions were then diluted in serial fashion to give concentrations of 0.0256, 0.064, 0.16, 0.4, and 0.6 mg/mL. A Perkin-Elmer Model LS 5 fluorescence spectrometer with the

Table 1. Ammonium sulfate concentrations used as environmental conditions for the determination of the thermal properties and surface hydrophobicity values of five different proteins.

Salt Conc.(M)	Ovalbumin	Legumin	Lysozyme	Ribonuclease	Aldolase
1.700	+	+	+	+	+
1.275	+	+	+	+	+
1.000	+	+	+	+	+
0.850	+	+	+	+	+
0.765	+	+	+	c	+
0.680	+	+	+	+	+
0.600	c		+	c	
0.510	c	+	+		+
0.380	c	+	c	t	+
0.310	t				
0.230			t		
0.000	+	+	+	+	+

[1]+ - concentration used in all studies.

[2]c - concentration used in chromatographic studies only.

[3]t - concentration used in thermal and fluoresence studies only.

slit width set to 5 nM and the fixed scale set at 1.0 was used to measure the relative fluoresence of all the samples. The temperature was maintained at 22 ^{o}C using a Haake DG-1 circulating water bath. Cis-parinaric acid (CPA; Calbiochem-Behring Corporation) was used as a probe to determine the effective aliphatic surface hydrophobicity. Using the method of Kato and Nakai (1980), 3.6 x 10^{-3} M cis-parinaric acid in ethanol was prepared containing an equal molar concentration of butylated hydroxytoluene as an antioxidant. All glassware used was cleaned by boiling in nitric acid. Two milliliters of a protein solution was dispensed into a cuvette; intrinsic fluoresence of the protein was then measured using an excitation wavelength of 390 nM and an emission wavelength of 470 nM. The total fluoresence was determined by adding 10 uL of the cis-parinaric solution to the cuvette, mixing, and reading the fluoresence emission. The relative fluoresence was calculated by subtracting intrinsic fluoresence from total fluoresence. The hydrophobicity was given by the initial slope of the plot of protein concentration as a function of relative fluoresence intensity. The aromatic hydrophobicty was also determined using 1-anilino-8-napthalenesulfonate (ANS; Sigma Chemical Company) as the extrinsic probe. As described by Hayakawa and Nakai(1985) the ANS solution was 8 mM in 0.02 M phosphate buffer, pH 7.4. The excitation wavelength was 325 nM and the emmission wavelength was 420 nM. All measurements were carried out using the methodology given for CPA.

3.5 Protein Physical Properties

The amino acid content of the legumin was determined according to the method of Bidlingmeyer et al. (1984). Legumin samples were dissolved in 0.1 N HCl (20 ug/mL). A sample volume corresponding to 0.1 to 5.0 ug was pipetted into a 50x60 mm tube; the tube was subsequently placed into a vacuum vial. The vial was then attached to the Waters Pico.Tag Workstation and the solvent was removed under vacuum. When dry, 200 uL of 6 N HCl containing 1% liquid phenol was added to the vacuum vial. The vial was then replaced on the workstation and air was removed by alternately evacuating the vial and then flushing it with nitrogen. The final step in the hydrolysis was to reduce the pressure to 1-2 Torr and to place the sample in the workstation oven at 105^{o} C for 24 hrs. After hydrolysis, the acid solution was removed under vacuum, then redried by adding 10 uL of a freshly prepared solution of triethylamine (TEA), ethanol and water (2:2:1) to the sample tube and then drying it again under vacuum.

The sample was derivatized with phenylisothiocyanate (PITC). The derivatizing reagent, prepared fresh daily, consisted of ethanol, TEA, water and PITC (7:1:1:1). The PITC amino acids were prepared by adding 20 uL of solution to the sample tube and allowing it to stand for 20 min at room temperature. At the end of this time the remaining

solution was removed under vacuum and the sample was diluted with 200 uL of diluent solution containing 2mM disodium hydrogen phosphate and 5% acetonitrile at pH 7.4.

The sample was analyzed on the HPLC system described previously using a Waters Pico.Tag reversed-phase column (15 cm x 39 mm). The column was maintained at a temperature of 38^{o} C by means of a Waters Temperature Control Module. The gradient described in Table 2 was used to elute the derivatized amino acids. Mobile phase A consisted of 0.134 M sodium acetate containing 0.05% TEA and 6% acetonitrile and adjusted to pH 6.40. Mobile Phase B was 60 % acetonitrile in water. Quantitation was carried out by means of external standards. Amino acid standard mix H from Pierce Chemical Company was used. The standards (5uL) were placed in a 50x60 mm tube and the same derivatization procedure used for the legumin was followed. The identification and quantitation of the amino acids was carried out using the Shimadzu CR34 Integrator. Since tryptophan is destroyed by acid hydrolysis, the spectrophotometric method of Messino and Mussaro (1972) for the determination of free and bound tryptophan was used. The average hydrophobicity of legumin was determined from the amino acid concentrations using the method of Bigelow (1967).

Table 2. Elution gradient used for the Pico.Tag analysis of legumin.

Time (min.)	Flow (mL/min.)	%A[1]	%B[2]	Curve
initial	1.0	100	0	*[3]
10.0	1.0	54	46	5[4]
10.5	1.0	0	100	6[5]
11.5	1.0	0	100	6[5]
12.0	1.5	0	100	6[5]
12.5	1.5	100	0	6[5]
20.0	1.5	100	0	6[5]
20.5	1.0	100	0	6[5]

[1]% Mobile phase A pumped.

[2]% Mobile phase B pumped.

[3]no curve involved

[4]slightly convex gradient curve

[5]linear gradient

3.6 Statistical Analysis of Data

The data presented in this study were subjected to analysis of variance and then examined by a Duncan's test at the 5% level of confidence to determine which values were significantly different. All data given in the form of graphs were tested for correlation with the plotted curves using analysis of variance. Both linear and fourth degree polynomial fits were tested. The equations for these curves together with the related correlation coefficents are presented in Appendix A.

4 RESULTS AND DISCUSSION

Several factors were considered when selecting the proteins to be used in this study. Since hydrophobic interactions form the basis upon which hydrophobic interaction chromatography (HIC) retains proteins, compounds were selected to give a wide variation in average hydrophobicity (see Table 3). The proteins were also selected to have a wide range of molecular weights since both average hydrophobicity and surface hydrophobicity are connected in some degree to the size of the molecule. Also, lysozyme, ribonuclease A and ovalbumin are monomers while aldolase is a tetramer (Penhoet et al., 1967) and legumin is believed to be a hexamer (Wright and Boulter, 1974). Because fluoresence was to be used as part of the methodology the proteins could not contain metallic ions since these ions have been shown to quench fluoresence. Finally the proteins chosen had to be soluble within the solvent systems used in this study.

4.1 Determination of Legumin Hydrophobicity

The Bigelow hydrophobicity for legumin was not available from the literature and was therefore determined

Table 3. The molecular weights and Bigelow hydrophobicities of five different proteins used in this study.

Protein	Mol. Wt.	$\bar{H}$ (cal./AA res.)	Reference
Ribonuclease	13,700	780	Yankulov (1970).
Lysozyme	14,000	890	Canfield and Anfinsen (1963).
Ovalbumin	44,000	980	Lewis *et al*. (1950).
Aldolase	160,000	960	Anderson *et al*. (1969).
Legumin	363,000		Georgiou (1987).
		834	Present Study.

experimentally. Pico.Tag analysis was used to determine the amino acid composition of legumin. Tryptophan levels were measured by the method of Messineo and Musarra (1972). The results of both these analyses are given in Table 4. These values were then used to calculate the hydrophobicity (HΦ) of legumin according to the method of Bigelow (1967). A value of 834 calories per amino acid residue was obtained. This value was thought to be quite low considering that legumin has a molecular weight of 363,000 (Georgiou, 1987). In comparison, lysozyme which has a molecular weight of 44,000 has a HΦ value of 890 calories per amino acid residue.

In order to confirm the HΦ value obtained for legumin the amino acid content of legumin for five different cultivars of Vicia faba, as determined by Utsumi et al. (1980), were used to obtain HΦ values as a basis for comparison. The results of these calculations are presented in Table 5. Values ranging from 848 to 877 cal/AA residue were obtained using the data of Utsumi et al. On the basis of these data the value presented in this study (834 cal./AA residue) appeared reasonable.

4.2 Hydrophobic Interaction Chromatography

The initial operating conditions of the HIC column for each protein were described previously. Under these conditions lysozyme, ovalbumin and ribonuclease A eluted at

Table 4. Amino acid content of legumin from *Vicia faba*.

Amino Acid	Concentration (Moles/100 G)
Glutamic Acid	1246.26
Aspartic Acid	706.46
Serine	441.10
Threonine	320.74
Arganine	436.57
Lysine	284.47
Histidine	174.68
Tyrosine	204.08
Tryptophan	17.63
Phenylalanine	244.73
Proline	434.06
Glycine	508.52
Alanine	345.68
Valine	287.11
Isoleucine	182.09
Leucine	176.07
Methionine	40.68
Cystine	22.14

Table 5. Average hydrophobicities as determined from the amino acid analysis of legumin from six cultivars of *Vicia faba*.

Vicia faba cultivar	H⌀ (cal./AA residue)
Sanuki-Nagasaya[1]	876.63
S-N-Wase[1]	847.99
Gifu-WAse[1]	848.64
Kumamot-Churyu[1]	865.94
Issun[1]	862.52
Diana[2]	834.17

[1]Amino acid composition taken from Utsumi *et al*. (1980).

[2]Present study.

17.25, 16.19 and 13.15 min respectively. Since it has been shown (Kato et al., 1983) that the lyotropic salt $(NH_4)_2SO_4$ induces hydrophobic interactions between the protein and column, the order of elution was indicitive of the strength of hydrophobic interaction between the individual proteins and the column surface. As the initial concentration of ammonium sulfate was decreased the retention time for these proteins decreased due to the reduced strength of the hydrophobic interaction (Tables 6, 7 and 8 respectively). It might be expected that the order of elution would agree with the Bigelow hydrophobicities given in Table 3. As can be seen the H$\bar{\Phi}$ values for these proteins in the order of elution are 890, 980 and 780. Clearly there was no agreement between retention time and overall Bigelow hydrophobicity. This observation supported the belief that only the hydrophobic sites located at the surface of the protein are important for retention considerations.

The ammonium sulfate concentration at the time of elution (C_E) was calculated and is also presented in tables 6, 7 and 8. This value indicated the solvent environment of the protein at the time of elution. It was interesting to note that the C_E value equalled 0.0 M for lysozyme when the initial salt concentration was 1.7 M, rose to a maximum value of 0.619 M when the starting ammonium sulfate concentration was 0.765 M, then returned to a value of 0.0 M as the initial salt concentration approached 0.0 M. The reason for the original C_E value of 0.0 M was that the

Table 6. High-peformance hydrophobic interaction chromatographic data for lysozyme.

Initial $(NH_4)_2SO_4$ Conc. (M)	Retention Time (t_R;Min.)	Adjusted Retention Time (t'_R;Min.)	Capacity Factor (k')	Elution Salt Conc. (C_E;M)
1.700	17.27±0.61	15.00±0.39	6.82±0.99	0.00±0.01
1.275	14.02±0.00	11.62±0.00	4.84±0.00[a]	0.29±0.00
1.000	9.70±0.01	7.50±0.01	3.40±0.01[a]	0.50±0.00
0.850	6.73±0.00	4.23±0.00	1.70±0.00[a]	0.61±0.00[a]
0.765	5.40±0.01	2.96±0.09	1.13±0.08[b]	0.62±0.01
0.680	4.29±0.09[a]	1.94±0.01[a]	0.83±0.02[b]	0.59±0.00[a]
0.600	4.20±0.17[a]	1.75±0.08[a]	0.85±0.10[b]	0.52±0.00
0.510	3.72±0.00[a,b]	1.13±0.00[b]	0.44±0.00[b]	0.47±0.00
0.380	3.39±0.00[b]	0.89±0.00[b]	0.36±0.00[b]	0.36±0.00
0.000	2.41±0.00	0.00±0.00	0.00±0.00	0.00±0.00

[1]Column values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 7. High-peformance hydrophobic interaction chromatographic data for ovalbumin.

Initial $(NH_4)_2SO_4$ Conc. (M)	Retention Time (t_R;Min.)	Adjusted Retention Time (t'_R;Min.)	Capacity Factor (k')	Elution Salt Conc. (C_E;M)
1.700	16.19±0.71	13.88±0.49[a]	6.03±0.26	0.13±0.07
1.275	13.77±0.00	11.27±0.00[a]	4.51±0.00	0.32±0.00
1.000	4.56±0.21	2.53±0.49[b]	1.25±0.72	0.83±0.03[a]
0.850	3.49±0.52[a]	1.05±0.05[b]	0.43±0.20[a]	0.79±0.02[a,b]
0.765	3.14±0.00[a]	0.74±0.00[b]	0.31±0.00[a]	0.73±0.00[a,b]
0.680	2.84±0.12[a]	0.49±0.25[b]	0.21±0.13[a]	0.66±0.01[c]
0.600	2.51±0.12[a]	0.38±0.07[b]	0.18±0.02[a]	0.59±0.00
0.510	2.40±0.00[a]	0.00±0.00	0.00±0.00	0.51±0.00
0.380	2.40±0.00[a]	0.00±0.00	0.00±0.00	0.38±0.00
0.000	2.40±0.00[a]	0.00±0.00	0.00±0.00	0.00±0.00

[1]Column values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 8. High-peformance hydrophobic interaction chromatographic data for ribonuclease A.

Initial $(NH_4)_2SO_4$ Conc. (M)	Retention Time (t_R;Min.)	Adjusted Retention Time (t'_R;Min)	Capacity Factor (k')	Elution Salt Conc. (C_E;M)
1.700	14.21±0.05	10.68±0.06[a]	4.32±0.04	0.49±0.01
1.275	5.35±0.03	2.84±0.04[a]	1.13±0.02	1.03±0.00
1.000	3.34±0.01	0.86±0.01[a]	0.35±0.00	0.94±0.00
0.850	3.08±0.00	0.58±0.00[a]	0.23±0.00[a]	0.82±0.00
0.765	2.83±0.01	0.32±0.01[a]	0.13±0.01[b]	0.75±0.01
0.680	2.74±0.00	0.43±0.00[a]	0.19±0.00[a,b]	0.66±0.00
0.600	2.40±0.00	0.00±0.00	0.00±0.00	0.60±0.00
0.000	2.40±0.00	0.00±0.00	0.00±0.00	0.00±0.00

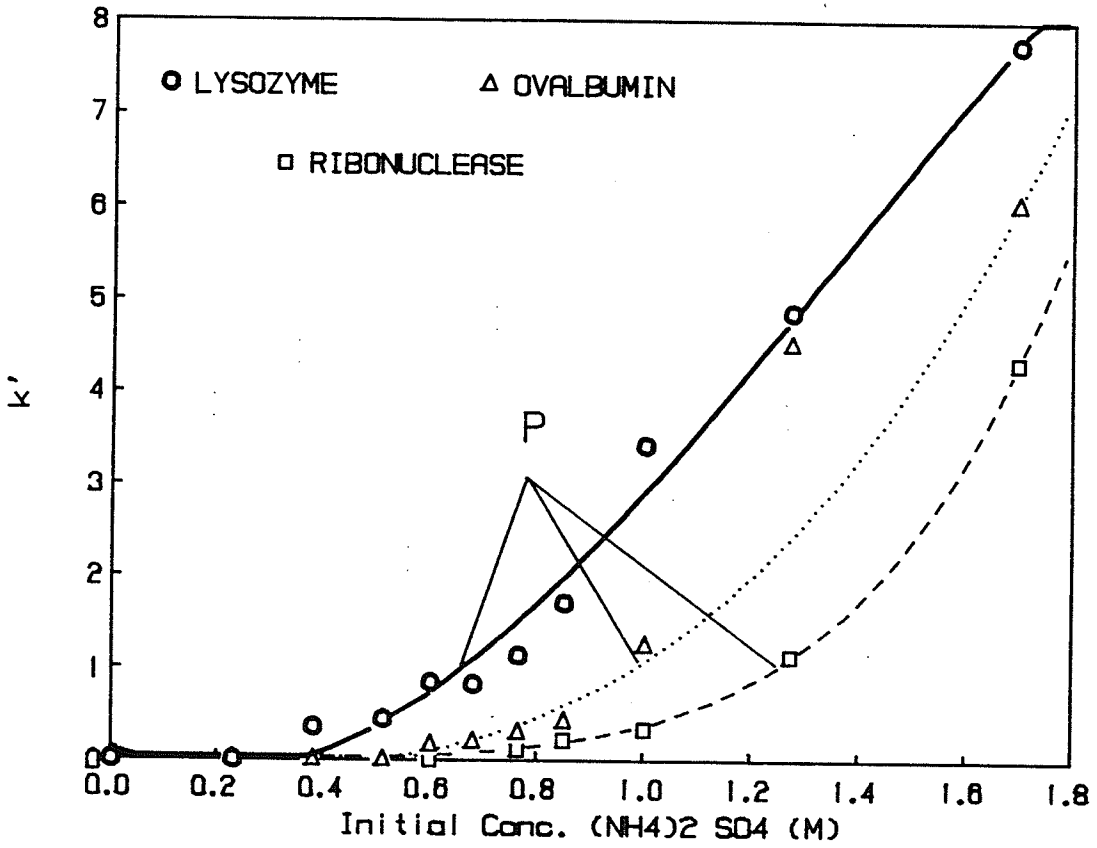
[1]Column values followed by the same letter are not significantly different ($P \leq 0.05$).

strength of the protein-column interaction was such that the protein was eluted after the 15 min gradient was complete and all the salt had been washed from the column. While ovalbumin and ribonuclease did not give initial C_E values of 0.0 M under these conditions they did begin at a low level, rose to a maximum, and then returned to 0.0 M. The maximum C_E value corresponded to a different initial salt concentration for each protein. Lysozyme had the lowest C_E of 0.62, ovalbumin had a value of 0.83 and ribonuclease had a value of 1.03. As the strength of the protein-column interaction decreased a higher initial salt concentration was required to retain the protein and to produce a maximum C_E and hence the greater the value of C_E itself. A further discussion of the pattern presented by the C_E values will be undertaken following an examination of the capacity factors calculated from the chromatography of lysozyme, ovalbumin and ribonuclease.

The column capacity factor (k') is a widely accepted measure of the ability of a column to retain a compound. This was used by Kato et al. (1983) (using silica based phenyl columns) to show the dependence of retention time on the initial concentration of ammonium sulphate in the mobile phase. Capacity factors were calculated for lysozyme, ovalbumin and ribonuclease and are also presented in tables 6, 7 and 8. As can be seen from these values, the ability of the column to retain these proteins decreased with a decrease in the initial salt concentration until a point was

reached where the column could no longer retain the solute. The point at which this occurred varied directly with the strength of the protein-column interaction. The k' for each of the three proteins was plotted as a function of the initial salt concentration in Figure 1. As can be seen from this graph the relationships were non-linear. Statistical analyses showed that lines drawn through the data using linear regression had correlation constants of 0.943, 0.876 and 0.799 for lysozyme, ovalbumin and ribonuclease respectively. Curves determined by fourth degree polynomials had correlations of 0.995, 0.995 and 0.9995 respectively (Appendex A). A linear relationship would represent the most direct relationship between the initial salt concentration and k'. The greater the slope of that line the more rapid the increase in retention of the protein. As the salt concentration increased, a threshold was reached for each protein above which k' rose above zero, indicating that the protein was being retained. From this point the value of k' increased at a rate which depended on the strength of the protein's interaction with the column. Thus the slopes for the curves showing k' as a function of initial ammonium sulfate concentration (Figure 1) were ordered as follows: lysozyme > ovalbumin > ribonuclease A. On each curve there exsisted a point, beyond which, the slope increased more abrubtly. The trend was less obvious for lysozyme since the relationship between salt concentration and k' closely approximated a straight line. However, the change in slope

Figure 1. The effect of initial ammonium sulfate concentration on the retention of lysozyme, ovalbumin and ribonuclease using a TSK-Phenyl-5-PW column.


LYSOZYME
OVALBUMIN
RIBONUCLEASE
P
k'
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
1
2
3
4
5
6
7
8
Initial Conc. (NH4)2 SO4 (M)

was quite obvious for both ovalbumin and ribonuclease. For the purposes of this study this point was referred to as the critical point (P). It is possible that a biphasic relationship may exsist between the data before and after P in which the data on either side of this point might conform to a straight line. Each of these lines would have a distinct slope. The exsistance of such a relationship would be strong evidence of a distinct change in the protein-column interaction. Proof of such a relationship would require more data than could be aquired in this study. This is particularly true at concentrations above P in Figure 1 where there are only three data points for ovalbumin and two for ribonuclease.

For each of the above mentioned three proteins P corresponded to an approximate k' value of 1. Since the capacity factor is the ratio of the time spent in the stationary phase to the time spent in the mobile phase, a k' value of 1 would represent that point where the two periods were equal. Initial salt concentrations which would give values of k'= 1 for lysozyme, ovalbumin and ribonuclease A were determined from the curve equations. Ammonium sulfate concentrations of 0.66, 0.98 and 1.24 respectively were obtained. They increased inversely with the stength of the hydrophobic interaction between the protein and the column. Lysozyme formed the strongest interactions and was followed by ovalbumin and ribonuclease.

The fact that the C_E value rose to a maximum and then decreased was discussed earlier. These values were plotted as a function of the k' values in Figure 2 and it can be seen from this graph that the maximum value of C_E corresponded to k'=1. As the initial salt concentration increased k' remained at zero until the salt content was great enough to initiate binding between the column and the protein. The salt concentration required depended on the surface hydrophobicity of the protein, with lysozyme being bound first followed by ovalbumin and ribonuclease respectively. This was supported by the thermal and fluoresence data (reported later) which indicated a steady increase in hydrophobicity over this range of initial salt concentration with no abrupt changes in stability. Figure 3 gives C_E as a function of initial salt concentration. The period during which no binding occurred was characterized by an initial slope of 1 since the protein eluted with the solvent front and as a result C_E was the same as the initial salt concentration. The slope of the line decreased once binding began ($k' > 0$) since the gradient reduced the value of C_E while the protein was in the stationary phase. During this portion of the graph the increase in initial salt concentration was greater than the increase in C_E. At P (the critical point) C_E attained a maximum. This value became successively higher for lysozyme (0.62), ovalbumin (0.83) and ribonuclease (1.03) since a higher initial salt concentration was needed to bind the proteins as the surface hydrophobicity decreased from one protein to the next. It is

Figure 2. The effect of retention on the elution environment of lysozyme, ovalbumin and ribonuclease.

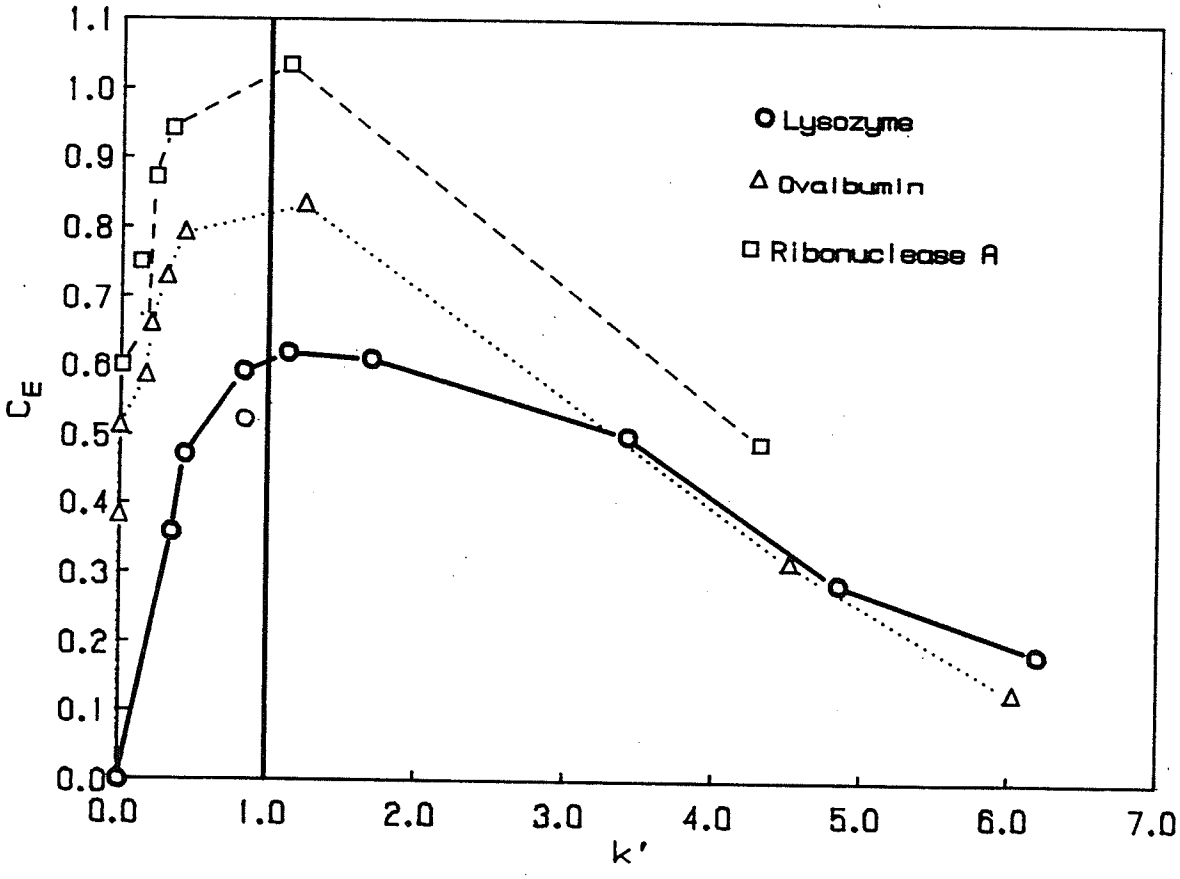
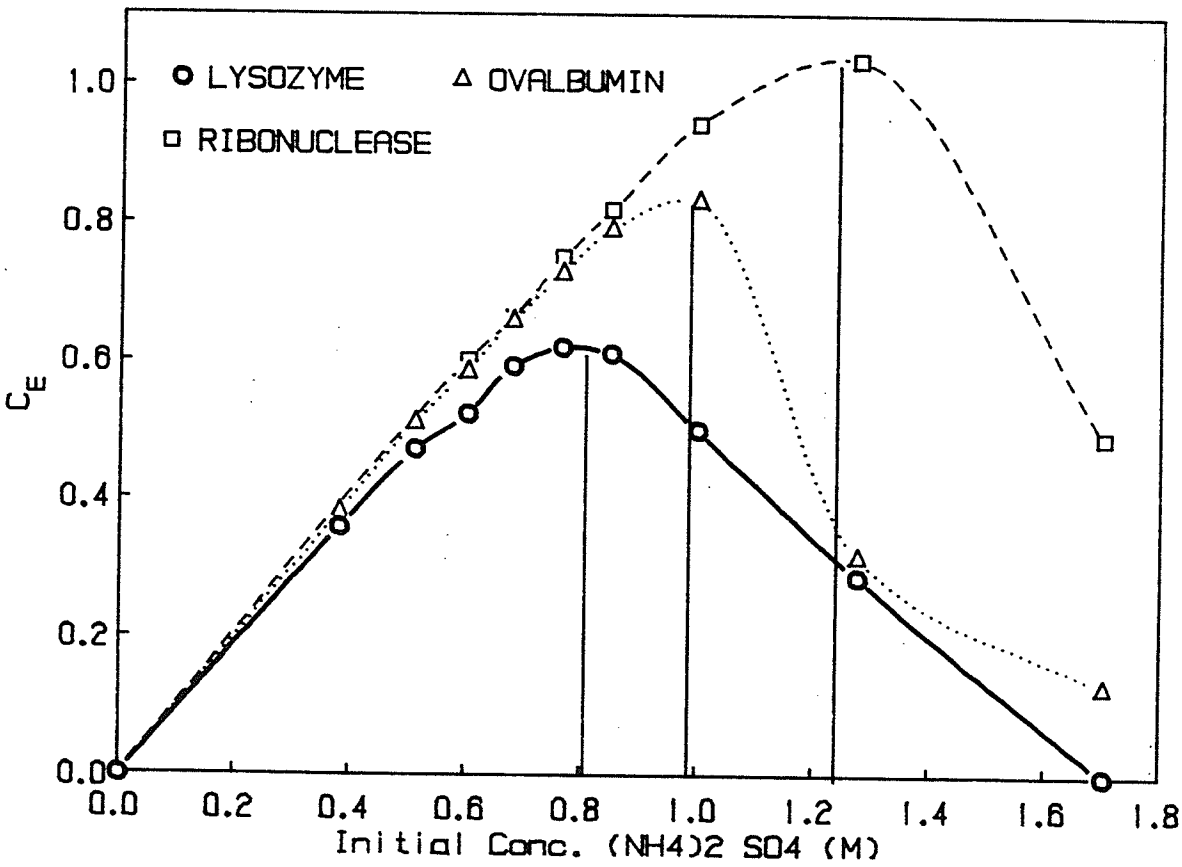

Lysozyme
Ovalbumin
Ribonuclease A
C_E
k'
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0

Figure 3. The effect of initial ammonium sulfate concentration on the elution environment of lysozyme, ovalbumin and ribonuclease.


LYSOZYME
OVALBUMIN
RIBONUCLEASE
C_E
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Initial Conc. (NH4)2 SO4 (M)

illustrated in Figure 1 that k' increased rapidly following P. The rate of increase in aromatic surface hydrophobicity (discussed later) also rose sharply following the initial salt concentration which gave rise to P for each of the three proteins, indicating a corresponding increase in the potential for the proteins to form hydrophobic associations. At the same time the curve of C_E (Figure 2) attained a negative slope and rapidly approached zero. During the period of residence on the column the HIC gradient reduced the level of ammonium sulfate eluting from the column. Since an increase in retention meant that the proteins were held on the column longer the gradient was able to reduce the salt content of the elution environment (C_E). The increase in the rate at which the retention changed following P suggests that there is an important change in the binding mechanism at this point.

Since both the maximum value of C_E and k'=1 were centered around the same point in the elution of a protein and since the retention of the protein on the column was dependent on its surface hydrophobicity, it was possible to speculate that a change in the stability and/or the conformation of the protein takes place at this point. However, the literature on hydrophobic interaction chromatography does not address these features. In an attempt to clarify what had happened to the protein conformation and stability between the ammonium sulfate concentrations of 1.7 and 0.0 M and, more particularily, at

the point where k'=1, DSC and fluoresence data were obtained for all five proteins under the same salt concentrations used in the HIC portion of the study.

Aldolase and legumin were chromatographed under the same initial conditions as the above mentioned proteins but they eluted at the void volume. As a result they were not retained at all by this column. This result persisted even when the inital ammonium sulfate concentration was increased to 2 M. The possiblity that these proteins were excluded from the pores of the column due to their large size (legumin-363,000 and aldolase-160,000) does not seem likely because TSKgel Phenyl-5PW was manufactured from TSKgel G5000PW through the addition of phenyl groups. This original material is described by Kato et al. (1984a) as a hydrophilic-polymer-based material of large pore size for high-performance gel filtration. They go on to state that the resulting HIC column is macroreticular having pores with a diameter of several thousand angstroms into which even very large molecules can easily penetrate. This column then is adequate for proteins with very high molecular weights.

Since the pore size of the column used here would appear to be large enough to accommodate legumin and aldolase, the lack of retention must be caused by surface features of the proteins. Such features would include surface charge and polarity as well as the accessibility of

these features to the active sites on the HPLC column. Since the column material contains very little if any charged character, the presence or lack of charge on the protein surface would not have a significant influence on retention. The amount of surface hydrophobicity and its accessibility to the column remain as the most logical factors affecting the premature elution of these proteins.

Legumin and aldolase have large molecular weights as compared to the other proteins in this study, (Table 3). Chothia (1976) showed that the proportion of polar surface buried when a protein folded remained constant (75 ± 4%) as molecular weight increased. On the other hand the proportion of non-polar surface buried increased from 60% for pancreatic trypsin inhibitor (M.W. 6,000) to 75% for carboxypeptidase (M.W. 34,000). This argument would indicate that a low surface hydrophobicity could be expected for legumin and aldolase.

Kato et al., 1983 suggested that HIC columns separate proteins on the basis of relative surface hydrophobicity rather than molecular weight. The results of this study suggest that, while surface hydrophobicity played a central role, the mechanism of retention was more complex. Table 9 contains a comparison of molecular weights, aromatic surface hydrophobicities, the ratio (B) of hydrophobicity to molecular weight and the retention time for each of the proteins studied using 1.7 M as the initial salt

Table 9. A comparison of surface hydrophobicity, molecular weight and adjusted retention time for legumin, aldolase, lysozyme, ovalbumin and ribonuclease A.

Protein	S_o[1]	Mol. Wt	B[2]	t'_R[3] (min.)
Legumin	1966	363,000	5.46×10^{-3}	0
Aldolase	316	160,000	1.97×10^{-3}	0
Ribonuclease	10	13,700	7.12×10^{-4}	10.68
Ovalbumin	705	44,000	1.60×10^{-2}	13.88
Lysozyme	224	14,000	1.60×10^{-2}	15.00

[1] S_o - the ANS surface hydrophobicity of the protein in 1.7 M ammonium sulfate as described in the fluoresence section of this study.

[2] B - the ratio of S_o/mol. wt.

[3] t'_R - the adjusted retention time of the protein on the HIC column used in this study with an initial ammonium sulfate concentration of 1.7 M.

concentration. Aliphatic surface hydrophobicity was not used since the results obtained in this study were not available in many cases. This comparison revealed that the ANS hydrophobicity data did not correlate with the retention data. When the values of B were considered it was seen that the values for lysozyme and ovalbumin, two proteins that were strongly retained, were ten times greater than for legumin and aldolase which were unretained. Ribonuclease alone did not conform to this pattern. This anomaly may be explained by the fact that ribonuclease does not contain tryptophan, the main aromatic component in proteins. ANS is aromatic and may bind more easily to tryptophan than to aliphatic side chains. Supporting this argument was the fact that the CPA hydrophobicity for ribonuclease appeared higher than for lysozyme or ovalbumin (Tables 10-12). The latter two proteins had the same B values but differed in retention times, 15.00 and 13.88 min respectively. It is possible that this small difference in retention may be attributed to the difference in accessibility of the hydropbobic sites to the bound column ligands as opposed to the ANS molecules which were free to move.

The evidence given by Clothia (1976) that proteins bury a relatively larger proportion of hydrophobic residues at high molecular weights and the indication given by this study that retention is governed by a balance of molecular weight, surface hydrophobicity and the acessibility of the hydrophobic sites to the column ligand suggests that legumin

Table 10. Differential scanning calorimetric and fluorometric data for lysozyme.

Initial Salt Conc.(M)	Td[1] °C	ΔH[2] J/g	S_o[3] CPA	S_o[4] ANS
1.700	78.45±1.13	5.77±3.09[a]	23.53±7.44[a]	224.23±14.26
1.275	77.51±0.23[a]	14.44±1.24	63.09±35.87[a]	47.05±2.07[a]
1.000	77.04±0.04[a,b]	11.31±10.07[a]	-[5]	33.26±0.86[a,b]
0.850	76.10±0.35[b]	1.95±0.09[a]	40.46±1.40[a]	31.21±3.58[b,c]
0.765	75.89±0.14[b,c]	4.72±0.14[a]	54.21±42.35[a]	16.90±1.60[c,d]
0.680	75.49±0.07[c,d]	1.88±0.13[a]	20.78±9.40[a]	13.58±0.41[d]
0.600	74.81±0.06[c,d]	2.18±0.20[a]	10.59±0.47[a]	12.32±0.41[d]
0.510	74.44±0.06[d,e]	1.65±0.00[a]	16.96±5.49[a]	10.22±0.76[d]
0.230	73.25±0.07[e,f]	1.85±0.04[a]	18.30±1.40[a]	7.25±1.24[d]
0.000	71.11±0.26[f]	2.53±0.17[a]	5.98±2.54[a]	4.60±0.01[d]

[1] Td - Denaturation temperature.

[2] ΔH - Entalpy of denaturation.

[3] S_o - Surface hydrophobicity (Kato and Nakai, 1980).

[4] S_o - Surface hydrophobicity (Hayakawa and Nakai, 1985).

[5] - Data not available.

[6] - Column values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 11. Differential scanning calorimetric and fluorometric data for ovalbumin.

Initial Salt Conc.(M)	Td[1] °C	ΔH[2] J/g	S_o[3] CPA	S_o[4] ANS
1.700	103.04±0.17	11.06±0.02[a]	24.63±1.13[a]	704.62±102.78
1.275	100.82±0.73	9.42±0.54[a]	13.02±2.29[c,d]	344.32±33.69[a]
1.000	99.49±0.13	9.69±0.12[a]	20.55±1.11[a]	254.94±19.35[a,b]
0.850	98.14±0.02	10.42±0.11[a]	12.89±0.17[c,d]	204.90±8.04[a,b,c]
0.765	96.45±0.23[a]	11.36±0.78[a]	15.34±2.67[b,c,d]	225.80±41.22[a,b,c]
0.680	96.62±0.26[a]	10.37±0.05[a]	11.01±0.89[d]	165.33±8.53[b,c]
0.310	93.29±0.05	12.04±0.13[a]	11.22±1.81[d]	109.85±2.48[b,c]
0.000	90.55±0.19	13.20±0.46[a]	18.04±2.51[b,c]	79.97±1.62[c]

[1]Td - Denaturation temperature.

[2]ΔH - Enthalpy of denaturation.

[3]S_o - Surface hydrophobicity (Kayto and Nakai, 1980).

[4]S_o - Surface hydrophobicity (Hayakawa and Nakai, 1985).

[5] - Column values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 12. Differential scanning calorimetric and fluorometric data for ribonuclease A.

Initial Salt Conc.(M)	Td[1] °C	ΔH[2] J/g	S_o[3] CPA	S_o[4] ANS
1.700	75.60±0.07	1.80±0.03[a]	72.12±18.75[a]	9.75±0.97
1.275	74.75±0.02	18.99±3.70[b]	-[5]	7.03±0.19
1.000	72.76±0.07	2.83±0.07[a]	62.99±2.08[a]	4.67±0.55[a]
0.850	71.64±0.09[a]	2.77±0.01[a]	210.51±157.12[a]	3.96±0.36[a,b]
0.680	71.40±0.27[a]	21.01±4.89[b]	123.80±0.00[a]	3.66±0.09[a,b,c]
0.380	68.78±0.17	2.54±0.13[a]	115.29±89.75[a]	2.72±0.05[b,c]
0.000	66.04±0.05	2.87±0.15[a]	7.60±0.76	1.66±0.11[c]

[1] Td - Temperature of denaturation.

[2] ΔH - Enthalpy of denaturation.

[3] S_o - Surface hydrophobicity (Kato and Nakai, 1980).

[4] S_o - Surface hydrophobicity (Hayakawa and Nakai, 1985).

[5] - - data not available.

[6] - Column values followed by the same letter are not significantly different ($P \leq 0.05$).

and aldolase were unable to interact sufficently with this column to be retained. The strength of the column - protein interaction could be increased by using a more hydrophobic column, that is to say, one with either a more hydrophobic ligand or one that has a higher density of phenyl groups.

4.3 Differential Scanning Calorimetry

Arntfield et al. (1986) and Ismond et al. (1986a) have used differential scanning calorimetry (DSC) to follow changes in protein stability with environmental manipulation. They have shown by this means that as a protein became more stable the denaturation temperature (Td) became higher. Some salts such as sodium chloride increased stability while others such as sodium thiocyanate reduced the stability. The results obtained in this study for the effect of increasing amounts of ammonium sulfate on the conformation of lysozyme, ovalbumin, ribonuclease, aldolase and legumin (Tables 10 - 14) supports their findings for lyotropic salts. In all cases an increased concentration of the salt produced a higher Td and thus greater protein stability. Ismond et al. (1986a) also found that the enthalpy of denaturation (Δ H) increased when some stabilizing anions (including SO_4^{-2}) were present. There were no marked trends found in the denaturation enthalpies which could be related directly to the patterns noted with regard to the Td values. However, there were some significant changes in ΔH under the salt conditions used in this study.

Table 13. Differential scanning calorimetric and fluorometric data for aldolase.

Initial Salt Conc.(M)	Td[1] °C	ΔH[2] J/g	S_o[3] CPA	S_o[4] ANS
1.700	67.21±0.17	13.60±0.34[a,b,c]	66.00±0.11	–[5]
1.275	65.96±0.22[a]	12.90±1.43[b,c]	–[5]	432.00±81.00[a]
1.000	66.55±0.06	13.32±0.01[a,b,c]	–[5]	335.00±53.85[a,b]
0.850	66.06±0.02[a]	14.42±0.35[a,b,c]	–[5]	287.98±25.38[b]
0.765	66.06±0.02[a]	15.15±0.59[a]	–[5]	301.60±4.55[b,c]
0.680	65.58±0.03	13.44±1.13[a,b,c]	–[5]	279.50±13.30[b,c]
0.510	64.53±0.07	12.81±0.09[a,b,c]	–[5]	218.45±3.05[b,c]
0.380	64.05±0.04	12.18±1.07[c]	–[5]	187.20±3.50[c,d]
0.000	63.19±0.17	14.84±1.31[a,b]	20.06±0.90	77.55±0.65[d]

[1] Td - Denaturation temperature.

[2] ΔH - Enthalpy of denaturation.

[3] S_o - Surface hydrophobicity (Kato and Nakai, 1980).

[4] S_o - Surface hydrophobicity (Hayakawa and Nakai, 1985).

[5] – - Data not available.

[6] - Column values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 14. Differential scanning calorimetric and fluorometric data for legumin.

Initial Salt Conc.(M)	Td[1] °C	ΔH[2] J/g	S_o[3] CPA	S_o[4] ANS
1.700	128.49±1.01	27.42±0.29[a]	50.04±2.81	2337.05±55.21
1.275	123.82±0.08	27.33±0.21[a]	-[5]	2154.08±0.00[a]
1.000	119.98±0.42	25.97±1.69[a]	-[5]	2050.72±0.00[a]
0.850	114.26±0.08[a]	23.25±0.38[a]	71.78±3.36	1297.58±5.57
0.765	113.31±0.12[a,b]	20.09±1.11[a,b]	-[5]	-[5]
0.680	112.90±0.01[b]	23.17±1.70[a]	-[5]	1799.00±11.92
0.510	108.59±0.28	13.22±7.20[b]	-[5]	1561.00±53.28
0.380	94.73±0.08[c]	13.84±0.72[b]	-[5]	601.65±11.5
0.000	94.83±0.00[c]	12.13±0.29[b]	42.99±14.82	181.30±4.70

[1]Td - Denaturation temperature.

[2]ΔH - Enthalpy of denaturation.

[3]S_o - Surface hydrophobicity (Kato and Nakai, 1980).

[4]S_o - Surface hydrophobicity (Hayakawa and Nakai, 1985).

[5]- - Data not available.

[6] - Column values followed by the same letter are not significantly different ($P \leq 0.05$).

At 1.275 M ammonium sulfate the H value for lysozyme increased from 1.95 J/g at 0.85 M salt to 14.44 J/g at 1.275 M ammonium sulfate indicating a sharp increase in stability at this point. Similarily, ribonuclease showed significant increases in ΔH at 1.275 M (18.99 J/g), at 0.765 M (21.64 J/g) and 0.68 M (21.01 J/g). A complete listing of these values is given in tables 10 - 12.

The denaturation temperatures for lysozyme, ovalbumin and ribonuclease have been plotted as a function of initial salt concentration in figures 4, 5 and 6, respectively. Low concentrations of ammonium sulfate produced a linear relationship with Td values but at higher concentrations the rate at which Td increased was significantly reduced. This trend was most evident for lysozyme (fig. 4) since the shift in rates occurred at a lower salt concentration. However, the same pattern could be observed for ovalbumin and ribonuclease. Regression lines were plotted through the linear portions of the data where the correlation coefficents were greater than 98%. The resulting plot resembled the biphasic model employed by Arntfield et al. (1986) except that the break point did not occur at one specific salt concentration for all the proteins tested. The break point observed here occurred at the same salt concentration associated with the critical point (P) described in the discussion on k' and C_E (Figure 1) indicating that the abrupt increase in retention was associated with the decrease in the rate of protein

Figure 4. The effect of ammonium sulfate concentration on the denaturation temperature of lysozyme.

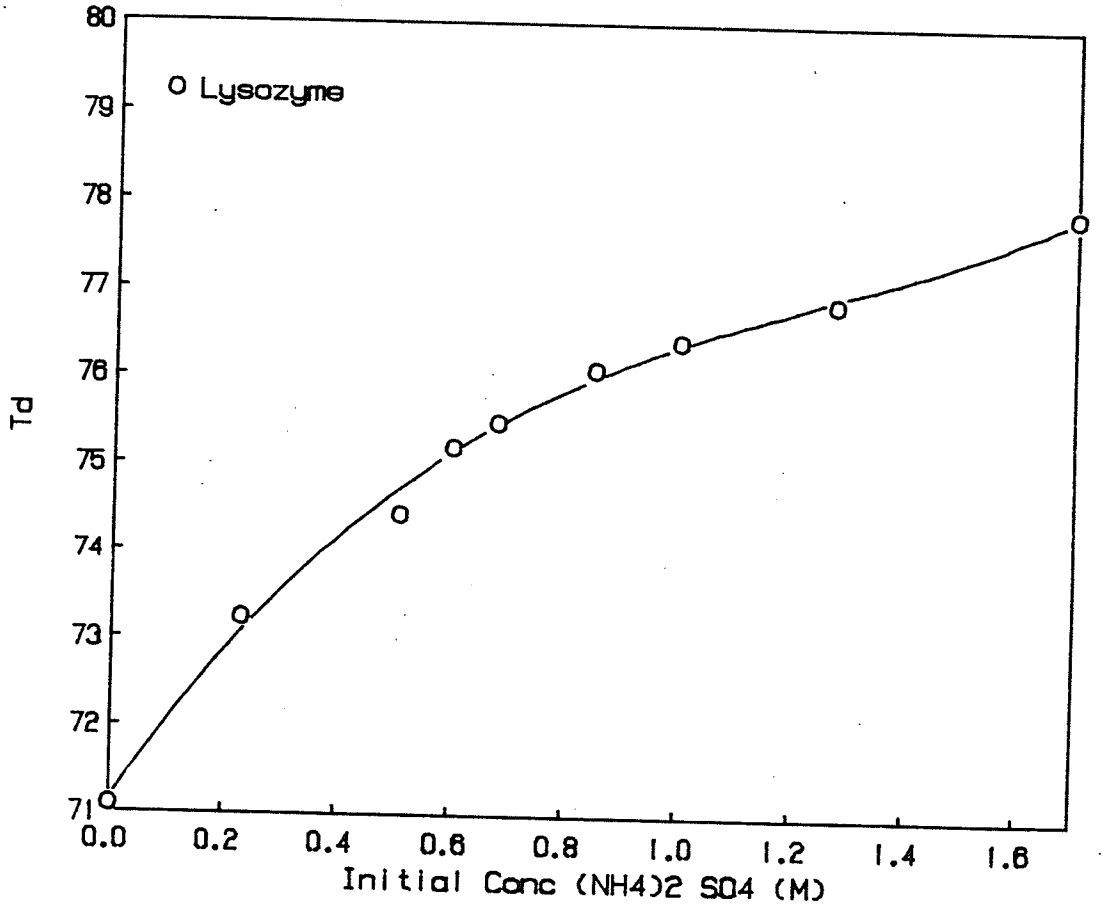

80
79
78
77
76
75
74
73
72
71
Td
O Lysozyme
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc (NH4)2 SO4 (M)

Figure 5. The effect of ammonium sulfate concentration on the denaturation temperature of ovalbumin.

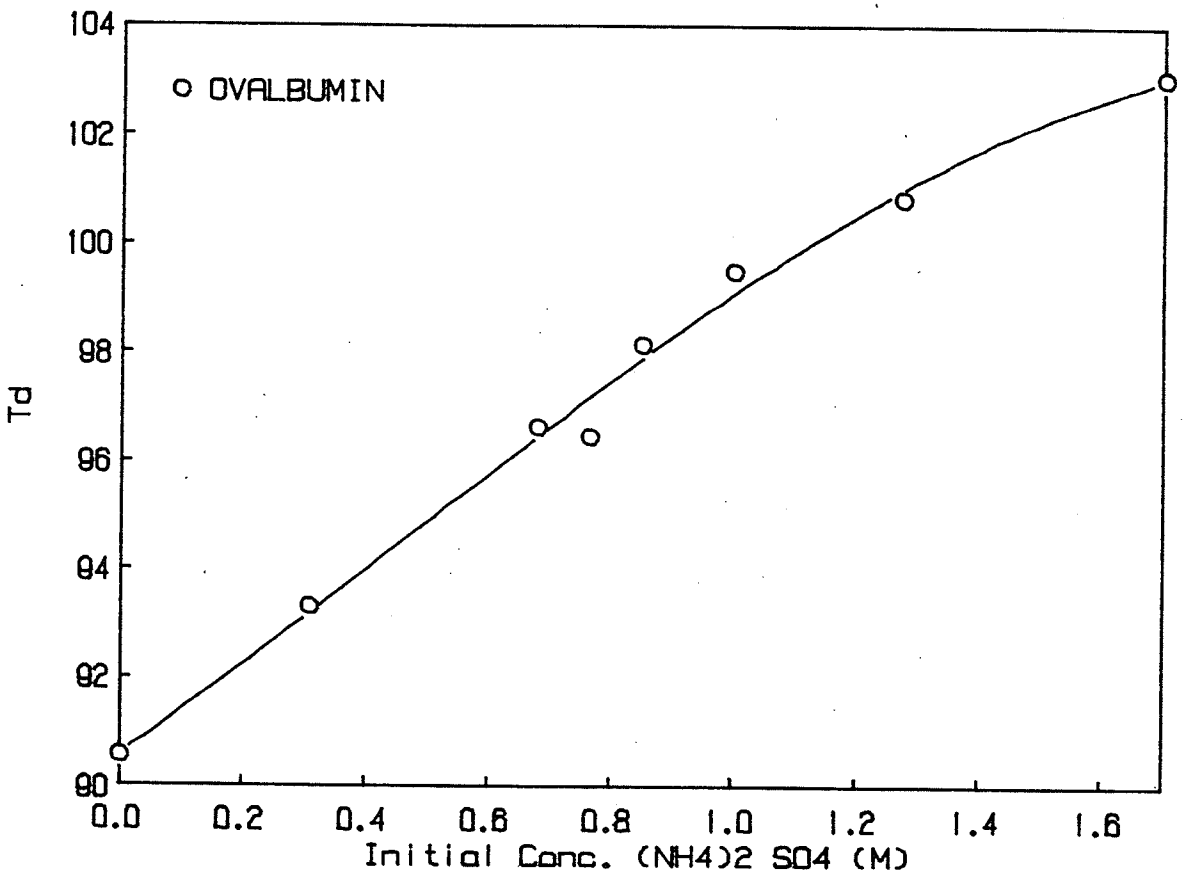
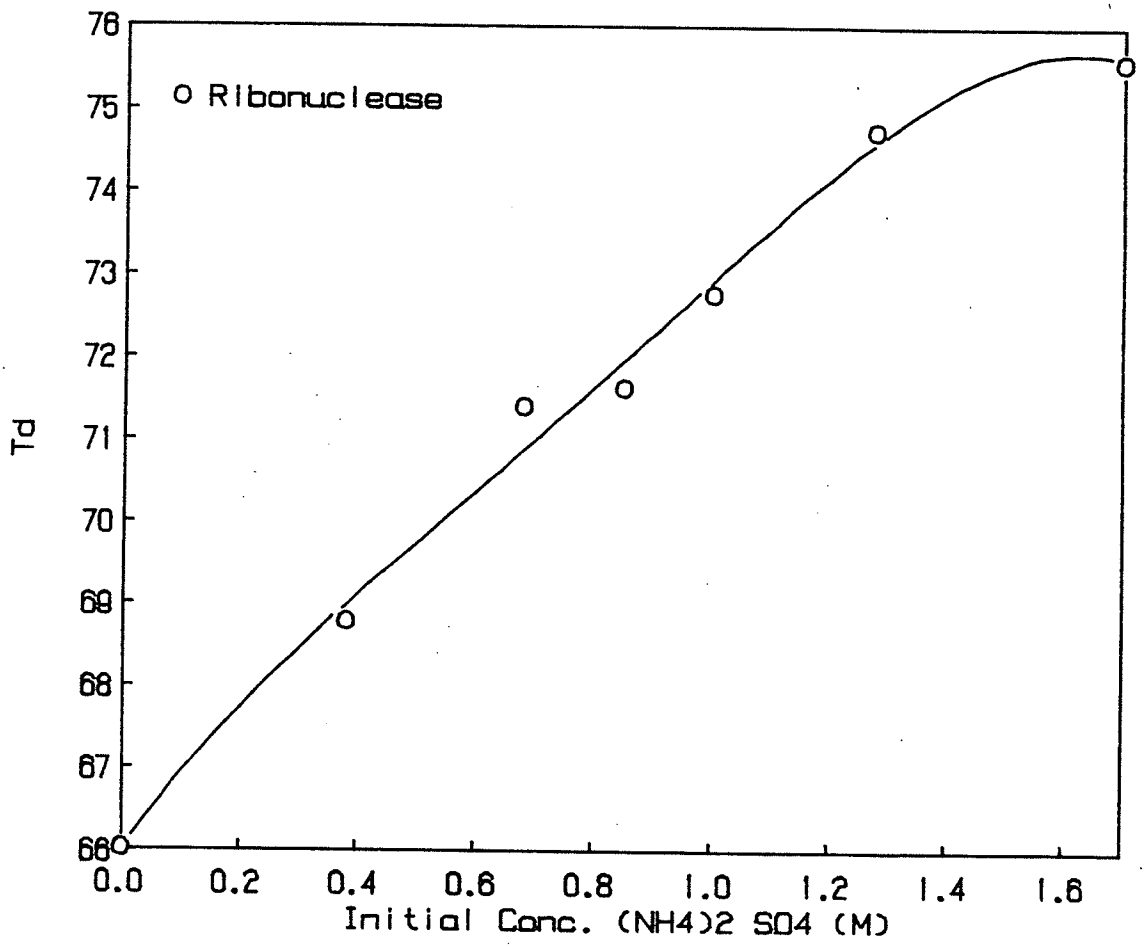

104
102
100
98
96
94
92
90
Td
o OVALBUMIN
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc. (NH4)2 SO4 (M)

Figure 6. The effect of ammonium sulfate concentration on the denaturation temperature of ribonuclease.


o Ribonuclease
Td
76
75
74
73
72
71
70
69
68
67
66
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc. (NH4)2 SO4 (M)

stabilization. It is also possible that the data reported here were biphasic at these salt concentrations but as in the case of the biphasic nature of k' more data would be required for conformation of this model.

Arakawa and Timasheff (1982) indicated that the presence of hydrophobic areas on the protein surface in contact with water causes an ordering of the water molecules next to the protein. This would be a thermodynamically unfavorable situation since it would cause a large decrease in entropy. A lyotropic salt such as ammonium sulfate would be preferentially hydrated by the water thus drawing water molecules away from the protein hydrophobic sidechains. As water molecules are withdrawn, the hydrophobic areas of the protein would have a greater ability to form hydrophobic interactions with other hydrophobic sites on the protein, with hydrophobic sites on the HIC column or with hydrophobic probes such as those used in the fuoresence portion of this study. Such interactions would be thermodynamically more favorable than the water-protein interface. As the initial salt concentration was increased more water would be removed from the vicinity of the protein and more hydrophobic interactions would be possible.

In the DSC analysis of the proteins in this study the only hydrophobic sites present were those on the proteins themselves, therefore, as the salt concentration was increased the number of intra-molecular hydrophobic

interactions increased. These interactions stabilized the proteins and higher Td values were recorded. The linear portions of Figures 4-6 are evidence of this occurrance. However, each protein must reach a characteristic point at which intra-molecular interactions would become more difficult due to steric considerations. As this situation developed, more energy would be required to form such associations. In Figures 4-6 this can be seen as a decrease in the slope since a larger increase in salt concentration was required to bring about an increase in Td. The characteristic point at which the rate of increase in Td (slope) began to decline were 0.765 M, 1.000 M and 1.275 M for lysozyme, ovalbumin and ribonuclease, respectively. These concentrations correlate closely with the initial salt concentrations which produced the critical point (P).

In an environment in which hydrophobic sites other than those on the protein surface were present in solution there would be competition as to whether intra- and inter-molecular hydrophobic interactions would form. Such was the case during the HIC and fluoresence analyses. At initial salt concentrations below P in Figure 1 the slope of the curves is a result of the competition between intra- and intermolecular hydrophobic interactions. Above P intra-molecular associations required more energy and fewer were formed. As a result inter-molecular hydrophobic interactions between the column and the protein increased more rapidly

causing k' to increase sharply. In a similar manner the binding of ANS to the protein increased sharply once the intra-molecular hydrophobic interactions were inhibited. The binding of ANS will be discussed more fully later.

Even though aldolase and legumin were not retained on the HIC column they were analysed by DSC using the same salt concentrations used for the other proteins. The results obtained are presented in tables 13 and 14 while plots of Td as a function of initial salt concentration are given in figures 7 and 8. Unlike the monomers discussed above, the graphs of these polymers did not show a rapid increase in stability at low salt concentrations. In the 0.000 - 0.380 M range legumin remained relatively unchanged as indicated by Td and ΔH values. Aldolase increased slightly in stability between 0.000 and 0.510 M as indicated by the increase in denaturation temperature (63.19 - 64.53 ^{o}C). As the initial salt concentration increased to 0.850 M both Td and ΔH values increased in a linear fashion. Between 0.850 M and 1.000 M the rate of increase in stability for aldolase began to decrease in a manner similar to that seen in the retained proteins with the exception of a significant decrease in stability at 1.275 M. Legumin increased sharply in stability between 0.380 M and 0.510 M initial salt then decreased the rate of change in stabilty between 0.765 M and 1.700 M salt concentrations. Once again there was a period of decreased stability, in this case between 0.765 M and 1.000 M. It would be reasonable to speculate that the differences

Figure 7. The effect of ammonium sulfate concentration on the denaturation temperature of aldolase.

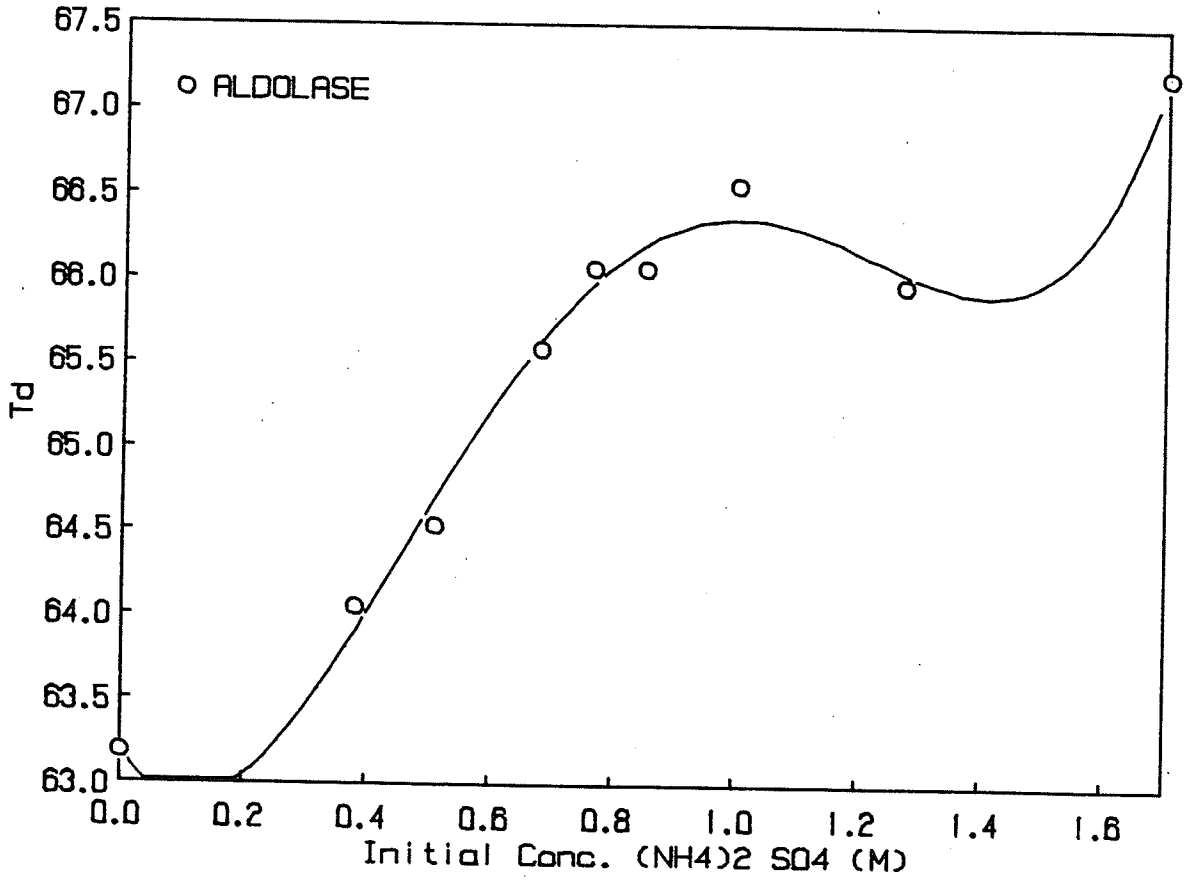
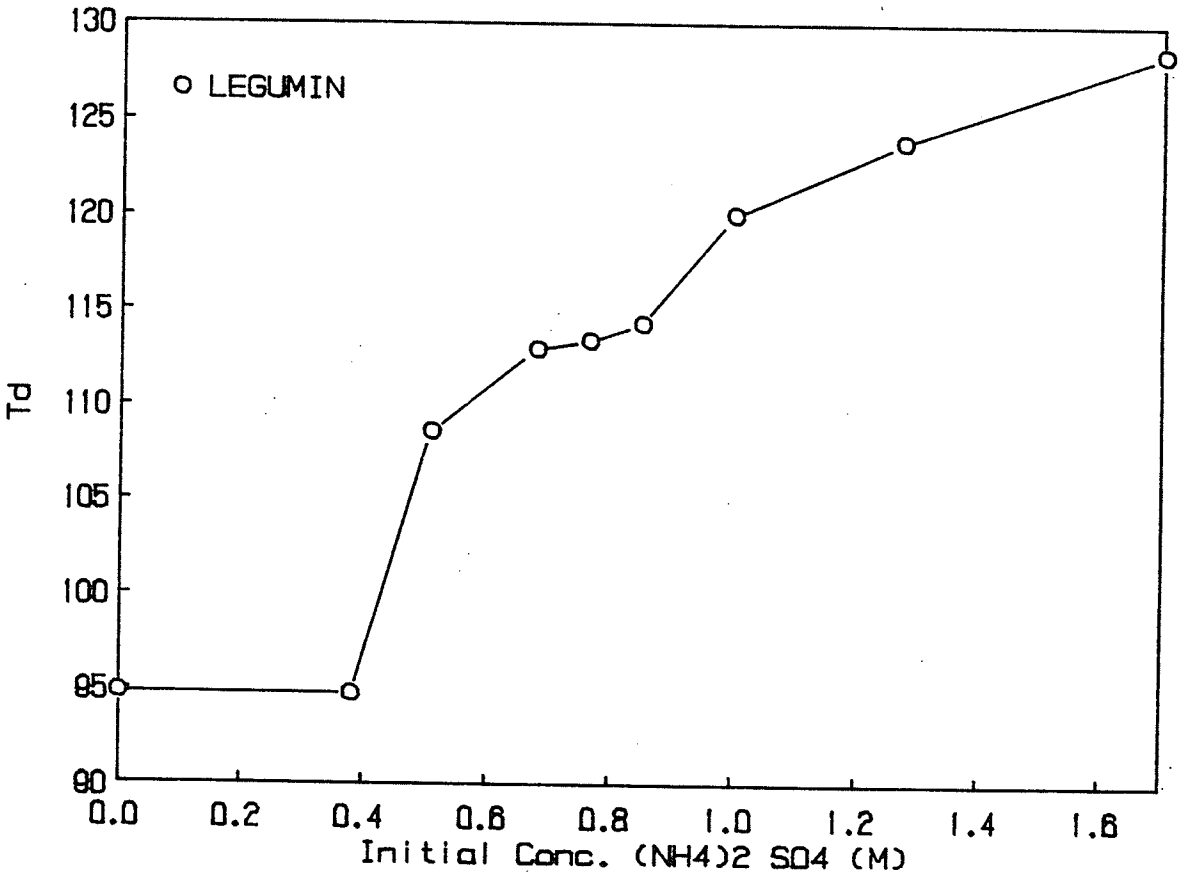

67.5
67.0
66.5
66.0
65.5
65.0
64.5
64.0
63.5
63.0
Td
ALDOLASE
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc. (NH4)2 SO4 (M)

Figure 8. The effect of ammonium sulfate concentration on the denaturation temperature of legumin.


130
125
120
115
110
105
100
95
90
Td
o LEGUMIN
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc. (NH4)2 SO4 (M)

displayed by aldolase and legumin as compared to lysozyme, ovalbumin and ribonuclease occurred due to changes in the bonding status of the subunits. However, investigation into this concept would require a separate study.

An examination of the range of denaturation temperatures exhibited by these proteins over the salt concentration range studied revealed considerable variation. The total changes in Td observed from 0 to 1.7 M ammonium sulfate were; lysozyme-5.10 ^{o}C, ovalbumin-14.21 ^{o}C, ribonuclease-9.56 ^{o}C, aldolase-3.00 ^{o}C and legumin-37.06 ^{o}C. Since an increase in Td represents a corresponding increase in stability, those proteins with a larger change in Td must have increased more in stability. It is shown in table 9 that lysozyme and ovalbumin have the same value for B and yet ovalbumin elutes from the HIC column more than a minute earlier than lysozyme. If the larger decrease in Td values reflected a greater decrease in stability of ovalbumin due to an increased openness of structure the difference in retention may have been due to a greater exclusion from the packing material or a reduced access to hydrophobic sites on the column. However, confirmation of this observation was beyond the scope of this study.

4.4 Fluoresence

Changes in protein conformation due to thermal

denaturation have been followed by Kato and Nakai (1980) using fluoresence spectrophotometry with cisparinaric acid (CPA) as a probe for aliphatic hydrophobicity. Hayakawa and Nakai (1985) have used ANS in a similar manner to assess aromatic hydrophobicity. Ismond et al. (1985) followed conformational changes in vicilin brought about by electrostatic surface modifications, also using CPA. Denaturation of the protein, in these cases, resulted in increased exposure of hydrophobic sites which in turn produced an increase in the fluoresence exhibited. It would be expected that an increase in stability would correspond to a decrease in fluoresence since hydrophobic sidechains would become buried inside the protein.

The proteins used in this research were examined for fluoresence activity at the same ammonium sulfate levels used in the study already described. Intrinsic fluoresence was first measured and then the fluoresence achieved by means of a probe. The increase in fluoresence (relative fluoresence intensity) represented the effect caused by the binding of the probe. Relative fluoresence was plotted as a function of protein concentration as described previously. The slope of the regression line produced for this plot gave a measure of the surface hydrophobicity (S_o) for the protein under the conditions tested (Kato and Nakai, 1980). For each protein the surface hydrophobicity was determined several times at each salt concentration. In order to be considered acceptable the individual readings in two consecutive

determinations of the surface hydrophobicity were required to possess not less than 92 % correlation to the regression line . Both ANS and CPA probes were used and the results are presented in tables 10 - 14.

The ANS hydrophobicities for the five proteins studied were plotted as a function of initial salt concentration and are presented in figures 9 - 13. The feature of these graphs that stood out, considering the above discussion, is that the hydrophobicities increased with increased salt levels. It has been shown through DSC data that increased levels of ammonium sulfate produced a more stable protein and fewer hydrophobic sites would be expected to be available to bind ANS since the sites would be occupied in the stabilization process. Nevertheless, as the initial level of ammonium sulfate increased the surface hydrophobicity, as measured by ANS, also increased. Studies using constant concentrations of individual amino acids and increasing amounts of ammonium sulfate revealed no increase in fluoresence. This indicated that an increase in the amount of probe bound to the protein and not the amount of salt in itself was the cause of the change in fluoresence. Moreover, between the point where the rate of Td change decreased and the 1.7 M maximum salt level the hydrophobicity increased sharply for the three proteins retained by the column. Hofstee (1975) showed that lyotropic salts such as ammonium sulfate enhance intermolecular and intramolecular hydrophobic interactions. It is believed that

Figure 9. The effect of ammonium sulfate concentration on the aromatic surface hydrophobicity of lysozyme.

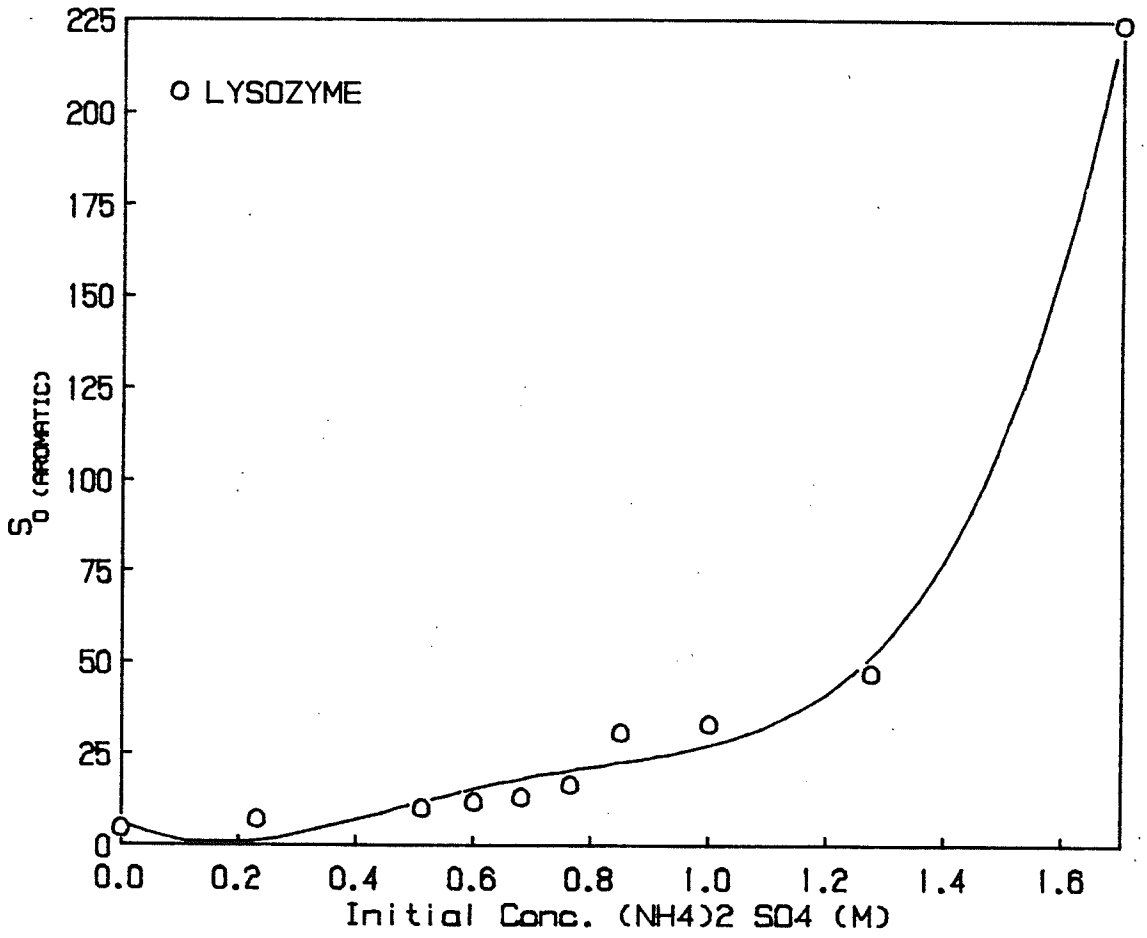

O LYSOZYME
S_0 (AROMATIC)
225
200
175
150
125
100
75
50
25
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc. (NH4)2 SO4 (M)

Figure 10. The effect of ammonium sulfate concentration on the aromatic surface hydrophobicity of ovalbumin.

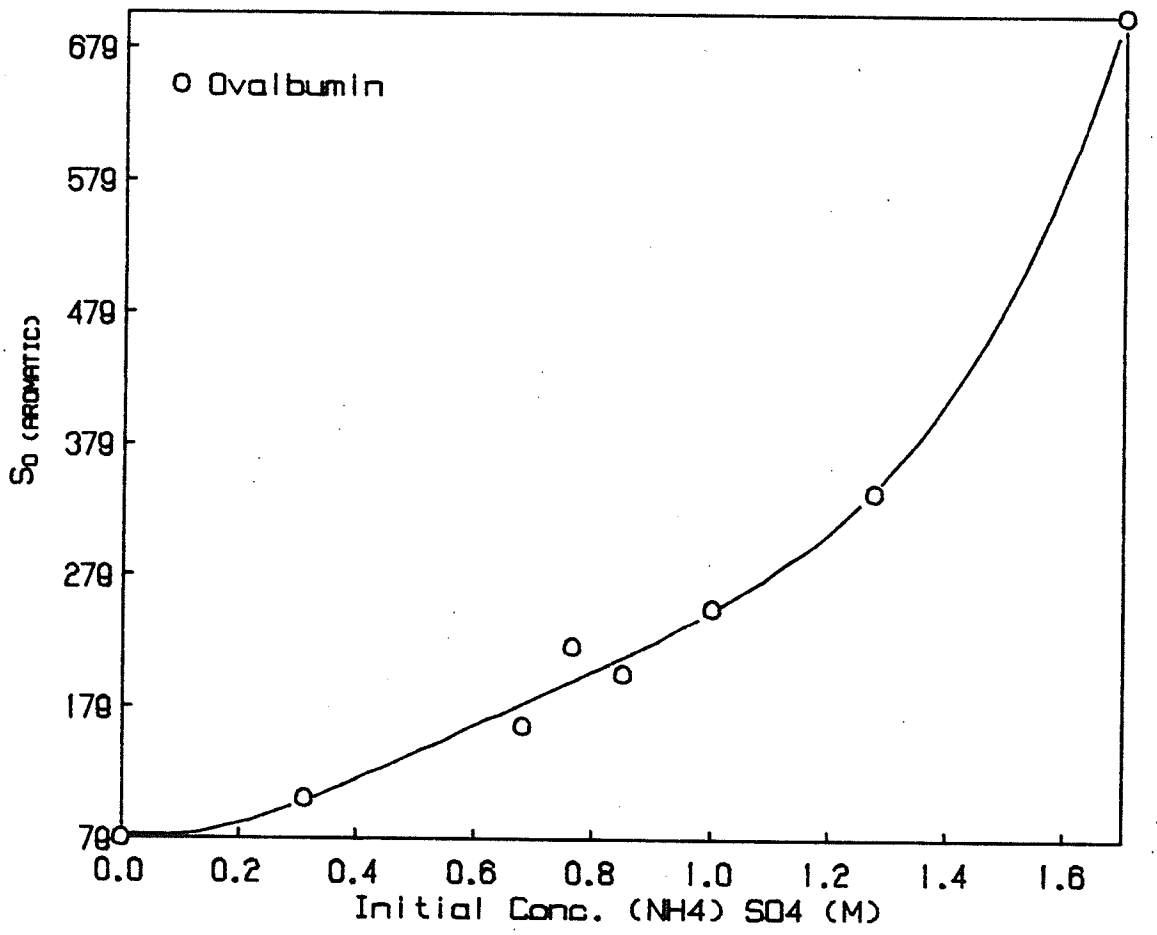

o Ovalbumin
679
579
479
379
279
179
79
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
S0 (AROMATIC)
Initial Conc. (NH4) SO4 (M)

Figure 11. The effect of ammonium sulfate concentration on the aromatic surface hydrophobicity of ribonuclease.

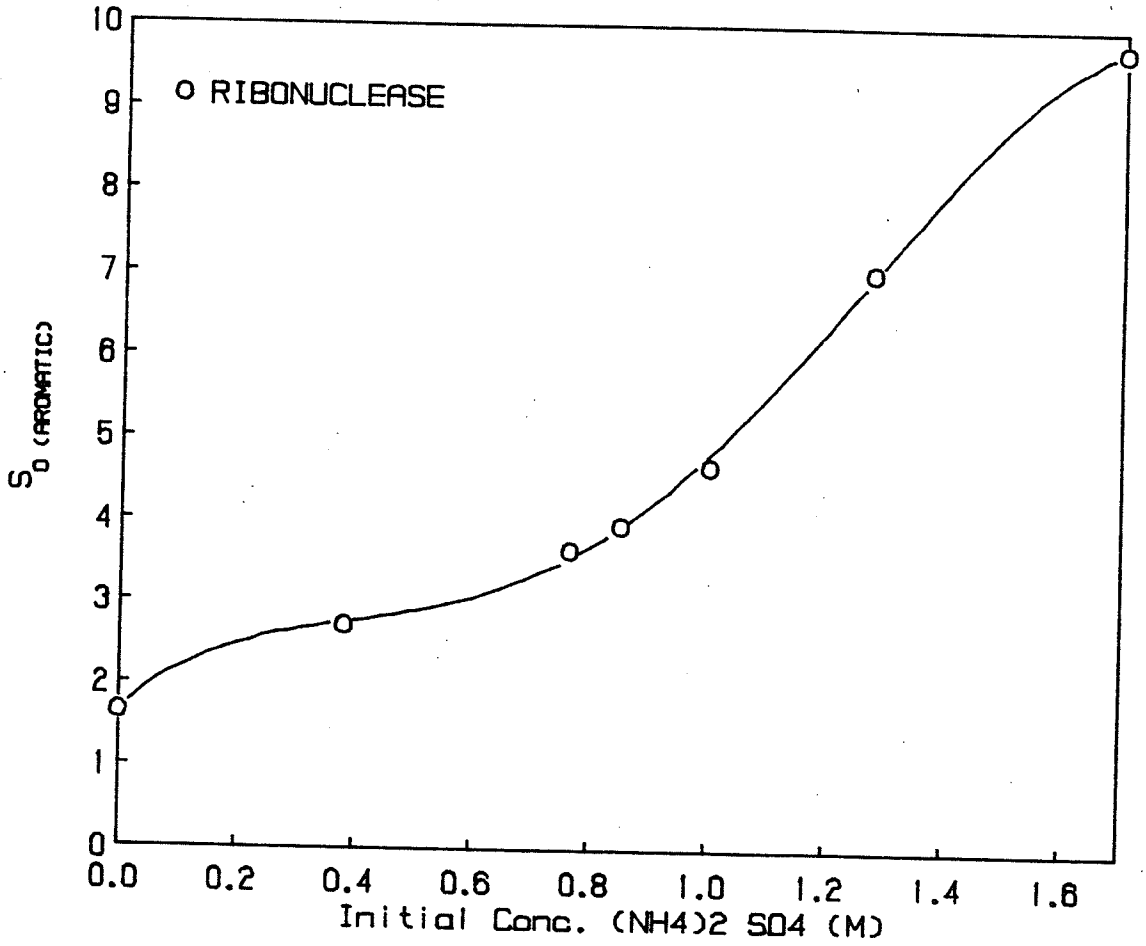

O RIBONUCLEASE
$S_{0\ (AROMATIC)}$
10
9
8
7
6
5
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Initial Conc. (NH4)2 SO4 (M)

Figure 12. The effect of ammonium sulfate concentration on the aromatic surface hydrophobicity of aldolase.

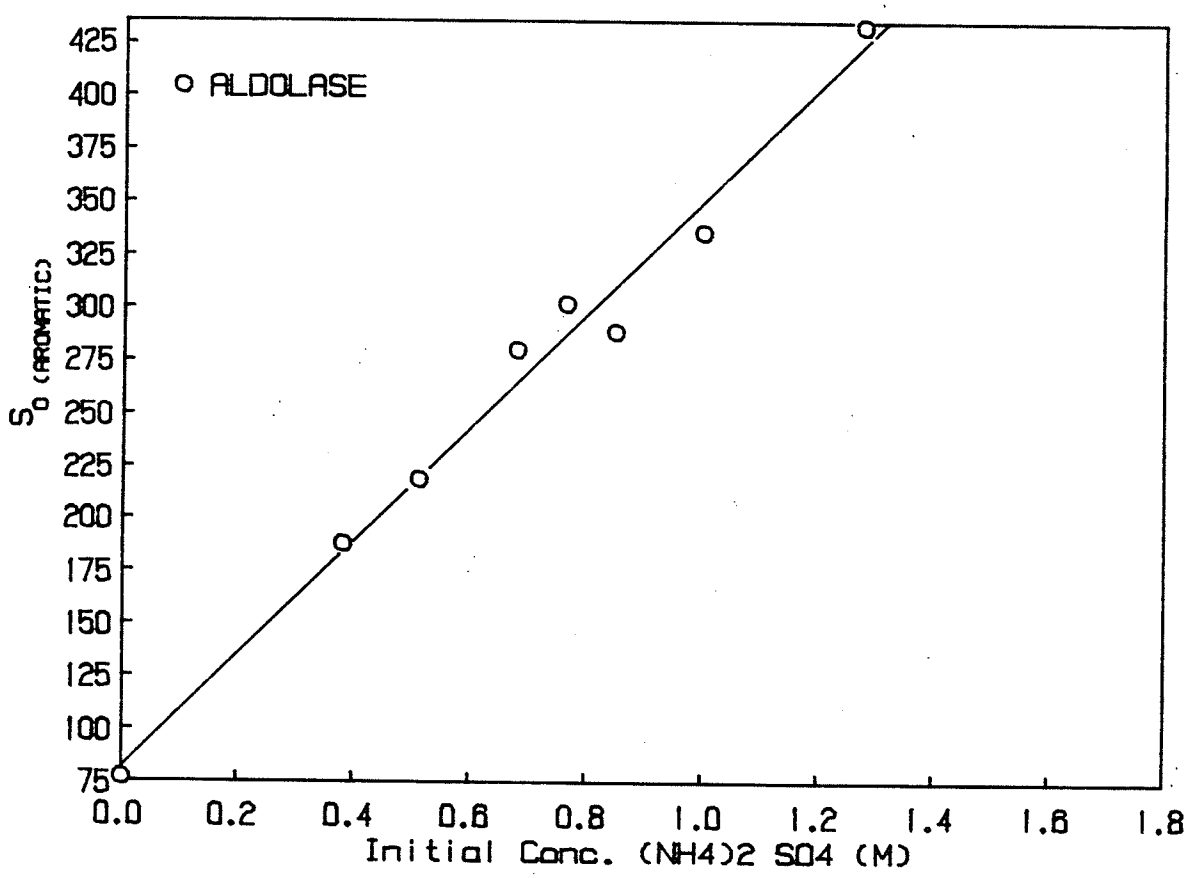
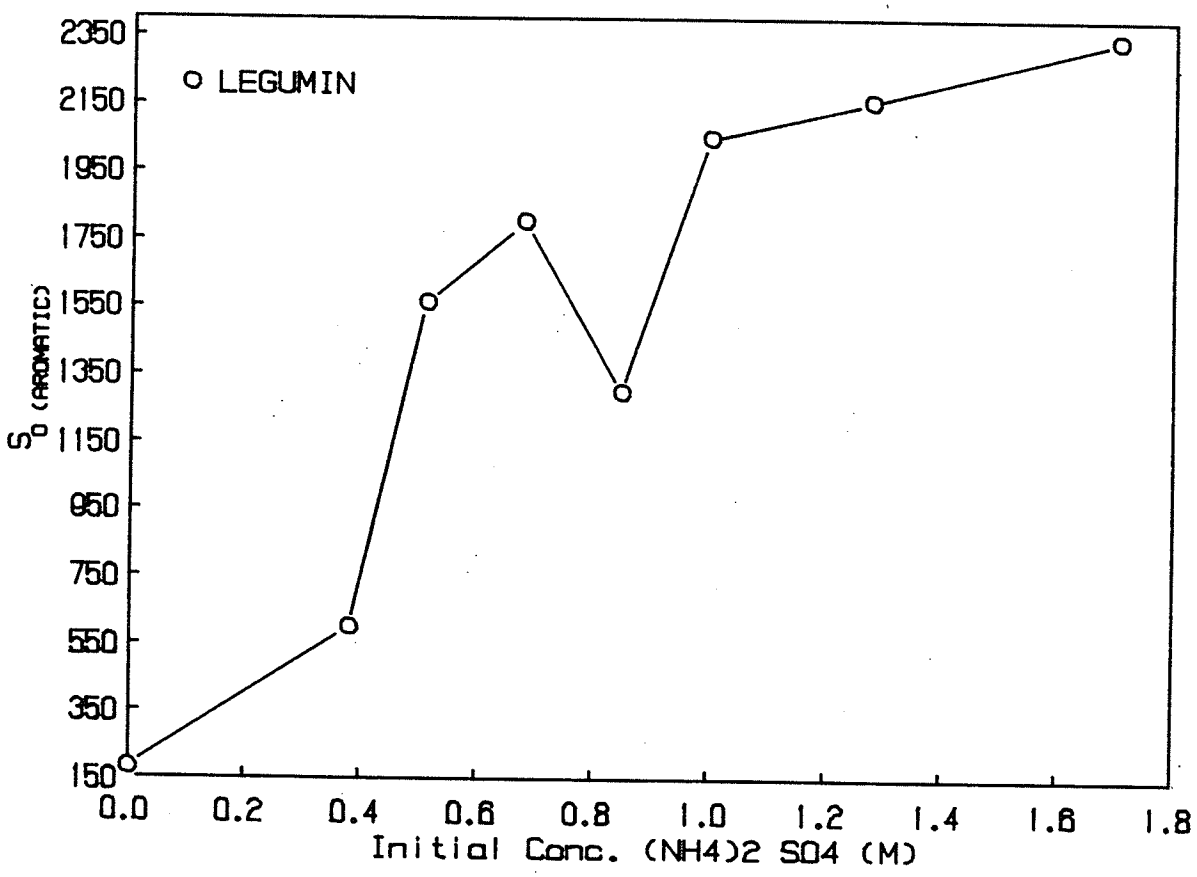

ALDOLASE
S_0 (AROMATIC)
425
400
375
350
325
300
275
250
225
200
175
150
125
100
75
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Initial Conc. (NH4)2 SO4 (M)

Figure 13. The effect of ammonium sulfate concentration on the aromatic surface hydrophobicity of legumin.


LEGUMIN
S_0 (AROMATIC)
2350
2150
1950
1750
1550
1350
1150
950
750
550
350
150
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Initial Conc. (NH4)2 SO4 (M)

this occurs due to preferential solvation of the salt thus removing water from the area of hydrophobic interface, (Arakawa and Timasheff, 1982). When both a protein and ANS are present in solution with ammonium sulfate, hydrophobic interactions take place within the protein and between the protein and ANS. At some point steric and charge limitations would slow and finally halt intramolecular hydrophobic interactions. Beyond this point competition for hydrophobic sites on the protein surface between ANS and other protein oriented hydrophobic sites would begin to decrease and the rate at which ANS was bound to the protein would increase significantly. It should be kept in mind that when an increase in surface hydrophobicity was discussed as a result of an increase in lyotropic salt concentration the phenomena that was being reported was assumed to be an increase in the number of hydrophobic interactions and not the number of hydrophobic sites on the protein surface. This assumption was made because the number of hydrophobic sites on the protein surface would be expected to decrease slightly due to intra-molecular interactions. Such interactions could cause the protein to contract slightly thus removing hydrophobic sites from the surface.

The fluoresence activity of aldolase and legumin were also studied under the same conditions of initial salt as those used for lysozyme. The results are included in tables 13 and 14 while the graphs of ANS surface hydrophobicity are given in figures 12 and 13. Aldolase exhibited a constant

increase in the amount of ANS bound as the initial ammonium sulfate concentration was increased. There was no evidence of a break point such as those noted for lysozyme, ovalbumin and ribonuclease. Possible reasons why this was not noted were that it occurred at a salt concentration greater than 1.7 M or that one did not exsist. The ANS surface hydrophobicity for legumin followed much the same pattern as the plot of Td as a function of initial ammonium sulfate concentration. Between 0.0 and 0.38 M the hydrophobicity increased gradually. This segment was followed by a rapid increase to 0.51 M. From this point on the rate of increase in S_o gradually decreased with the exception of an abrupt decrease in hydrophobicity of approximately 500 units at 0.85 M. This area of sudden depression in S_o was accompanied by a decrease in both denaturation temperature and enthalpy of denaturation (see table 14). These results suggested a significant structural change at this point. It has been suggested by Wright and Boulter (1974) that some of legumin's subunits are covalently bound and others are held by hydrophobic interactions. It is possible that the shift detected here was associated with this latter group but further research would be required to evaluate this hypothesis.

Cis-parinaric acid has been used successfully as a fluoresence probe for the determination of surface hydrophobicity by several researchers. Kato and Nakai (1980)

and Kato et al. (1981) both used this method to study the surface hydrophobicity of lysozyme and ovalbumin. While these studies used 0.01 M phosphate buffer exclusively, Ismond et al. (1986a) measured the surface hydrophobicity of vicilin in the presence of several salts using this method. However, the results obtained in this study using cis-parinaric acid were very erratic. In some instances where results are not reported (see tables 10 - 14), a slope could not be calculated across the normal protein concentration range and as a result surface hydrophobicity data could not be obtained. Where CPA hydrophobicities are reported they have met the criteria of correlation and repeatability described in the methods section. However, the variation which occurred from one salt concentration to another and from one protein to another made it impossible to make any meaningful interpretation from the data.

4.5 Relationship of HIC, DSC and Fluoresence Results

As discussed in the HIC portion of this study the initial ammonium sulfate concentration at which C_E attained a maximum was also the concentration at which k' equalled 1. This held true for all proteins retained by the column in this study. When the initial salt concentration was increased further, k' increased more rapidly than before while C_E approached zero. Analysis of the DSC data indicated that protein stability increased at a fairly constant rate until P was approached. Beyond P the stability

increased at a significantly slower rate. ANS surface hydrophobicity also increased prior to P despite a theoretical decrease in hydrophobic sites due to the intramolecular interactions that occur with increased protein stability. At higher initial salt concentrations ANS surface hydrophobicity increased sharply. Ammonium sulfate's ability to induce hydrophobic interactions was demonstrated in Figure 1 and dramatically emphasized by the plots of ANS hydrophobicity as a function of initial salt concentration (Figures 10 - 14). The binding of ANS to the protein was perhaps more dramatic than the binding of the protein (k') to the column since ANS was always present in excess and was mobile whereas the column phenyl groups were fixed to the column matrix.

Comparisons of the plots of k' and C_E as a function of initial salt concentration revealed that the former gave a better indication of the retention characteristics of the protein on the column since C_E gave zero values once the gradient was completed. The C_E plot gave the best graphical representation of the location of P, marked the end of gradient effects and represented the environment of the protein on elution from the column.

In terms of the protein conformation under changing salt conditions, P appeared to be of significant importance since it was here that protein stability began to decrease and the rate of change in hydrophobic interactions

increased. This point was clearly defined by the HIC data and by the C_E plots in particular. In addition both P and the point where k' no longer equaled zero were indications of the relative surface hydrophobicity displayed by the proteins. These points occurred at higher salt concentrations as surface hydrophobicity decreased.

The DSC data have shown, for the retained proteins, that there is a characteristic salt concentration for each protein where the rate of increase in stability declines. This salt concentration correlates well with both the critical point (P) and the salt concentration at which ANS binding also increases. The results indicate that as the rate at which intra-molecular hydrophobic interactions (which promote stability) decreases the rate of inter-molecular hydrophobic interactions (which cause binding) increases. This salt concentration can be established by any of the three techniques used in this study. In addition to the importance this salt concentration has when attempting to understand the retention mechanism it is possible that this concentration may act as an indicator as to how well a protein will be retained by the column. As the strength of the hydrophobic interaction between the column and the protein decreases, as in the case of lysozyme, ovalbumin, and ribonuclease respectively, the critical salt concentration increases. Therefore, if the critical point occurs at a low salt concentration the protein in question

will be strongly retained by the column. It must be emphasized that polymeric proteins such as legumin and aldolase did not appear to follow the same pattern and more study is required for such proteins. Also only one column was used in this study and further study is indicated in this area as well.

A second feature of this study which may be useful in assessing the retention of proteins on HIC columns is the B ratio discussed earlier. It was shown that this ratio was ten times smaller, for proteins such as legumin and aldolase which did not bind to the column, than the ratio of proteins, such as lysozyme and ovalbumin, which did bind to the column. If the B ratio were to be used for this purpose however, it would be necessary to consider the method used to determine surface hydrophobicity in relation to the protein structure. Ribonuclease served as an example in this regard, giving a low B ratio in spite of being well retained on the HIC column. As was discussed earlier, the reason for the low value of B was that ANS did not bind well to proteins, such as ribonuclease, which contains no tryptophan.

5 CONCLUSIONS AND RECOMMENDATIONS

As the initial concentration of ammonium sulfate is increased during HIC two concentrations will be reached which are unique to the retained proteins. The first of these was previously known and is the concentration at which proteins begin to be retained by the column ($k'>0$). The second point occurs when $k'=1$. From a chromatographic perspective, it is at this point where the rate of change in retention increases due to a rapid increase in the number of hydrophobic interactions between hydrophobic sites on the column and hydrophobic sites on the protein surface. DSC and ANS fluoresence data indicated that this increase was due to a decrease in the number of hydrophobic interactions between hydrophobic sites on the protein surface. At the same initial salt concentration that initiated this increase in retention the elution environment (C_E) reached a maximum salt level. This second point (P) formed the focal point of this study.

An analysis of the DSC data indicated that protein stability increased with increasing ammonium sulfate concentration. After P was reached the rate of change in Td decreased. Since the change in stability was brought about through an increase in hydrophobic interactions among amino

acid side chains on the surface of the proteins it was concluded that at concentrations beyond P there was a growing resistence to intra-molecular interactions possibly due to steric limitations. Although some significant changes in the enthalpy of denaturation did occur they could not be linked in any meaningful way to the other results that were observed in this study.

The proteins unretained by HIC, aldolase and legumin, were also studied by means of DSC. Although the plots of Td as a function of intial salt concentration did resemble those of the other three proteins, depressions occurred in the curve following P. It was speculated that these depressions were related to the polymeric nature of these two proteins. It was also speculated that the differences in the ranges covered by the Td values for all five proteins might be related to the relative compactness of the native protein configuration. A large change in Td values could be associated with a protein having a relatively loose structure while a small change in Td may be associated with a more compact structure.

Fluoresence data using an ANS probe revealed that surface hydrophobicity increased with increasing salt concentration despite the above mentioned increase in protein stability. The ANS hydrophobicity increased at a steady rate until the salt concentration was reached which gave rise to P, following this concentration the ANS

hydrophobicity increased more sharply in a similar manner to column retention. The reason for the increase in the rate of binding was due to a decrease in the competition between intra- and inter-molecular hydrophobic interactions for the hydrophobic sites on the protein. This held true for the monomers; however, legumin followed a pattern which was closely related to that of the Td values. Aliphatic (CPA) hydrophobicities followed no descernable trends and in many cases were unusable due to inconsistant results within individual determinations.

The retention data did not correlate well with either molecular weight or ANS surface hydrophobicities. The ratio (B) of hydrophobicity to molecular weight seemed to give a better correlation. This evidence would tend to support the argument that retention depends upon not only the number of surface hydrophobic sites but also upon the number of sites that are accessible to the fixed column ligands and upon the mass of the protein.

It is possible that two of the terms developed in this study may be used to predict the relative retention of proteins on HIC columns. These are the ratio (B) and the initial salt concentration at which the critical point (P) occurs. High values of B and low values of P indicate that the protein should be strongly retained on the column used in this study. The use of both these terms for this purpose

requires further development since their application has been tested with a limited number of proteins under one set of operating cconditions.

The results reported in this study and the conclusions which have been drawn are based on only five proteins and one chromatographic column. Only one solvent system has been used. As a result much more work is required to confirm the suggestions that have been presented here. Syncropac has marketed a series of HIC silica based columns having ligands ranging from methyl to pentyl that could be compared to the TSK column used here. Other salts such as sodium chloride and potassium chloride could be used. Finally there is a vast number of proteins to examine, differing not only in hydrophobicity and molecular weight but also in their susceptability to the effects of salt, temperature, and the competing hydrophobicities of the column.

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APPENDIX A

This appendix contains the equations for the fit of the data in this study to linear and fourth degree polynomial lines. Included with each equation is the correlation coefficient (r) for the fit of the data to the given line.

Lysozyme:

K' as a function of initial saltconcentration:

Linear: $Y=-1.446 + 4.489\ X$

$r=.943$

Polynomial: $Y=1.849E-02 + 1.550\ X - 6.372\ X^2 + 12.102\ X^3 - 4.418\ X^4$

$r=.995$

CE as a function of initial salt concentration:

Polynomial: $Y=5.272E-03 - 4.765\ X\ 17.463\ X^2 - 16.636\ X^3 + 4.727\ X^4$

$r=.961$

CE as a function of K':

Polynomial: $Y=.058 + .933\ X - .465\ X^2 + .084\ X^3 - 5.316\ X^4$

$r=.979$

Td as a function of initial salt concentration:

Linear: $Y=7.200 + 4.22\ X$

$r=.964$

Lysozyme:

Polynomial: $Y=71.190 + 9.140\ X - 6.54\ X^2 + 4.091\ X^3 - 1.138\ X^4$

r=.996

ANS as a function on initial salt concentration:

Linear: $Y=-41.577 + 107.279\ X$

r=.796

Polynomial: $Y=3.536 - 83.480\ X + 351.318\ X^2 - 408.576\ X^3 + 161.754\ X^4$

r=.997

Ovalbumin:

K' as a function if initial salt concentration:

Linear: $Y=-1.798 + 3.980\ X$

r=.876

Polynomial: $Y=3.487E-02 + 7.566\ X - 29.267\ X^2 + 34.071\ X^3 - 10.726\ X^4$

r=.995

CE as a function of initial salt concentration:

Polynomial: $Y=-4.673E-02 + 5.361\ X\ 20.272\ X^2 + 25.620\ X^3 - 9.121\ X^4$

r=.934

CE as a function of K':

Polynomial: $Y=2.523E-02 + 3.119\ X - 2.843\ X^2 + 0.759\ X^3 - 6.191\ X^4$

r=.986

Td as a function of initial salt concentration:

Linear: $Y=91.124 + 7.510\ X$

r=.989

Ovalbumin:

Polynomial: $Y=90.576 + 7.946\ X + 2.056\ X^2 - 1.587\ X^3 + 9.484\ X^4$

r=.996

Ribonuclease A:

K' as a function of initial salt concentration:

Linear: $Y=-1.226 + 2.349\ X$

r=.799

Polynomial: $Y= -1.010E-03 - 0.491\ X + 2.015\ X^2 - 2.558\ X^3 + 1.424\ X^4$

r=.9995

CE as a function of initial salt concentration:

Polynomial: $Y=-3.424E-03 - 6.277\ X + 18.813\ X^2 -15.295\ X^3 + 3.825\ X^4$

r=.947

CE as a function of K':

Polynomial: $Y=.014 + 6.415\ X - 14.414\ X^2 + 10.380\ X^3 - 1.710\ X^4$

r=.986

Td as a function of initial salt concentration:

Linear: $Y=66.801 + 5.771\ X$

r=.978

Polynomial: $Y=66.008 + 9.393\ X - 5.709\ X^2 + 5.115\ X^3 - 1.794\ X^4$

r=.995

Aldolase:

Td as a function of initial salt concentration:

Linear: $Y=63.589 + 2.359\ X$

r=.915

Aldolase:

Polynomial: $Y=63.204 - 5.523\ X\ 29.183\ X^2$
$29.169\ X^3 + 8.663\ X^4$

r=.995

ΔH as a function of initial salt concentration:

Linear: $Y=13.847 - 0.275\ X$

Polynomial: $Y=-14.283 - 25.144\ X + 69.424\ X^2$
$63.025\ X^3 + 18.026\ X^4$

r=.874

ANS as a function of initial salt concentration:

Linear: $Y=137.332 + 167.565\ X$

r=.834

Polynomial: $Y=76.078 + 526.076\ X - 875..045\ X^2$
$+ 972.199\ X^3 - 347.247\ X^4$

r=.990

Legumin:

Td as a function of initial salt concentration:

Linear: $Y=94.768 + 22.066\ X$

r=.945

Polynomial: $Y=94.494 - 32.075\ X + 162.514\ X^2$
$- 143.445\ X^3 + 38.762\ X^4$

r=.977

ΔH as a function of initial salt concentration:

Linear: $Y=11.932 + 11.038\ X$

r=.891

Polynomial: $Y=12.172 - 26.680\ X + 103.297\ X^2$
$- 83.848\ X^3 + 20.835\ X^4$

r=.965

Legumin:

ANS as a function of initial salt concentration:

Linear: $Y=618.188 + 766.902\ X$

$r=.471$

Polynomial: $Y=184.661 - 6255.996\ X\ 32097.08\ X^2 - 36449.82\ X^3 + 11864.06\ Xt4$

$r=.922$