A CHROMATOGRAPHIC METHOD FOR ESTIMATING HYDROPHOBIC AND ELECTROSTATIC SURFACE PROPERTIES OF SOLUBLE PROTEINS

By

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ABSTRACT

In this research experiments were carried out to estimate hydrophobic and electrostatic interactions in soluble proteins. Five proteins, lysozyme, lactalbumin, ovalbumin, myoglobin and ribonuclease-A were chromatographed isocratically on a HIC column at several molalities (0.3-1.3m) of each of three different neutral salts, ferrous sulfate, ammonium sulfate and sodium sulfate. The calculated retention coefficients were then fitted to a recently developed chromatographic model in two ways. a) Multiple regression analysis was conducted to estimate $C$ values according to the non-linear model ($\log k = A + B \log m + C m$). b) Simple regression analysis was conducted to estimate $C'$ values according to the linear model ($\log k = A' + C'm$) at higher salt concentrations (above 0.3m). Results indicated that $C'$ values better estimate the hydrophobic interactions than $C$ values, in experiments conducted only at higher salt concentrations.

The comparison of $C$ and $C'$ values with ANS, CPA, and Bigelow's average hydrophobicity indices showed no clear correlations. But, omission of ovalbumin improved the correlation coefficient of $C'$ with ANS. Both parameters indicated straight line relationships with molal surface tension increment of salts.

Further, the same model was used to estimate the
hydrophobic and electrostatic interactions in protein-protein interactions. Lysozyme and avidin were chromatographed on a lysozyme immobilized affinity column. Lysozyme-lysozyme interaction showed more affinity for hydrophobic interactions at low pH values. Avidin-lysozyme interaction showed both hydrophobic and electrostatic interactions. Both interactions showed a greater change in the strength of hydrophobic interaction rather than the surface area of interaction, to changing pH.
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1. INTRODUCTION

The molecular surface properties of proteins, including electrostatic and hydrophobic characteristics, play a major role in a wide range of non-covalent interactions of proteins with other compounds: lipids, fatty acids and peptides. Such properties are of central interest in the study of protein attributes such as solubility, antigenicity, surface activity, aggregation, foaming and gelation. However, the quantification of hydrophobic properties of proteins still remains a problem. In a relatively few cases of proteins with known three dimensional structures, the estimate of surface hydrophobic regions is possible by calculating exposure of hydrophobic amino acids (Lipman, 1987). In the case of less well defined proteins, the measure of actual non-polar surface area requires a probe, and the measured hydrophobicity is related to the contact area between the molecule and the non-polar surface area of the probe. Probes which exhibited changes in fluorescence in relation to the hydrophobicity of their environment have been widely used for this purpose.

Recently, a chromatographic model \( \log k = A + B \log m + C m \) was proposed by El-Rassi and Horvath (1986a) to estimate both hydrophobic \((C)\) and electrostatic \((B)\) interaction parameters between a protein and a chromatographic stationary phase. The model is based on the observation that log capacity factors \((\log k)\) decrease linearly with log salt
molality (log m) when retention is due to electrostatic interaction, and increase linearly with salt molality when retention is due to hydrophobic interaction. Parameters B and C are the limiting slopes of log k vs log m and log k vs m relationships respectively. Parameter C is believed to be closely related to the molecular surface area of hydrophobic interaction and the molal surface tension increment of salts.

The advantage of using this method is that the consideration of capacity ratios over a wide range of salt molalities overcomes the errors that could simply result from estimation of hydrophobicity as the capacity ratio at a given salt molality. Furthermore, as the hydrophobic surface of the protein interacts directly with the stationary phase, an actual estimate of the hydrophobic surface area of contact between a protein and the HIC stationary phase may be possible.

The model also shows that there are two ways of estimating hydrophobic interaction parameters. a) By fitting data to the above non-linear model and calculating C values by multiple regression analysis. b) By fitting data to the linear model (i.e. electrostatic interaction parameters become insignificant at higher salt concentrations and the model reduces to, log k = A' + C'm where, C' is the hydrophobic interaction parameter at higher salt concentrations and A' is a constant) and calculating C' values by linear regression analysis.

This model also has potential for investigating the
forces involved in protein-protein interactions. This can be achieved by covalently immobilizing a protein to the stationary phase and passing a soluble protein through the column several times with varying salt molalities in the mobile phase.

In this research the investigations were carried out to,

1. Correlate hydrophobic interaction parameters (C and C') of lysozyme, lactalbumin, ovalbumin, myoglobin, and ribonuclease with ANS, CPA and Bigelow's average hydrophobicity indices.

2. Correlate hydrophobic interaction parameters (C and C') with molal surface tension increments of neutral salts (ferrous sulfate, ammonium sulfate, sodium sulfate).

3. Explain lysozyme dimerization in terms of experimental hydrophobic (C and C') and electrostatic (B) interaction parameters.

4. Explain avidin-lysozyme interaction in terms of experimental C, C' and B parameters.
2. LITERATURE REVIEW

2.1 HYDROPHOBIC INTERACTION

The attraction between hydrophobic polymers (e.g., proteins) and hydrophobic surfaces of other compounds while immersed in aqueous media is generally referred to as "hydrophobic interaction" (Van Oss et al., 1986). This same phenomenon is also referred to as "hydrophobic effect" or even as "hydrophobic bonds". Hydrophobicity is a structural feature of a compound that has important implications for biological activity. The theoretical framework for explaining hydrophobic interactions of proteins in solution is somewhat controversial at this time. There exist, two completely different interpretations concerning this driving force between two non-polar molecules. One approach is to explain hydrophobic interaction as an entropic force which is called the entropic approach (Tanford, 1980). The other is to consider hydrophobicity as a special case of van der Waals attraction (Van Oss et al., 1986). This is called the solvent approach.

2.1.1 Entropic approach

In this theory, hydrophobic effect is said to arise due to the unfavourable interactions between non-polar surfaces and water molecules. Therefore, it is mainly the solvent that forces molecules to associate, rather than the attraction between them (Horvath et al., 1976). When a non-polar molecule
is introduced into water, both enthalpy and entropy changes occur in the system. This unfavourable enthalpy change is caused by the creation of a hole in the hydrogen bonded structure of water to accommodate the non-polar molecule. The resulting formation is a "cage" of more or less fully H-bonded water molecules immediately surrounding the non-polar molecule. This structure is called an "iceberg" (Frank and Evans, 1945). Formation of the "cage" causes the entropy of the system to decrease. To minimize this unfavourable entropy effect, non-polar molecules are forced to self-associate in water. In doing so, the total non-polar surface area in contact with the solvent is decreased and the unfavourable entropy decrease is minimized. To a large extent this unfavourable change is offset by London forces between the non-polar molecules and water (Tanford, 1980). Thus the aggregation of non-polar solutes in water is "entropy driven" and no "hydrophobic bond" exists as such. Therefore according to the above theory, the correct term is "hydrophobic effect" and not "hydrophobic bond".

2.1.2 Solvent approach

This general approach considers hydrophobic interaction as a special case of van der Waals and hydrogen bonds. In addition to the attraction between hydrophobic regions, solvent-solvent interactions and solute-solvent interactions are also considered (Van Oss et al., 1986; Nakai and Li-Chan, 1987). Therefore the hydrophobic effect is attributed to the
interplay of all three intermolecular interactions.

There are two groups of surface thermodynamic interactions.

(1) Long-range Lifshitz van der Waals interactions (LW) of the London (dispersion), Keesom (orientation), and Debye (induction) varieties.

(2) Short-range (SR) hydrogen bonds. Energy of H-bonds decay rapidly with distance and it drops to zero at a distance of 2Å, or so beyond the normal bond length. Therefore these are called short range interactions.

According to Hamarker's combining rule, the potential of van der Waals interaction between two bodies of materials, one and two, across a medium three is given by the sum of geometric terms that are influenced by the size and shape of each body. Thus the Hamarker coefficient $A_{132}$ is given by:

$$A_{132} = A_{12} + A_{33} - A_{13} - A_{23} \quad .......(2.1)$$

Hydrophobic bond prevails when $A_{33} > (A_{13} + A_{23})$. The total free energy of adhesion ($\Delta G_{132}^{\text{TOT}}$), between non-polar surfaces due to hydrophobic interaction when distance $d_0$ = 1.35 Å is given by:

$$\Delta G_{132}^{\text{TOT}} = \Delta G_{132}^{\text{LW}} + \Delta G_{132}^{\text{SR}} \quad .................(2.2)$$

Therefore, all that is necessary for a hydrophobic bond to occur is $A_{12}^{\text{LW}} + A_{13}^{\text{SR}}$ to be positive (which happens when $\Delta G_{132}^{\text{LW}} + \Delta G_{132}^{\text{SR}}$ is negative). As shown by Van Oss et al. (1986) LW forces
between hydrophobic moieties and aqueous solvent are always attractive, and SR forces between the same entities are either zero or attractive, but never repulsive. As such, the hydrophobic effect is an attraction between macromolecules by which some of the more hydrophobic sites preferentially interact with each other by van der Waals interaction. Therefore Tanford’s definition of hydrophobic effect based upon repulsion by the solvent, instead of attractive forces at the site of organization is misleading. Moreover, the solvent approach proposes that the term "interfacial force" is more appropriate than the term "hydrophobic effect", as LW and SR forces are interfacial forces.

It has been argued that changes in water structure do not play a major role in solution processes of hydrocarbons, because the major component of the free energy of transfer stems from van der Waals interaction. On the other hand, the unusually large changes in entropy and heat capacity that are observed in accompanying the transfer of non-polar solutes to water cannot be explained only by van der Waals interactions. Therefore, the changes in water structure must also be considered in understanding the hydrophobic effect (Nakai and Li-Chan, 1987).

2.2 Importance of Hydrophobicity

Hydrophobic interactions are known to be of essential importance for the stability, conformation, and function of cells and biological macromolecules (eg. proteins). Proteins
are amphipathic molecules and are composed of amino acids that have a strongly hydrophobic peptide group and either hydrophobic or hydrophilic side chains. Recently Rose et al. (1985b) divided amino acids into three groups, such as very polar (serine, proline, aspartate, asparagine, glutamate, lysine, and arginine), moderately polar (alanine, threonine, histidine, and tyrosine), and hydrophobic (glycine, cysteine, valine, isoleucine, leucine, methionine, phenylalanine, and tryptophan). Therefore all proteins are characterized by a certain degree of hydrophobicity (Mann and Moreno, 1984).

Protein folding was thought to be related to the hydrophobicity of amino acids and side chains. The charged groups of the protein molecule were assumed to be at the protein/solvent interface of the native structure and most of the non-polar parts to be in the interior of the native structure (Tanford, 1962). However, the analysis of three dimensional protein structures recently revealed that 40-55% of the surface area of each protein is occupied by non-polar atoms (Lee and Richard, 1970).

Some relationships that have been observed between secondary structure and hydrophobicity are:

1. Alpha helices that lie at protein surfaces tend to have one face projecting mainly hydrophobic residues and an opposite face projecting hydrophilic residues.

2. Beta sheets which frequently occur in the interior of the globular proteins tend to be particularly rich in hydrophobic residues.
3. Beta turns and other abrupt bends that reverse chain directions at the surface of globular proteins tend to be especially hydrophilic (Sweet and Eisenberg, 1983).

Hydrophobicity is not only important in stability and conformation of proteins. It is a molecular surface property that plays a major role in a wide range of non-covalent interactions of proteins with other compounds such as peptides, proteins, lipids, etc. These properties are of central interest in studies of protein solubility, surface activity, antigenicity, foaming, aggregation, gelation, etc. Solubility of a compound is related to its polarity. If polarity of solvent and solute matches, solute easily dissolves in solvent. "Hydrophilic" is a synonym for "polar" and "hydrophobic" is a synonym for "non-polar". Therefore solubility is related to hydrophobicity. The Hydrophile Lipophile Balance (HLB) concept which is used to select proper surfactants is another expression of polarity. HLB is defined as, \[\text{HLB} = \frac{\text{[polar radicals]}}{\text{[polar] + [non-polar]}}\] and hydrophobicity is defined as, \[\text{protein hydrophobicity} = 1 - \text{HLB}\]. Therefore, protein hydrophobicity = 1 - [HLB]. In such a way hydrophobicity, solubility and emulsification are all related to each other (Nakai and Li-Chan, 1987). Significant correlations have been experimentally observed among these properties of proteins. Therefore many attempts have been made to quantitate the hydrophobic effect. As a result, a large number of hydrophobicity scales have evolved within a given class of biological substances. Fendler et al.
(1975) developed a scale based on the free energy of transfer of amino acids from water to hexane. Bull and Breeze (1974) measured the tendency of amino acids near their iso-electric point (pI) to be transferred from bulk to the surface. Chothia (1976) generated a list of "residue accessibilities" of individual amino acids that are found to be inaccessible to solvent over 95% of their respective surface areas in the structures of globular proteins. Using the hydrophobicity values of individual amino acids, Tanford (1962) calculated the total hydrophobicity of several proteins, and using the Nozaki Tanford scale, Bigelow (1967) calculated the average hydrophobicity ($H^\phi_{ave} = \text{total hydro./# of residues}$) of several proteins.

These scales can be classified as solution measurements, empirical scales, or some combination of the two (Rose et al., 1985b; Nakai and Li-Chan, 1987). Solution scales are based on distribution coefficients between an aqueous phase and a suitably chosen organic phase. Empirical scales are based on partitioning between the solvent accessible surface and the buried interior in the proteins of known structures. Some of these scales are discussed in detail in the following section.
2.3 HYDROPHOBICITY SCALES

2.3.1 Solution scales

2.3.1.1 Nozaki and Tanford (1971) scale

The difference in solvation free energies in water and 100% ethanol or other non aqueous media has been used to establish a hydrophobicity scale. The free energy of transfer \( (\Delta G^*_t) \) of an amino acid near its iso-electric point (pI) was defined as the change in chemical potential of solute "i" in going from water to any other solvent at the same mole fraction, at the limit of infinite dilution. In this scale, ethanol was considered as a model for the organic interior of a globular protein. If solubility of a certain amino acid, for example was \( N_{\text{HOH}} \) in water and \( N_{\text{EtOH}} \) in ethyl alcohol, then the free energy of transfer of one mole of the amino acid from water to ethanol was given by:

\[
\Delta G^*_t = -RT \ln \frac{N_{\text{EtOH}}}{N_{\text{HOH}}} \tag{2.3}
\]

Negative values of hydrophobicity meant that the side chain preferred water to ethanol and would tend to occur in the outer shell of the protein molecule. Tanford also pointed out that \( \Delta G^*_t \) is a roughly additive function of molecular structure. According to this scale, histidine and glycine were located at the hydrophilic end and tyrosine and tryptophan were located at the hydrophobic end. Aromatic side chains were
more hydrophobic than aliphatic side chains. This was due to the larger size of the aromatic groups. But if $\Delta G_t^*$ per constituent atom was considered, then aliphatic chains become more hydrophobic. In this scale no data were provided for the five amino acids cysteine, cystine, histidine, glutamate and aspartate.

2.3.1.2 *Wolfenden et al. (1981)* scale

The tendency of a dissolved compound to leave water and enter an empty cavity that neither attracted nor repelled solutes was used to establish a hydrophobicity scale. The hydration potential ($\Delta G_h^*$) of an amino acid was defined as the free energy of transfer of an amino acid from the vapour phase to neutral aqueous solution at pH 7.0. Affinity of a compound to an aqueous environment was evaluated by determining its vapour pressure over dilute aqueous solution. According to this scale, the side chain of arginine was located at the hydrophilic extreme and glycine was located at the hydrophobic extreme. Tryptophan and tyrosine were in the mid range closer to the hydrophilic end.

2.3.2 *Empirical scales*

2.3.2.1. *Sweet and Eisenberg (1983)* scale

The development of this hydrophobicity scale was based on the theory that two amino acid sequences folding into similar three dimensional structures were likely to have
highly correlated hydrophobicities. Each amino acid was assigned with a numerical hydrophobicity value taken from the scale of Eisenberg (1982), in which hydrophobicity was measured as the free energy of transfer of an amino acid residue from the interior to the surface of a hydrated protein. Then the correlations between all pairs of residues in the two sequences were calculated. According to the discovery of Dayhoff et al. (1979), that amino acids that substitute for each other in related three dimensional proteins had similar chemical properties, Sweet and Eisenberg (1983) derived a set of optimum matching hydrophobicities (OMH) by considering the observed frequency of amino acid replacements among related structures. With this set of OMH’s significant correlations have been calculated for similar three dimensional structures even though the two sequences contain few identical residues. In this scale, phenylalanine and tyrosine were located at the hydrophobic extreme and histidine and glycine were located at the hydrophilic extreme.

2.3.2.2 Rose et al. (1985b) scale

This scale was based on the calculation of the area of a residue buried upon folding, when transferred from a defined standard state to a folded state. This area was proportional to its hydrophobic contribution to the conformational free energy $\Delta G_{\text{conf}}$. The mean area buried upon transfer was given by: $\AA <A>$ where $\AA$ is the solvent accessible surface area of a residue in the standard state and $<A>$ is the average solvent
accessible area of a residue in the folded protein. Therefore
the mean fractional area loss denoted by $f$ was given by:

$$f = 1 - \left( \frac{<A>}{\AA} \right) \quad \text{(2.4)}$$

According to this scale tryptophan and tyrosine were located
at the hydrophobic end and alanine and arginine were located
at the hydrophilic end.

2.3.3 Empirical and measured scales

2.3.3.1 Manavalan and Ponnaswamy (1978) scale

These researchers measured a parameter called "bulk
hydrophobicity" from an analysis of the surrounding
hydrophobic environment of the amino acid residues in the
protein. The surrounding hydrophobicity ($H_i$) of a residue was
defined as the sum of hydrophobic indices assigned to the
various residues that appear within an 8Å radius volume in the
protein crystal and is given by:

$$H_i = \sum_{j=1}^{20} L_{i,j} h_i \quad \text{(2.5)}$$

where $L_{i,j}$ is the total number of residues of the $i^{th}$ type
associated with the $j^{th}$ residue in a given protein and $h_i$ is
the hydrophobic index of the $i^{th}$ residue, calculated by
crystallographic data. The arithmetic average $<H>$ of all $H_i$
values that belong to the same type of residue in several
proteins were calculated and called the bulk hydrophobicity of that residue.

2.3.4 **Comparison of hydrophobicity scales**

Hydrophobicity values of amino acid residues obtained from several scales have been reported by Nakai and Li-Chan, (1987). Their results are reproduced in Table 2.1.

Significant differences exist among scales. Residues that are strongly hydrophobic in one scale may appear to be strongly hydrophilic in another scale. For example, tryptophan and tyrosine which seemed to be more hydrophobic in Nozaki Tanford scale are more hydrophilic in Wolfenden et al. (1981) scale. Even though both are measured scales, the correlation coefficient \( r^2 = 0.04 \) seemed to be quite low (Nakai and Li-Chan, 1987). But Wolfenden et al.'s scale correlated well with Chothia's and Janin's scales (Janin, 1979). Chothia (1976) showed that the correlation of hydrophobicity with the residue total surface area is excellent but the correlation with the residue area buried upon folding is very low. Surprisingly, this was a complete contradiction to the finding by Rose et al. (1985b). They found a strong correlation between hydrophobicity and the surface area residues buried upon folding.

In the method of Manavalan and Ponnaswamy (1978) a low hydrophobicity value was observed for tryptophan and tyrosine compared to Nozaki Tanford scale and valine had a hydrophobic value similar to tryptophan. But in the Nozaki Tanford scale,
Table 2.1: Hydrophobicity scale values of amino acid residues. (Nakai and Li-Chan, 1987).

<table>
<thead>
<tr>
<th>Residue</th>
<th>AC</th>
<th>AC'</th>
<th>x</th>
<th>P</th>
<th>C &lt;x&gt;</th>
<th>AC' &lt;x&gt;</th>
<th>H kcal</th>
<th>OMH</th>
<th>&lt;x&gt;</th>
<th>Fraction 50% buried</th>
<th>Hydrophobic index</th>
<th>Log P</th>
<th>Single residue parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>0.87</td>
<td>0.5</td>
<td>4.32</td>
<td>0.52</td>
<td>0.83</td>
<td>-1.0</td>
<td>1.2</td>
<td>12.97</td>
<td>-0.40</td>
<td>86.6</td>
<td>0.38</td>
<td>0.59</td>
<td>1.8</td>
</tr>
<tr>
<td>Arg</td>
<td>0.85</td>
<td>-</td>
<td>6.55</td>
<td>0.49</td>
<td>0.98</td>
<td>0.3</td>
<td>3.0</td>
<td>11.72</td>
<td>-0.59</td>
<td>166.2</td>
<td>0.01</td>
<td>-1.82</td>
<td>-4.5</td>
</tr>
<tr>
<td>Asn</td>
<td>0.09</td>
<td>-</td>
<td>6.24</td>
<td>0.42</td>
<td>0.98</td>
<td>0.7</td>
<td>0.2</td>
<td>11.42</td>
<td>-0.92</td>
<td>103.3</td>
<td>0.12</td>
<td>-0.55</td>
<td>-3.5</td>
</tr>
<tr>
<td>Asp</td>
<td>0.66</td>
<td>-</td>
<td>6.04</td>
<td>0.37</td>
<td>1.01</td>
<td>-1.2</td>
<td>2.5</td>
<td>10.85</td>
<td>-1.31</td>
<td>97.8</td>
<td>0.15</td>
<td>-0.69</td>
<td>-3.5</td>
</tr>
<tr>
<td>Cys</td>
<td>1.52</td>
<td>-</td>
<td>1.73</td>
<td>0.83</td>
<td>0.88</td>
<td>1.2</td>
<td>-1.0</td>
<td>14.63</td>
<td>0.97</td>
<td>132.3</td>
<td>0.45</td>
<td>1.44</td>
<td>2.5</td>
</tr>
<tr>
<td>Gin</td>
<td>0.00</td>
<td>-</td>
<td>6.13</td>
<td>0.35</td>
<td>1.02</td>
<td>-0.1</td>
<td>0.2</td>
<td>11.76</td>
<td>-0.91</td>
<td>139.2</td>
<td>0.07</td>
<td>-0.83</td>
<td>-3.5</td>
</tr>
<tr>
<td>Glu</td>
<td>0.67</td>
<td>-</td>
<td>6.17</td>
<td>0.38</td>
<td>1.02</td>
<td>-0.7</td>
<td>2.5</td>
<td>11.89</td>
<td>-1.22</td>
<td>133.9</td>
<td>0.18</td>
<td>-0.83</td>
<td>-3.5</td>
</tr>
<tr>
<td>Gly</td>
<td>0.10</td>
<td>0.6</td>
<td>6.09</td>
<td>0.41</td>
<td>1.00</td>
<td>0.0</td>
<td>0.0</td>
<td>12.43</td>
<td>-0.67</td>
<td>82.9</td>
<td>0.36</td>
<td>0.59</td>
<td>-0.4</td>
</tr>
<tr>
<td>His</td>
<td>0.87</td>
<td>0.5</td>
<td>5.66</td>
<td>0.70</td>
<td>0.89</td>
<td>1.1</td>
<td>-0.5</td>
<td>12.16</td>
<td>-0.64</td>
<td>155.8</td>
<td>0.17</td>
<td>0.02</td>
<td>-3.2</td>
</tr>
<tr>
<td>Ile</td>
<td>3.15</td>
<td>-</td>
<td>2.3</td>
<td>0.79</td>
<td>0.79</td>
<td>4.0</td>
<td>-1.8</td>
<td>15.67</td>
<td>1.25</td>
<td>135.0</td>
<td>0.60</td>
<td>1.16</td>
<td>4.5</td>
</tr>
<tr>
<td>Leu</td>
<td>2.17</td>
<td>-</td>
<td>1.8</td>
<td>3.95</td>
<td>0.77</td>
<td>0.85</td>
<td>2.6</td>
<td>-1.8</td>
<td>14.90</td>
<td>1.22</td>
<td>104.4</td>
<td>0.45</td>
<td>0.87</td>
</tr>
<tr>
<td>Lys</td>
<td>1.64</td>
<td>-</td>
<td>7.92</td>
<td>0.31</td>
<td>1.05</td>
<td>-0.9</td>
<td>3.0</td>
<td>11.36</td>
<td>-0.67</td>
<td>115.5</td>
<td>0.03</td>
<td>-2.39</td>
<td>-3.9</td>
</tr>
<tr>
<td>Met</td>
<td>1.67</td>
<td>1.3</td>
<td>2.41</td>
<td>0.76</td>
<td>0.84</td>
<td>1.8</td>
<td>-1.3</td>
<td>14.39</td>
<td>1.02</td>
<td>172.9</td>
<td>0.40</td>
<td>0.73</td>
<td>1.9</td>
</tr>
<tr>
<td>Phe</td>
<td>2.87</td>
<td>2.5</td>
<td>2.59</td>
<td>0.87</td>
<td>0.78</td>
<td>2.8</td>
<td>-2.5</td>
<td>14.00</td>
<td>1.92</td>
<td>194.1</td>
<td>0.50</td>
<td>0.87</td>
<td>2.8</td>
</tr>
<tr>
<td>Pro</td>
<td>2.77</td>
<td>7.19</td>
<td>0.35</td>
<td>1.00</td>
<td>0.4</td>
<td>-1.4</td>
<td>11.37</td>
<td>-0.49</td>
<td>92.9</td>
<td>0.18</td>
<td>-0.26</td>
<td>-1.6</td>
<td>0.54</td>
</tr>
<tr>
<td>Ser</td>
<td>0.07</td>
<td>0.3</td>
<td>5.37</td>
<td>0.49</td>
<td>1.02</td>
<td>-1.2</td>
<td>0.3</td>
<td>11.23</td>
<td>-0.55</td>
<td>85.6</td>
<td>0.22</td>
<td>0.02</td>
<td>-0.8</td>
</tr>
<tr>
<td>Thr</td>
<td>0.07</td>
<td>0.4</td>
<td>5.16</td>
<td>0.38</td>
<td>0.99</td>
<td>-0.5</td>
<td>-0.4</td>
<td>11.69</td>
<td>-0.28</td>
<td>100.5</td>
<td>0.23</td>
<td>-0.12</td>
<td>-0.7</td>
</tr>
<tr>
<td>Trp</td>
<td>3.77</td>
<td>3.4</td>
<td>2.78</td>
<td>0.86</td>
<td>0.83</td>
<td>3.0</td>
<td>-3.4</td>
<td>13.93</td>
<td>0.50</td>
<td>222.6</td>
<td>0.27</td>
<td>0.50</td>
<td>-0.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.67</td>
<td>2.3</td>
<td>3.58</td>
<td>0.64</td>
<td>0.93</td>
<td>2.1</td>
<td>-2.3</td>
<td>13.42</td>
<td>1.67</td>
<td>377.7</td>
<td>0.15</td>
<td>-0.40</td>
<td>-1.3</td>
</tr>
<tr>
<td>Val</td>
<td>1.87</td>
<td>1.5</td>
<td>3.31</td>
<td>0.72</td>
<td>0.81</td>
<td>1.4</td>
<td>-1.5</td>
<td>15.71</td>
<td>0.91</td>
<td>141.0</td>
<td>0.54</td>
<td>1.02</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Note: Reference numbers are in parentheses.
* 0.50 and 0.40 for Cys and 1/2 Cys, respectively.

valine had a value almost half that of tryptophan. The reason for the observed low values for tryptophan and tyrosine, as indicated by Manavalan and Ponnaswamy (1978) was that even in a large non-polar side chain, one polar atom was sufficient to cause a large reduction in the hydrophobic environment.

Such discrepancies between scales arise probably in part from differences between conditions chosen for distribution experiments. Unrecognized self-association of amino acids near their limits of solubility in organic solvents may have tended to interfere with the determination of free energies of transfer (Wolfenden et al., 1981).

A comprehensive study by Rose et al. (1985a, 1985b) compared fifteen different scales and was summarized by Nakai and Li-Chan (1987). This is given in Table 2.2. Scales 1-10 had relatively high correlations. Scales 11-14 made another group. But 15 and 16 did not belong to either group nor did they correlate with each other. Therefore it is important to find a reliable method to measure the actual hydrophobicity.

2.5 METHODS OF MEASURING HYDROPHOBICITY

Among the many methods of measuring hydrophobicity only the hydrophobic probe method, hydrophobic chromatography, and hydrophobic partitioning are discussed in this section.

2.5.1 Hydrophobic probe method

This is the simplest method of measuring protein hydrophobicity. Many different hydrophobic probes are
Table 2.2 Correlation matrix of hydrophobicity scales for amino acid residues. (Nakai and Li-Chan, 1987).

<table>
<thead>
<tr>
<th>Scale</th>
<th>Type&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
<th>(7)</th>
<th>(8)</th>
<th>(9)</th>
<th>(10)</th>
<th>(11)</th>
<th>(12)</th>
<th>(13)</th>
<th>(14)</th>
<th>(15)</th>
<th>(16)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Jones (&lt;1975)</td>
<td>M/E</td>
<td>0.99</td>
<td>0.53</td>
<td>0.66</td>
<td>0.68</td>
<td>0.83</td>
<td>0.70</td>
<td>0.63</td>
<td>0.75</td>
<td>0.72</td>
<td>0.41</td>
<td>0.35</td>
<td>0.47</td>
<td>0.34</td>
<td>0.47</td>
<td>0.40</td>
<td>0.60 ± 0.190</td>
<td></td>
</tr>
<tr>
<td>(2) Noskii and Tanford (&lt;1975)</td>
<td>M</td>
<td>0.81</td>
<td>0.82</td>
<td>0.75</td>
<td>0.91</td>
<td>0.99</td>
<td>0.66</td>
<td>0.78</td>
<td>0.91</td>
<td>0.20</td>
<td>0.28</td>
<td>0.24</td>
<td>0.04</td>
<td>0.89</td>
<td>0.63</td>
<td>0.66 ± 0.134</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Krivba and Komolys (&lt;1979)</td>
<td>E</td>
<td>0.89</td>
<td>0.87</td>
<td>0.78</td>
<td>0.74</td>
<td>0.86</td>
<td>0.78</td>
<td>0.61</td>
<td>0.79</td>
<td>0.81</td>
<td>0.79</td>
<td>0.63</td>
<td>0.15</td>
<td>0.04</td>
<td>0.67 ± 0.255</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) Wetl and Sheraga (&lt;1978)</td>
<td>E</td>
<td>0.93</td>
<td>0.88</td>
<td>0.72</td>
<td>0.85</td>
<td>0.80</td>
<td>0.78</td>
<td>0.71</td>
<td>0.74</td>
<td>0.71</td>
<td>0.49</td>
<td>0.35</td>
<td>0.15</td>
<td>0.15</td>
<td>0.70 ± 0.215</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5) Meliochich et al. (&lt;1980)</td>
<td>E</td>
<td>0.87</td>
<td>0.75</td>
<td>0.89</td>
<td>0.82</td>
<td>0.73</td>
<td>0.79</td>
<td>0.72</td>
<td>0.66</td>
<td>0.29</td>
<td>0.29</td>
<td>0.34</td>
<td>0.13</td>
<td>0.72 ± 0.188</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(6) Robson and Osguthorpe (&lt;1970)</td>
<td>E</td>
<td>0.74</td>
<td>0.83</td>
<td>0.84</td>
<td>0.80</td>
<td>0.65</td>
<td>0.64</td>
<td>0.64</td>
<td>0.48</td>
<td>0.49</td>
<td>0.21</td>
<td>0.70 ± 0.201</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7) Levitt (&lt;1974)</td>
<td>M/E</td>
<td>0.67</td>
<td>0.75</td>
<td>0.52</td>
<td>0.62</td>
<td>0.77</td>
<td>0.67</td>
<td>0.65</td>
<td>0.05</td>
<td>0.14</td>
<td>0.63 ± 0.240</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8) Manavalan and Ponnuswamy (&lt;1980)</td>
<td>M/E</td>
<td>0.82</td>
<td>0.58</td>
<td>0.86</td>
<td>0.76</td>
<td>0.71</td>
<td>0.25</td>
<td>0.23</td>
<td>0.82</td>
<td>0.70 ± 0.208</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(9) Sweet and Eisenberg (&lt;1987)</td>
<td>E</td>
<td>0.71</td>
<td>0.67</td>
<td>0.58</td>
<td>0.75</td>
<td>0.57</td>
<td>0.22</td>
<td>0.34</td>
<td>0.68 ± 0.182</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>(10) Rose et al. (&lt;1985&gt;)</td>
<td>E</td>
<td>0.24</td>
<td>0.23</td>
<td>0.27</td>
<td>0.06</td>
<td>0.66</td>
<td>0.58</td>
<td>0.56 ± 0.249</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>(11) Chothia (&lt;1986)</td>
<td>E</td>
<td>0.91</td>
<td>0.96</td>
<td>0.86</td>
<td>0.06</td>
<td>0.00</td>
<td>0.58 ± 0.319</td>
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<tr>
<td>(12) Jannin (&lt;1999)</td>
<td>E</td>
<td>0.87</td>
<td>0.83</td>
<td>0.22</td>
<td>0.15</td>
<td>0.59 ± 0.269</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>(13) Kyle and Doolittle (&lt;1982)</td>
<td>M/E</td>
<td>0.89</td>
<td>0.12</td>
<td>0.07</td>
<td>0.60 ± 0.290</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>(14) Wilesden et al. (&lt;1981)</td>
<td>M</td>
<td>0.30</td>
<td>0.10</td>
<td>0.47 ± 0.283</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(15) Younger and Cramer (&lt;1981)</td>
<td>M</td>
<td>0.43</td>
<td>0.32 ± 0.227</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(16) Nishikawa and Oni (&lt;1980)</td>
<td>E</td>
<td>0.29</td>
<td>0.241</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup>The absolute values of correlation coefficient are shown.

<sup>b</sup>M: solubility measurement, E: empirical calculation.
available. The most frequently used probe is 1-anilino naphthalene-8-sulfonate (ANS). The method is based on the fact that fluorescent emission of ANS is dependent on its environment. For example, the quantum yield of fluorescence of ANS exceeds 0.7 in a non-polar solvent, while it is only 0.0032 in water (Nakai and Li-Chan, 1987). ANS in solution has two different conformations. In one case the planes of benzene and naphthalene are perpendicular. In the other case the two rings are nearly coplanar due to hydrogen bonding between \(-\text{NH}\) and \(-\text{SO}_3\) groups. This second type of conformation is more rigid and fluorescent.

Another fluorescent probe is cis parinarate (CPA) (Sklar et al., 1977). CPA \((9,11,13,15, \text{ cis, trans, trans, cis-octadecatetraenoic acid})\) possess an aliphatic hydrocarbon chain and is structurally similar to fatty acids while ANS is composed of aromatic rings. Therefore, the CPA probe can be used to interpret naturally occurring fatty acid-protein and lipid-protein interactions.

Applications of the hydrophobic probe method in food chemistry have been investigated by many workers (Townsend and Nakai, 1983; Voutsinas et al., 1983; Li-Chan et al., 1984; etc.).

2.5.2 **Hydrophobic chromatography**

In earlier studies hydrophobicity of a solute is measured as the partition coefficient of a solute in an aqueous two phase system. The two phases used were octanol and
water and the partition coefficient \((P)\) of the solute was defined as:

\[
P = \frac{C_s}{C_w}
\]  \hspace{1cm} \text{(2.6)}

where \(C_s\) and \(C_w\) are the solute concentration at equilibrium in the organic and water phase respectively. Since, at equilibrium, the chemical potential of the solute is equal in both phases, the logarithm of the partition coefficient \((\log P_{o/w})\) is directly proportional to the free energy \((\Delta G)\) required to transport one mole of solute from one phase to another. This free energy of transfer of the solute can be split into fragments:

\[
\log P = \sum a_i f_i
\]  \hspace{1cm} \text{(2.7)}

where \(a_i\) represent the number of times a particular fragment "i" is found in a given molecule and \(f_i\) is the hydrophobic fragmental constant of fragment "i". Therefore a molecule can be visualized as a series of fragments each of which contributes to its hydrophobic character. The \(\log P\) value of a given substance can be calculated using the above equation and \(f_i\) values derived from the partition coefficients in octanol/water. Rekker (1977) calculated the \(\log P\) values for a series of compounds which are now called as Rekker fragmental constants.

Classically, determination of \(\log P\) has been carried
out by the "shake flask" method of Fujita et al. (1964). However this method has been found to be time consuming and expensive. Moreover, the sensitivity of the results to impurities of the sample added another drawback. Therefore many attempts have been made to evaluate log P values by means of chromatographic techniques. These techniques can be divided into two classes (Gago et al., 1987).

1. Octanol separation systems, where TLC plates or HPLC packing materials are covered with octanol and the eluent consists of octanol saturated buffer or water.

2. Reversed phase separation systems, where octa decyl silica is used as the stationary phase and binary mixtures of water and an organic modifier are used as eluents.

High correlations between log $P_{o/w}$ calculated by the method of Rekker (1977) and chromatographic capacity factor log k ($k = (t_R-t_0)/t_0$ where $t_R$ and $t_0$ are elution times of retained and unretained substances) have been reported for a series of compounds such as alkyl benzenes and alcohols (Gago et al., 1987), aromatic compounds (D’ambroise and Hanai, 1982) etc. The difference between log P and log k, as reported by Minick (1987) is that they describe two different equilibria, static equilibrium and dynamic equilibrium respectively.

With the development of high pressure pumps, sensitive detectors, and the attachment of different length alkyl and aryl groups to column material, reversed phase chromatography (RPC) became more popular and the shake flask method was gradually replaced by RPC methods.
The additive nature of hydrophobic retention parameters of peptides and proteins in RPC have been investigated by O'Hair and Nice (1979). They reported that the retention order of smaller compounds with less than fifteen amino acids generally correlated well with the sum of Rekker fragmental constants of the individual amino acids ($r^2=0.9194$). Also, the elution behaviour of peptides in RPC showed good correlation with the predicted hydrophobicity values computed by adding the hydrophobicity of constituent amino acid residues. Meek (1980) observed a correlation of 0.997 between predicted and observed retention times of peptides. All these observations recommend RPC as a tool for hydrophobicity measurement.

To elute solutes in RPC, the polarity of the eluent is decreased by increasing the proportion of miscible non-polar solvent in the aqueous eluent. The disadvantage of this method is that it can denature the solutes. But to elute solutes without denaturation, decreasing salt concentrations may also be used and this technique was called hydrophobic interaction chromatography (HIC).

2.5.3 Hydrophobic partitioning

As described in the earlier section, estimation of partition coefficients is a useful technique for determination of hydrophobicity of proteins. However, the use of water immiscible organic solvents may cause a problem since most proteins do not dissolve in organic solvents. Therefore Shanbhag and Axelsson (1975) used a two phase system
containing a dextran rich lower phase and poly ethylene glycol (PEG) rich upper phase for the partitioning of proteins. The partitioning coefficient of a protein in the system was defined as:

\[ K = \frac{C_u}{C_l} \]  

\( (2.8) \)

where \( C_u \) and \( C_l \) were the equilibrium concentrations of protein in the upper and lower phases respectively. The logarithm of the partitioning coefficient \( \log k \) was defined as the free energy required to transport one mole of solute from the dextran rich lower phase to the PEG rich upper phase. This \( \log k \) could be divided into two factors, \( \log k_0 \) and \( \log k_{el} \), using the relationship:

\[ \log k = \log k_0 + \log k_{el} \]  

\( (2.9) \)

where, \( \log k_0 \) was a measure of relative solvation of a substance in the two phases and may also include some specific interactions with some components in the system. \( \log k_{el} \) depends on the net charge (\( Z \)) of the partitioned substance and on the difference in electrical potential (\( \psi \)) between the phases. The latter would arise only when the system contained electrolytes or when the polymers themselves carry ionizable groups. Therefore \( \log k_{el} = f(Z, \psi) \) and this became zero when a substance was partitioned at its pI or when the salts included in the system did not increase interfacial tension (Shanbhag
Another way of eliminating this charge factor was by using the same concentration of polymers but with only one of the systems containing a polymer-bound, uncharged ligand. Thus the measure of hydrophobicity $\Delta \log k$ was computed as:

$$\Delta \log k = \log k'' - \log k'$$

$$= \log \left(\frac{C_u}{C_l}\right)'' - \left(\log \left(\frac{C_u}{C_l}\right)'\right)$$

(2.10)

where $k''$ was the partition coefficient in a system containing polymer bound ligand and $k'$ was the partition coefficient in the same system without the ligand.

$\log k_0$ in equation (2.9) is a measure of at least two processes. Both the hydrophilic and hydrophobic portions of the protein contribute to $\log k_0$. Thus $\Delta \log k$ would be negative when the hydrophilic part dominates, zero when two effects are equal and positive when the hydrophobic interaction dominates.

2.6 COMPARISON OF METHODS OF HYDROPHOBICITY MEASUREMENT

Among the various methods discussed above, the ANS and CPA hydrophobic probe methods are the most popular and widely used. They are less time consuming and have a long history of utilization, making such results easy to compare with other results. The CPA probe may have advantages over the ANS method due to its similarity to naturally occurring fatty acids. As ANS is an aromatic compound and CPA is an aliphatic compound.
it has been suggested that ANS measurements represent aromatic hydrophobicity and CPA measurements represent aliphatic hydrophobicity. Even though these two methods depend upon the same principle, they do not typically yield comparable or reproducible results.

Hydrocarbon partitioning methods are tedious and some proteins are insoluble in the non-polar phase and therefore float at the interface without attaining equilibrium (Nakai and Li-Chan, 1987). Therefore such methods are of little value in evaluation of food proteins.

Hydrophobic chromatography methods are said to be related to the surface hydrophobicity of solutes, as the surface of the solute is directly in contact with the chromatographic stationary phase (Molnar and Horvath, 1977). Their major disadvantage is the potential for denaturation of proteins. Even though HIC is milder than RPC, there is still no guarantee of measuring hydrophobicity of native proteins by this method. The need to have special equipment and experimental skills is a another disadvantage of this method (Nakai and Li-Chan, 1987).

Various methods of measuring hydrophobicity and the correlations between them have been discussed by Nakai and Li-Chan (1987). No method is entirely adequate and no generally accepted definition of protein hydrophobicity exists at present.
2.7 HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Hydrophobic interaction chromatography was introduced in 1957 under the name of salting out chromatography as a technique for separating substances according to their hydrophobicity (Kato, 1987). Conventional HIC has been carried out mainly on uncharged alkyl and aryl derivatives of agarose (Hjerten et al., 1974). As this type of column material cannot withstand high pressures, until the development of TSK-phenyl-5PW column, HIC had to be performed at low pressures (Kato et al., 1983). TSK-phenyl-5PW column stationary phases are packed with 10μ (1000Å) porous hydroxylated polyether supports with a low density of covalently bound surface phenyl groups. Conceptually HIC is similar to RPC. One main difference is that RPC stationary phases have more hydrophobic groups than HIC stationary phases. Thus the native structure of proteins is more likely to be maintained in HIC than RPC (Goheen and Engelhorn, 1984).

2.7.1 Salt effects on HIC

A characteristic feature of HIC is that retention increases with the salt concentration in the eluent due to the enhancement of hydrophobic interactions between the protein and the non-polar stationary phases. When different salts are used in the eluent, retention of proteins parallel the Hofmeister series (Melander and Horvath, 1977). This retention enhancing effect of neutral salts has been shown to represent the effect of salt in increasing the surface tension of
aqueous solution (Melander and Horvath, 1977). Structure forming salts enhance hydrophobic bonding through the salting out effect (Hofstee, 1975). Therefore, the principal parameters that determine the effect of salt on the retention time of solutes are salt molality and molal surface tension increment (Melander and Horvath, 1977; Melander et al., 1984; Melander et al., 1989).

As has been discussed earlier in this chapter, there are two schools of thought concerning the hydrophobic effect of solutes. From these two approaches, a variation of the solvent effect approach called the Solvophobic theory has been applied to the salting out of proteins in HIC and RPC by Melander and Horvath and their co-workers at Yale university. They developed a simple theory to describe the effect of salts on both electrostatic and hydrophobic interactions in HIC.

In this theory, the free energy change ($\Delta G^*$) associated with a transfer of a molecule from a hypothetical gas phase into solution is given by:

$$\Delta G^* = \Delta G_{\text{cav}} + \Delta G_{\text{es}} + \Delta G_{\text{vdw}} + RT \ln(RT/PV) \quad \ldots \ldots (2.11)$$

where $\Delta G_{\text{cav}}$ is the free energy change for formation of a cavity in the solvent, $\Delta G_{\text{es}}$ is the free energy associated with the electrostatic interactions between the solute and the solvent, $\Delta G_{\text{vdw}}$ is the free energy change due to van der Waals interaction between the solute and solvent, $R$ is the universal gas constant, $T$ is the absolute temperature, $P$ is the
pressure, and \( V \) is the mean molar volume of the solvent.

Application of above relationship to HIC gives the following equation for the isocratic retention factor \( k \) of a solute:

\[
\ln k = - \frac{1}{RT} (\Delta G_{\text{cav}} + \Delta G_{\text{es}} + \Delta G_{\text{vdw}} + \Delta G_{\text{assoc}} + \Delta G_{\text{red}}) + \ln(RT/\rho V) + \Theta \quad \ldots \ldots \quad (2.12)
\]

where \( \Delta G_{\text{assoc}} \) is the free energy change for ligate-eluate association in the absence of surrounding solvent, \( \Delta G_{\text{red}} \) is the reduction of free energy due to solvent-ligate and solvent-eluate interaction and \( \Theta \) is a constant related to the concentration of accessible ligates in the column. As only the salt concentration in the aqueous solvent changes, and there are no specific interactions between the solute and salts, the energetics of the transfer process is assumed to be only affected by the changes in \( \Delta G_{\text{cav}} \) and \( \Delta G_{\text{es}} \) parameters (Melander and Horvath, 1977). Therefore the above equation reduces to,

\[
\ln k = - \frac{1}{RT} (\Delta G_{\text{cav}} + \Delta G_{\text{es}}) + \text{cons.} \quad \ldots \ldots \quad (2.13)
\]

This equation can be further simplified if one considers \( \ln (k/k_0) \) instead of \( \ln k \) where \( k_0 \) is the retention factor at zero salt concentration.

The energy of cavity formation in the mobile phase is related to the surface tension (\( \gamma \)) and surface area of the molecule (\( A_s \)) and is given by:
\[ \Delta G_{\text{cav}} = [N\Lambda_s + 4.8N^{1/3}(K^e-1)V^{2/3}] \]  
\[ \text{where } K^e \text{ is a constant which corrects for the curvature of the cavity. } N \text{ is the Avogadro's number. The surface tension of aqueous salt solutions is a function of molal salt concentration,} \]
\[ \gamma = \gamma^o + \delta m \]

\[ \text{where } \delta \text{ is the molal surface tension increment of the salt, } \gamma^o \text{ is the surface tension of the pure water, and } m \text{ is the salt concentration. When equations (2.14) and (2.15) are combined,} \]
\[ \Delta G_{\text{cav}} = [N\Lambda_s + 4.8N^{1/3}(K^e-1)V^{2/3}]\gamma^o \]
\[ + [N\Lambda_s + 4.8N^{1/3}(K^e-1)V^{2/3}] \delta m \]

Thus overall energy of cavity formation will be,
\[ \Delta G_{\text{cav}} = -[N\Delta\Lambda_s + 4.8N^{1/3}(K^e-1)V^{2/3}] \delta m \]
\[ + \text{constant} \]

\[ \text{where, } \Delta\Lambda_s \text{ is the difference in surface area of ligate and protein exposed to the mobile phase.} \]

There are two theories to explain the electrostatic interactions.

1. At low ionic strength the protein molecule is usually assumed to be a simple ion and its electrostatic free
energy is calculated from Debye-Huckel theory.

2. At high ionic strength according to the Kirkwoods theory, the average distance between the charged ions become smaller and smaller and the concomitant ionic shielding makes the charged macromolecule behave as a neutral dipole.

Therefore, to explain $\Delta G_{es}$ over a wide range of salt concentrations both theories have to be considered and the free energy of electrostatic interaction is given by:

$$\Delta G_{es} = A - \frac{[B(m^{1/2})]}{[1 + C(m^{1/2})]} - D\mu m \ldots \ldots \ldots (2.18)$$

where $A$, $B$, $C$, and $D$ are constants and $\mu$ is the dipole moment of the protein. The first two terms of the above equation comes from the Debye-Huckle theory and the last term comes from the Kirkwoods theory. When protein has no net charge (i.e at its pI) the first two terms in the equation become negligible and the $\Delta G_{es}$ is expected to be propotional to the salt concentration. Away from pI, the relationship is non-linear at low ionic strength.

When equations (2.13), (2.17), and (2.18) are combined:

$$\ln \frac{k}{k_0} = -(1/RT) \frac{[Bm^{1/2}]}{[1+Cm^{1/2}]} + D\mu m)$$

$$+ \frac{(1/RT)}{[N\Delta \phi + 4.8N^{1/3}(K_e-1)V^{2/3}]\sigma m) \ldots \ldots (2.19)$$

In this equation, the total surface area of the protein ($A_s$) has been replaced by the non polar surface area of the
protein molecule ($\phi$). If, $\Lambda = Du/RT$ and

$$\Omega = [N \Delta \phi + 4.8 N^{1/3} (K^e - 1)^{2/3}] / RT$$

Then equation (2.19) will be,

$$\ln k/k_0 = -(B m^{1/2}) / RT (1 + C m^{1/2}) \Lambda m + \Omega \delta m \ldots \ldots (2.20)$$

According to the above equation, retention factors first decrease with increasing salt concentration as the electrostatic interaction parameters (1st and 2nd terms) are negative. But further increase in salt concentration will increase the retention factors and the dependence of log $k$ vs $m$ become linear at sufficiently high salt concentrations. Under such conditions, the limiting slope of log $k$ vs $m$ plots is given by $(\Omega \delta - \Lambda)$ which is termed the hydrophobic interaction parameter. According to the above equation slope is dependent on the hydrophobic contact area, the molal surface tension increment of the salt and an electrostatic parameter related to the dipole moment of the molecule. In hydrophobic chromatography $\Lambda$ is expected to be smaller than $\Omega \delta$. Therefore the magnitude of slope is mainly dependent on the non-polar contact area between the protein and the ligand.

Recently this equation and the theory of Manning's counter ion condensation were combined to develop a new model to evaluate hydrophobic and electrostatic interactions in ion exchange, metal chelate, and bio-polymer chromatography (Melander et al., 1989; El-Rassi and Horvath, 1986a). An equation of the following form has been proposed:
\[ \log k = A + B \log m + C m \]  \hspace{1cm} (2.21)

where \( k \) is the capacity factor, \( m \) is the salt molality, and "B" and "C" are the electrostatic and hydrophobic interaction parameters respectively. "A" is a constant encompassing all characteristic system parameters. According to the model, the magnitude of the hydrophobic interaction parameter \( C \) is determined by the hydrophobic contact area between the protein and the stationary phase surface and the properties of the salt as measured by its molal surface tension increment. At sufficiently high salt concentrations, the electrostatic interactions will approach a constant and the relationship is governed by the \( \log k \) vs \( m \) linear relationship of hydrophobic interaction. \( B \) on the other hand is the slope of the \( \log k \) vs \( \log m \) relationship at low salt concentrations and is a measure of the effect of salt on the magnitude of the electrostatic interaction.

Traditionally, the measurement of hydrophobic interaction parameters using chromatography have been carried out under fixed values of salt concentrations or by gradient elutions. But these models (2.20, 2.21), have shown that the salt concentration has a significant influence on the measured values of capacity factors. Also, to obtain a better understanding of these changes, isocratic elution may be more suitable than gradient elution. Therefore the above chromatographic models, which takes into account the effect of
salt concentration over a wide range, is more appropriate as a measure of hydrophobicity over the simple estimation of retention time in a single concentration of salt or salt gradients. Model (2.20) has been used by Él-Rassi and Horvath (1986a) in Metal Chelate Interaction Chromatography (MCIC), Durance and Nakai (1987) in HIC, and Melander et al. (1989) in bio-polymer chromatography.

2.8 PROTEIN-PROTEIN INTERACTIONS

Klotz (1953) classified the interactions of proteins into two groups,

1. Those in which electrostatic forces are of major importance

2. Those in which specific structural factors play a major role.

However, Frieden and Nichol (1981) categorized protein-protein interactions into three groups such as homogeneous protein-protein interactions, heterogeneous protein-protein interactions, and others (eg. isomerizations, acceptor-ligand binding, etc.). The forces involved in these interactions can be di-sulfide, intermolecular charge-charge interactions (salt bridges), intermolecular H-bonding, hydrophobic bonding, or electrostatic interactions (Von Hippel, 1974). These types of interactions are important in determining the structure and texture of food products.
2.8.1 Affinity chromatography

Association processes undoubtedly play a most important role in bio-chemical processes. Methods in which ligand-protein interactions are measured by direct analysis, such as ultrafiltration, gel filtration, equilibrium dialysis, etc. have now been supplemented by a new technique for the determination of dissociation constants based on affinity chromatography (Dunn and Chaiken, 1974; Larsson et al., 1981; Fassina and Chaiken, 1987). This technique was rapidly developed during the 1970's because of its spectacular success as a purification method for proteins.

Affinity chromatography has other important and exciting uses also. Immobilized ligands, substrates, or enzymes can be used to study the nature of biological interactions, the mechanism and kinetic constants of enzyme interactions, subunit interactions of oligomeric proteins, and immunological responses etc. (Parikh and Cuatricasas, 1985). It also has potential for studying the interactions between mobile and immobilized bio molecules (Dunn and Chaiken, 1974., Kasai and Ishii, 1975).

The separation processes of affinity chromatography can be varied according to circumstances, but are usually characterized by three steps: sample adsorption, washing of the column, and finally elution of the desired compound. An underlying assumption of affinity separation is that the mobile molecule binds to an affinity matrix through the complementary binding surfaces of the immobilized molecule,
but not to non-biospecific matrix sites. Given such bio-specific adsorption, affinity chromatographic elution behaviour of the mobile form can be expected to be a direct measure of quantitative interaction properties of mobile and immobilized interactants including equilibrium binding constants, and in more restricted circumstances, rate constants for associations and dissociations (Fassina and Chaiken, 1987). Several reviews have appeared on analytical affinity chromatography (Chaiken, 1979, 1986; Kasai et al., 1986). The most commonly used method of quantitative affinity chromatography is based on the elution of a biological macromolecular substance from an affinity matrix with soluble macromolecular solutions of various concentrations (Dunn and Chaiken, 1974).

The elution volume of a macromolecular substance is directly dependent on the concentration of the affinity ligand bound to a solid matrix, if the concentration of the soluble affinity ligand is constant. These dependences can be expressed by the following equations (Dunn and Chaiken, 1974):

$$P + \overline{M} \rightarrow \overline{MP}$$

$$P + \overline{M} \leftrightarrow \overline{LP}$$

where $P$ is the mobile macromolecule, $\overline{M}$ is the matrix immobilized interactant, and $L$ is the soluble interactant. Overbars represent species within the gel matrix. As shown in the above figure $\overline{MP}$, $\overline{LP}$ and $LP$ are the possible binary
complexes in the system and their dissociation constants are given by:

\[ K_{LP} = \frac{[L][P]}{[LP]} \] \hspace{1cm} (2.22)

\[ K_{LP}^p = \frac{[L][P]}{[LP]} \] \hspace{1cm} (2.23)

\[ K_{MP} = \frac{[M][P]}{[MP]} \] \hspace{1cm} (2.24)

For liquid chromatography (Freeman, 1972),

\[ V = V_m + D[V_s] \] \hspace{1cm} (2.25)

where \( V \) is the volume required to half elute the protein, \( V_m \) is the mobile phase volume (inside gel) and \( V_s \) is the stationary phase volume (inside gel) and \( D \) is the distribution coefficient for interactions with the gel. Then,

\[ D = \frac{(V - V_m)}{V_s} \] \hspace{1cm} (2.26)

The distribution coefficient relates to the ratio of protein species interacting with the matrix to those present free in solution. Thus,

\[ D = \frac{[\bar{P} + L\bar{P} + M\bar{P}]}{[P + L\bar{P}]} \] \hspace{1cm} (2.27)

Dividing (2.27) by \( P \) and using \( D_0 = \frac{\bar{P}}{P} \) (therefore \( P = \bar{P}/D_0 \)) for distribution of mobile macromolecule into the gel yields:
\[ D = \left( D_0 + D_0 \left( \frac{\bar{L}}{\bar{P}} \right) + D_0 \left( \frac{\bar{M}}{\bar{P}} \right) \right) / (1 + \frac{L}{P}) \]  

\[ \text{Since} \quad \frac{\bar{L}}{P} = \left[ \frac{\bar{L}}{P} \right] \]  

and \[ \frac{\bar{P}}{P} = \left[ \frac{\bar{P}}{P} \right] \]  

Thus all ratios in equation (2.28) may be converted to ratios of concentrations. Then substitution from equations (2.22) to (2.24) yields:

\[ D = D_0 \left( \frac{1 + [L]/K_{LP}}{1 + [L]/K_{LP}} \right) \]  

Then,

\[ \frac{(V - V_m)}{V_s} = D_0 \left( \frac{1 + [L]/K_{LP}}{1 + [L]/K_{LP}} \right) \]  

For \([M] = 0\) and \([L] = 0\)

\[ \frac{(V_0 - V_m)}{V_s} = D_0 \]  

where \(V_0\) is the elution volume of the protein in the absence of interaction with gel. Then,

\[ V = (V_0 - V_m) \left\{ \frac{1 + [L]/K_{LP}}{1 + [L]/K_{LP}} \right\} + \]  

\[ (V_0 - V_m) \left\{ \frac{[M]/K_{MP}}{1 + [L]/K_{LP}} \right\} + V_m \]
Since the gel is considered to be quite porous, both protein (P) and soluble inhibitors (L) will be diffusible throughout the system. Therefore a reasonable simplification to this system is the assumption that binding of soluble inhibitor is not affected by the presence of the gel. i.e., $K_{LP} = K_{LP}$

Therefore,

$$1 + \frac{[L]}{K_{LP}} = 1 + \frac{[L]}{K_{LP}}$$

Therefore equation (2.35) reduces to:

$$V = V_0 + \frac{(V_0 - V_m) ([M]/K_{MP})}{1 + \frac{[L]}{K_{LP}}} \quad (2.37)$$

By linearization of this equation:

$$\frac{1}{(V - V_0)} = \frac{K_{MP}}{(V_0 - V_m) [M]} + \frac{K_{MP} [L]}{K_{LP} (V_0 - V_m) [M]}$$

$$\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \四是
Thus $K_{MP}$ can be calculated independently of competitive ligand.

This relationship can therefore be used to determine dissociation constants of interactions between soluble macromolecules and immobilized ligands from chromatographic retention volumes.

2.8.2 Lysozyme

Lysozyme is an enzyme that lyses the microorganism *Micrococcus lysodeikticus* and is defined as 1,4 beta-N-acetyl-muramidase. It is a basic protein of low molecular weight (14,300) and is stable at acidic pH at fairly high temperatures but labile at alkaline pH. It accounts for 3% of the protein in egg white (Osuga and Feeny, 1974).

Self-association of lysozyme molecules has been reported earlier by Osuga and Feeny (1974). The pH dependence of lysozyme dimerization has been studied by Sophianopoulos and Van Holde (1964). According to their observations dimerization of lysozyme occurs in the pH range of 5-9 and polymerization occurs above pH 9.0 (Fig 2.1). Also, it has been proposed that the dimer formation in solutions of lysozyme at alkaline pH directly obscured one or two binding sites and the extent of association is critically dependent on the contribution of net electrostatic repulsive forces. Thus
Fig. 2.1: Variation of the apparent molecular weight with pH at 20°C. Protein concentration approximately 1.4 g/100 mL in 0.15M KCl. (Sophianopoulos and Van Holde, 1964).
at pH 5.0, where the net charge of the lysozyme monomer (MW 14,300) is about +11.5 only a little if any self association is detected in solutions of ionic strength 0.15. At pH 8.0 where the net charge of lysozyme has decreased to +7.3, significant proportions of higher polymers co-exist in equilibrium with the monomers (Frieden and Nichol, 1981). However, yet the type of bonding is not known.

Lysozyme not only self associates, it also makes heterogeneous complexes with other egg white proteins such as ovomucoid (Matsuda et al., 1982), ovomucin (Kato et al., 1975., Garibaldi et al. 1968), conalbumin (Ehrenpreis and Warner, 1956) and ovalbumin (Forsythe and Foster, 1950). The possibility of avidin interacting with lysozyme has been suggested by Durance (1987). This will be further investigated in the present study.

2.8.3 **Avidin**

Avidin is a glyco-protein widely found in eggs of avian (0.05 g/l) and other oviparous vertibrates. It is a basic secretory oviduct protein (Hertz et al., 1943) with an pI of 10.0 (Green, 1975). Molecular weight is about 68,000, much higher than the molecular weight of lysozyme. In its native form avidin is a tetramer. This protein is remarkably stable over a wide range of pH and temperature (Wei and Wright, 1964).
3. MATERIALS AND METHODS

3.1 HIGH PRESSURE HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HPHIC)

3.1.1 Materials

Bovine pancreas (5x crystallized type-1-AS protease free) ribonuclease-A, chicken egg white Grade-1 (3x crystallized dialized and lyophilized) lysozyme, bovine milk type-3 alpha-lactalbumin, crystallized lyophilized type-3 whale skeletal muscle myoglobin, crystallized lyophilized type-5 ovalbumin, sodium azide, Trizma base [tris (hydroxy methyl) amino methane] and type-3 sialic acid (N-acetyl neuraminic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate (BDH assured) was obtained from BDH Chemical Co. (W. Germany). Ferrous sulfate and sodium sulfate were purchased from Fisher Scientific (Pittsburg, PA, USA). Distilled water was prepared with a Barnsted distillation apparatus. Millipore (Bedford, MA, USA) all glass filtering apparatus was used to prepare HPLC grade high purity water and buffers. Millipore protein filtering unit was used to filter the protein samples.

3.1.2 Instruments

HPLC chromatographic system employed consists of a Varian (Palo Alto, CA, USA) 5000 liquid chromatograph, 20 μL
sample loop, Bio gel-TSK-Phenyl-5PW 75x7.5 mm I.D hydrophobic column purchased from Bio-Rad (Richmond, CA, USA), Varian model 50 UV detector and Varian CDS 401 chromatographic data system.

3.1.3 Buffers

A series of buffers were prepared from 0.025m Tris-HCl at pH 8.0 with varying concentrations (0.3-1.3m) of ammonium sulfate, sodium sulfate and ferrous sulfpate neutral salts.

3.1.4 Estimation of $t_0$

Several methods of obtaining $t_0$ in HPHIC were carried out in this research. 20 μL of sodium nitrate (0.001% w/v), sialic acid (0.1% w/v) and methanol were injected into the column and detected at 235 nm. Distilled water was also used as a method of measuring $t_0$ and detected as a negative peak at 210 nm.

3.1.5 Chromatography

Chromatograms were obtained under isocratic conditions at room temperature at a flow rate of 1 mL/min. After each change of mobile phase 30 min was allowed for equilibration. Protein samples (2 mg) were dissolved in 2 mL of the same buffer and injected into the column. Elution time was taken as the point of maximum $A_{280}$ for all the proteins. The mobile phase hold up time was taken as the maximum point of $A_{235}$ with sialic acid (1% w/v). The capacity factor k was calculated using the equation: 
where $t_R$ is the elution time of protein and $t_0$ is the elution time of sialic acid. At least two reproducible chromatograms for each compound were used to calculate "$k$". The chromatograms were considered to be reproducible when the deviation in elution time is less than 0.01 min.

3.1.6 Data Analysis

Logarithms of the capacity factors (log $k$) were plotted against the salt molality (m) for all three neutral salts and solid lines were drawn by fitting the data points according to the following equation.

$$\log k = A + B \log m + C m$$

(3.2)

Results were examined by evaluating the $C$ values obtained by multiple regression analysis and the slopes ($C'$) of log $k$ vs m relationships obtained by simple regression analysis, performed with Statgraf Data System (Statistical Graphics Co.).

3.2 HYDROPHOBIC PROBE MEASUREMENTS (ANS and CPA methods)

3.2.1 Materials

Mono-sodium hydrogen phosphate and di-sodium hydrogen phosphate were obtained from Fisher Scientific (Pittsburg, PA,
USA). Butylated hydroxy anisole (BHA) and 1-anilino naphthalene-8-sulfonate (ANS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cis parinaric acid (CPA) was purchased from Molecular Probe Inc. (Eugene, OR, USA). n-Decane, ethanol and methanol were obtained from BDH Chemical Co. (W. Germany).

3.2.2 Instruments

Fluorescent measurements were made on a Shimadzu RF-540 recording spectrofluorophotometer (Kyoto, Japan). A Varian (Palo Alto, CA, USA) Cary model 210 spectrophotometer was used to measure the absorbance of proteins at 280 nm.

3.2.3 Method

ANS method

An ANS solution was prepared by dissolving 12.42 mg of ANS in 5 mL of 0.05M Tris HCl (pH 8.0) buffer. A series of protein concentrations ranging from 0.005-0.030% were obtained by diluting a concentrated protein stock solution with the same buffer. Concentration of the diluted protein solutions was calculated using the extinction coefficient ($E_{1cm}^{1%}$) at 280 nm (Table 3.1). Then 10 μL of ANS solution was added to 2 mL of each protein solution and relative fluorescence intensity (RFI) was measured by a spectrofluorophotometer at excitation and emission wave lengths of 390 and 470 nm respectively. The excitation and emission slit widths were 5 nm. The RFI reading was standardized by adjusting the reading of the fluorometer to 30% full scale for 10 μL of ANS in 2 mL of methanol. The
Table 3.1: Extinction coefficients of proteins used in HPHIC

<table>
<thead>
<tr>
<th>Protein</th>
<th>$E_{1cm}^{1%}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>7.50</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>20.90</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>26.00</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>10.00</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>6.95</td>
</tr>
</tbody>
</table>

Ref. Sorber and Harte (1970)
net RFI of each protein concentration was measured by subtracting RFI of each solution without the probe from that with the probe.

**CPA method**

CPA solution was prepared by dissolving 10 mg of CPA and 100 µg of BHA in 10 mL of absolute ethanol. Same method described in the ANS procedure was carried out to prepare the protein dilutions. Then 10 µL of CPA was added to 2 mL of protein dilutions and RFI was measured at wave lengths of excitation and emission, 325 and 420 nm respectively. The RFI reading was standardized by adjusting the reading of the fluorometer to 75% full scale for 10 µL of CPA in 2 mL of n-Decane.

### 3.3 AFFINITY CHROMATOGRAPHY

#### 3.3.1 Materials

Grade-1 3x crystallized hen egg white lysozyme, avidin, Ficoll 70,000, Ficoll 400,000, and sodium azide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BDH assured ammonium sulfate, di-sodium hydrogen orthophosphate, and citric acid monohydrate were obtained from BDH Chemical Co. (W. Germany). Activated immuno-affinity supports (Affigel-10) were purchased from Bio-Rad Chemical Co. (Richmond, CA, USA).
3.3.2 Instruments

A liquid chromatograph apparatus was assembled from a Gilson Miniplus-3-peristaltic pump, a Rheodyne type 50 teflon rotary valve equipped with a 1 mL sample loop, Gilson model 112 UV detector, and model 203 Gilson fraction collector purchased from Gilson Medical Electronics (USA). Kontes flex columns and column flow adapters were obtained from Mandel Scientific Co. (Toronto, Canada). The JCL 6000 chromatography data system obtained from the same company was used to process the signal from the UV detector to obtain the peak. Refractive index of the eluted fractions of Ficoll were measured by a ABBE Mark 2 digital refractometer (Cambridge Instruments Inc., Buffalo, NY, USA).

3.3.3 Buffers

A series of mobile phase buffers were prepared with 0.01m citric phosphate buffer with varying molalities of ammonium sulphate (0.001-1.7m) in four pH values (3.2, 5.0, 6.8, 7.2) for the lysozyme-lysozyme interaction. pH of the buffers used for avidin-lysozyme interaction were 6.5 and 7.8.

3.3.4 Coupling of lysozyme to Affi gel-10 column

Ligand solution (lysozyme) was prepared by dissolving lysozyme (30 mg lys/mL gel) in 0.01m phosphate buffer. 25 mL of Affi gel 10 suspension was transferred to a small buchner funnel and allowed the supernatent solvent to drain. Then the gel bed was washed with three to four times the volume of 4°C
deionized water. The moist gel remaining on the funnel was then transferred to a conical flask and mixed with the cold (4°C) ligand solution (0.5 mL of ligand solution/mL gel). Agitation was continued for 1 hr at 4°C. At the end, 1m glycine ethyl ester (pH 8) blocking agent per mL gel was added to the ligand solution and kept for another one hour for completion of the blocking reaction. Finally the gel was transferred to a column and washed with the same buffer (0.01m phosphate) until it is free from reactants.

3.3.5 Chromatography

To study the lysozyme-lysozyme interaction 1 mL of 0.2% (w/v) soluble lysozyme was injected into the pre saturated lysozyme immobilized column in buffers of 0.001 to 1.7m ammonium sulfate in pH values of 3.2, 5.0, 6.5 and 7.2. To study the avidin-lysozyme interaction, same procedure was carried out with soluble avidin (0.2% w/v) in buffers of 0.001 to 1.3m ammonium sulfate in pH values of 6.5 and 7.8. The experiment was done at room temperature at a flow rate of 0.65 mL/min. Retention times of the proteins with isocratic elution were measured by a UV detector at 280 nm. Ficoll 70,000 and Ficoll 400,000 were applied to the column to measure the void volume and the mobile phase volume respectively. Elution volume of Ficoll was taken as the point of maximum refractive index. To measure the immobilized ligand concentration in the column, procedure given in section 3.3.7 was carried out.
3.3.6 Data analysis

Logarithmic capacity factors (log k) of proteins were calculated from k in the equation (3.1). The data were analysed by performing multiple regression as well as linear regression analysis (for points above 0.3m) according to equation 3.2. To calculate the apparent dissociation equilibrium constants ($K_{\text{MP}}^e$) of lysozyme-lysozyme and avidin-lysozyme interactions data were fitted to the following equation at 1.1m and 0.9m salt molalities respectively.

$$\frac{1}{(V-V_0)} = \frac{K_{\text{MP}}^e}{(V-V_0)[M]} \quad \text{...............(3.3)}$$

3.3.7 Measurement of ligand concentration in stationary phase

Bio-Rad protein assay method was used to measure the ligand concentration in stationary phase. Dye reagent was prepared as described in the Bio-Rad manual. Several dilutions of lysozyme containing 0.2-1.4 mg/mL were taken as the protein standards. 5 mL of diluted dye reagent was added to 0.1 mL of lysozyme standard and 0.1 mL of sample buffer (0.01m phosphate buffer). Only 0.1 mL of sample buffer and 5 mL of dye reagent was used as the blank. The solutions were mixed several times and after a period of 5 min to 1 hr, $OD_{595}$ vs reagent blank was measured in a spectrophotometer. The plot of $OD_{595}$ vs protein concentration (μg) was used as the standard curve.

To measure the ligand concentration in the column 0.1 g of drained lysozyme immobilized gel was suspended in 5 mL of dye and the absorbance was measured at $OD_{595}$. Then the ligand
concentration was read from the standard curve.
4.0 RESULTS AND DISCUSSION

This chapter presents the investigation of the surface hydrophobicity of proteins and the behaviour of hydrophobic and electrostatic interaction parameters in protein-protein interactions, conducted on high pressure hydrophobic interaction chromatography (HPHIC) and low pressure affinity chromatography with increasing and decreasing salt molalities.

4.1 HIGH PRESSURE HYDROPHOBIC INTERACTION CHROMATOGRAPHY

4.1.1 Measurement of hold up time \( (t_0) \) in HPHIC

The hold up time \( t_0 \) is a crucial parameter that is needed for the accurate determination of retention factors \( (k) \). The correct measurement of \( t_0 \) is an intensely active research area (Smith et al., 1986) and no general agreement exists. In the present study, several methods of obtaining \( t_0 \) were examined (section 3.1.4).

The elution times obtained with methanol and acetone were higher than the elution time of myoglobin at low salt concentrations and tended to deviate with the changing salt molalities. Distilled water gave retention times similar to or greater than myoglobin and sometimes eluted as flat peaks that the computer program was unable to identify. The results obtained with sialic acid were fairly constant with no noticeable deviations from expected behaviour under all
experimental conditions. The average retention time was 2.157 min and the standard deviation was 0.02. Therefore in view of the failure of other techniques this retention time was used to calculate $t_0$ and thus the capacity ratios.

4.1.2 Retention of proteins in HPHIC

Effect of salts on the retention behaviour of proteins in the electrostatic and hydrophobic interaction chromatography has been described by a three parameter model:

$$\log k = A + B \log m + C m$$ ................................(4.1)

The model allows the estimation of both electrostatic ($B$) and hydrophobic ($C$) binding parameters concurrently, between a solute and a resin, by quantitatively examining the effect of neutral salt concentrations in the mobile phase on solute retention (El-Rassi and Horvath, 1986a; Melander and Horvath, 1989). In the present study, the capacity factors ($\log k$) of proteins were calculated from $k$ values derived from equation (3.1) at several salt molalities and the plots of salt molality ($m$) vs $\log k$ are given in Figs. 4.1 to 4.3. According to the above equation (4.1), $C$ is the limiting slope at sufficiently high salt concentrations, which is a measure of solute resin interaction in conditions which maximize hydrophobic interactions and minimize electrostatic interactions. On the other hand, $B$ is the limiting slope at sufficiently low salt concentrations. Thus the plots of $\log k$
Fig 4.1: Plot of the logarithmic retention factor of proteins against salt molality of ferrous sulfate in the mobile phase.
Fig. 4.2; Plot of the logarithmic retention factor of proteins against salt molality of ammonium sulfate in the mobile phase.
Fig. 4.3; Plot of the logarithmic retention factor of proteins against salt molality of sodium sulfate in the mobile phase.
vs m should yield straight lines at sufficiently high salt concentrations, with slopes representing hydrophobic interaction parameters, as the electrostatic interactions are insignificant at higher salt concentrations.

From the Figures 4.1 to 4.3 it can be observed that ovalbumin generally has a more curvilinear nature than the other four proteins. This can be explained by considering the charges on the protein molecule. Table 4.1 gives the isoelectric points (pI) and the charges of the five proteins studied in HPHIC. Charges were reproduced from Bigelow (1967). It is clear from this table that the charge frequency is independent on the pI values. Also, the two proteins ovalbumin and lactalbumin have close pI values and charge frequencies. But lactalbumin gave straight line relationships in this study with all three salt types. Therefore, the reason for the curvilinear nature of ovalbumin is not due to a significant fraction of charge residues on the protein molecule. It can be a reason of protein denaturation at higher salt concentrations that make ovalbumin to behave as a very hydrophobic protein in HPHIC. On the other hand it has been reported, that lactalbumin is a protein which possesses unusually large amount of tryptophan (5.6% per mole) (Gordon, 1971). Tryptophan is a hydrophobic amino acid. Therefore it may make lactalbumin behave as a very hydrophobic protein.

In this experimental program, the hydrophobic interaction parameters were calculated by two methods. In one method, the retention data measured at different salt
Table 4.1: The molecular weights, iso-electric points, and the charges of proteins used in HPHIC.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Wt.</th>
<th>pI</th>
<th>Charges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>44,000*</td>
<td>4.7</td>
<td>0.24</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>17,400**</td>
<td>4.8</td>
<td>0.28</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14,000*</td>
<td>11.0</td>
<td>0.14</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17,500*</td>
<td>7.1</td>
<td>0.34</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>13,700*</td>
<td>9.4</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Note: * Ref. Sober and Harte (1970)
** Ref. Gordon (1971)
concentrations in the eluent were fitted to the above three parameter model (4.1) to obtain C values. In the other method, the data were examined by linear regression analysis (log k = A + C'm) and the slopes (C') were evaluated. In the case of ovalbumin, linear regression analysis was conducted for the points above 0.8m. These results are presented in Tables 4.2 and 4.3 respectively. According to the theory both these values are measures of hydrophobicity. Therefore they should be in good agreement. But the correlation coefficients calculated between these two parameters for the three salt types, ferrous sulfate, ammonium sulfate, and sodium sulfate were 0.12, 0.88, and 0.79 respectively.

When C' values given in Table 4.3 are considered, ovalbumin and lactalbumin had higher values compared to myoglobin and ribonuclease with all three salt types. Lysozyme possessed the third place in ammonium and sodium sulfate salts. This order of hydrophobicity was observed in C values, only with ammonium sulfate. However from the C' values (Table 4.3) it seems that ovalbumin, lactalbumin and lysozyme are more hydrophobic than myoglobin and ribonuclease. El-Rassi and Horvath (1986b) have found that the slopes of log k vs m plots of ovalbumin and lysozyme increase with the chain length of the stationary phase ligates, indicating an increase in the hydrophobic contact area upon increasing the length of bound polyether chains. On the other hand, the hydrophobic interaction parameter of ribonuclease was particularly independent of the length of bound polyether ligates. These
Table 4.2: Hydrophobic interaction parameters (C) of the plots of log k vs m of ferrous sulfate, ammonium sulfate and sodium sulfate in the mobile phase.

<table>
<thead>
<tr>
<th>Protein</th>
<th>FeSO₄</th>
<th></th>
<th></th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>-1.7559</td>
<td>1.3644</td>
<td>1.2496</td>
<td>0.978</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>-1.4692</td>
<td>-0.4626</td>
<td>1.1930</td>
<td>0.967</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>-1.4673</td>
<td>0.6463</td>
<td>1.7616</td>
<td>0.987</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>-0.8521</td>
<td>2.3542</td>
<td>0.3822</td>
<td>0.973</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>-5.3657</td>
<td>-5.7783</td>
<td>1.4942</td>
<td>0.945</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>(NH₄)₂SO₄</th>
<th></th>
<th></th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>-4.4503</td>
<td>-3.0816</td>
<td>4.5266</td>
<td>0.996</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>-2.3281</td>
<td>-0.8954</td>
<td>3.0582</td>
<td>0.998</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>-2.6126</td>
<td>-1.9504</td>
<td>3.0491</td>
<td>0.995</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>-3.3434</td>
<td>-1.9504</td>
<td>2.6928</td>
<td>0.941</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>-2.2880</td>
<td>-0.4268</td>
<td>1.9637</td>
<td>0.997</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Na₂SO₄</th>
<th></th>
<th></th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>-6.5094</td>
<td>-5.9782</td>
<td>7.6953</td>
<td>0.996</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>-2.6889</td>
<td>-1.3313</td>
<td>3.8418</td>
<td>0.999</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>-3.1767</td>
<td>-2.9090</td>
<td>4.5047</td>
<td>0.999</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>-4.0717</td>
<td>-3.2149</td>
<td>4.2156</td>
<td>0.975</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>-2.8498</td>
<td>-1.7118</td>
<td>3.1308</td>
<td>0.981</td>
</tr>
</tbody>
</table>
Table 4.3: Slopes (C') and intercepts of the plots of log k vs salt molality of ferrous sulfate, ammonium sulfate and sodium sulfate in the mobile phase.

| Protein          | FeSO₄          |            |         |          |           |          |          |
|------------------|---------------|------------|---------|----------|-----------|---------|
|                  | slope (C')    | intercept  | r²      |          |           |         |
| Ovalbumin        | 2.1895        | -2.7303    | 0.998   |          |           |         |
| (>0.8m)          | 1.9769        | -2.5602    | 0.993   |          |           |         |
| (all points)     | 1.9640        | -2.0969    | 0.994   |          |           |         |
| Lactalbumin      | 1.4779        | -1.1504    | 0.993   |          |           |         |
| Lysozyme         | 1.8202        | -2.1862    | 0.982   |          |           |         |
| Myoglobin        | 1.4942        | -1.8472    | 0.950   |          |           |         |
| Ribonuclease     | 3.3586        | -2.4085    | 0.997   |          |           |         |
| (NH₄)₂SO₄        | 2.4379        | -2.4085    | 0.993   |          |           |         |
| Lactalbumin      | 2.3554        | -1.6826    | 0.998   |          |           |         |
| Lysozyme         | 1.8832        | -1.4170    | 0.988   |          |           |         |
| Myoglobin        | 1.8228        | -2.4513    | 0.990   |          |           |         |
| Ribonuclease     | 1.7086        | -2.0264    | 0.998   |          |           |         |
| Na₂SO₄           | 4.1710        | -2.4241    | 0.999   |          |           |         |
| Ovalbumin        | 3.2230        | -2.4241    | 0.988   |          |           |         |
| (>0.8m)          | 2.7659        | -1.7394    | 0.999   |          |           |         |
| (all points)     | 2.1537        | -1.1014    | 0.999   |          |           |         |
| Lactalbumin      | 1.8361        | -1.8317    | 0.976   |          |           |         |
| Lysozyme         | 1.9374        | -1.7265    | 0.988   |          |           |         |
observations are in agreement with the present study showing that lysozyme and ovalbumin are more hydrophobic than ribonuclease.

Slope, which is the tangent value, is sensitive to small changes in inclination. Thus, even though the curves fitted using multiple linear relationship and simple linear relationships overlap at higher salt concentrations, the values of $C$ and $C'$ are noted to be quite different. Therefore a question arises as to which parameter is best. The coefficients of correlation ($r$) observed in these analyses are also given in Tables 4.2 and 4.3. The correlations were better in simple linear regression analysis ($C'$) than in multiple regression analysis ($C$) except for ovalbumin. When all the points were considered, ovalbumin had a higher $R^2$ value with multiple regression analysis. Therefore it appears that simple regression analysis is more suitable for the interpretation of the data conducted at higher salt concentrations. El-Rassi and Horvath (1986b) have also used, both the multiple and linear regression analysis to evaluate their data.

The $C$ and $C'$ values of chromatographic retention are believed to be related to the hydrophobic contact area between a protein and the chromatographic stationary phase, and the intercepts of log $k$ vs $m$ relationships are believed to be related to the strength of hydrophobic interaction (Fausnaugh, et al, 1986). As illustrated in Table 4.3, with all three salt types, lysozyme had the highest intercept value and ovalbumin had the lowest intercept value. Therefore, if the above
statement is true, the strength of hydrophobic interaction of lysozyme with the stationary phase is higher than the strength of the hydrophobic interaction of ovalbumin with the stationary phase even though ovalbumin possess higher C and C' values. Therefore these results suggest that the surface hydrophobic area is not proportional to the strength of hydrophobic interaction.

As given in Tables 4.1, 4.2 and 4.3 molecular weights of the proteins do not show any correlation with the hydrophobic interaction parameters. For example, in this experiment, although lysozyme and ribonuclease-A have similar molecular weights, lysozyme has higher C and C' values. But a relationship between molecular weights and the slopes of log k vs m graphs has been reported by El-Rassi and Horvath (1986b). As previously indicated, an increase in the alkyl chain length and the ligand density caused an increased retention of certain proteins on HIC columns. The extent of this effect could be dependent on the size of proteins, if one assumes that proteins are spherical molecules. Smaller proteins would interact with the support over a smaller surface area. Small proteins with less surface area of contact with the column material have shown a decrease in protein retention, which levelled off at lower ligand densities. Larger proteins with higher contact surface area exhibited a continuous decrease in protein retention with decreasing ligand density (Fausnaugh et al., 1984). Thus, even though size can be a contributing factor to protein retention in HIC, surface
4.1.3 **Comparison of C and C′ with ANS and CPA hydrophobicity indices**

ANS and CPA hydrophobic probes are the most widely used methods of measuring surface hydrophobicity. Although such probes are useful as measures of relative hydrophobicity, the precise meaning of the measurement in terms of protein structures has not yet been defined. As an actual estimate of the surface area of hydrophobic contact between a protein and the HIC stationary phase may be possible, correlation coefficients were calculated to find out a relationship between C and C′ parameters with ANS and CPA indices. Figs. 4.4 and 4.5 show the plots of C and C′ vs ANS and CPA measurements respectively for experiments with sodium sulfate in the mobile phase. It appears from these figures that ovalbumin C and C′ values are in greatest disagreement with the ANS results. Ovalbumin is a protein known to be susceptible to surface denaturation (Warner, 1954). This may explain the higher C and C′ values obtained for ovalbumin, even though it possesses a lower value for ANS.

Correlation coefficient values of ANS vs C and ANS vs C′, with ferrous sulfate, ammonium sulfate, and sodium sulfate were 0.0068, 0.0043, 0.035 and 0.1297, 0.0413, 0.0487 respectively. When ovalbumin was not considered r² value of ANS vs C′ improved tremendously. For ammonium sulfate the
Fig. 4.4; Graph illustrating the lack of dependence of C and C' (sodium sulfate in the mobile phase) on ANS.
Fig. 4.5; Graph illustrating the lack of dependence of C and C' (sodium sulfate in the mobile phase) on CPA.
value changed from 0.048 to 0.914. Also the analysis of variance showed that these values are statistically significant at 5% level. But the omission of ovalbumin does not improve the $r^2$ values of ANS vs C. There were no significant correlations between CPA vs C or C'.

Another attempt was made to correlate C' values with Bigelow's average hydrophobicity measurements (Fig.4.6). However, no significant improvements in the $r^2$ values were observed by omitting ovalbumin. Bigelow's average hydrophobicity is a measure of total hydrophobicity. Therefore $r^2$ values were not expected to improve with the omission of ovalbumin. In the literature it has been stated that hydrophobic chromatography does not determine the total content of the non-polar amino acids in the molecule but rather measures the ability of proteins to take part in hydrophobic interactions (Keshavarz and Nakai, 1979).

4.1.4 Relationship of C and C' to molal surface tension increment

"Lyotropy" is a term which describes the order of effectiveness of ions in influencing a large number of chemical and physical phenomena (Robinson and Jencks, 1965). Although many attempts have been made to elucidate the nature of lyotropy, by evoking hydration theories, electrostatic effects, and van der Waals forces none have satisfactorily accounted for experimental observations (Melander and Horvath, 1977). However the molal surface tension increments (δ) of
Fig. 4.6; Graph illustrating the lack of dependence of C' (sodium sulfate in the mobile phase) on Bigelow's average hydrophobicity.
salts, calculated by the above workers provided a strong theoretical support to the lyotropic series. There are two different types of salts, "salting in" and "salting out". "Salting in" salts depress the surface tension of water and "salting out" salts increase it. Therefore a "salting out" salt at high ionic strength increases the van der Waals attraction between proteins and adsorbent across the medium, leading to association, while "salting in" salts reduce it (Nakai and Li-Chan, 1987). A molal surface tension increment value of about $1.5(\text{dyne g/cm mol})^{10^3}$ marks the dividing line between these two salt types in the lyotropic series.

In this experiment, only salts of the "salting out" type ($\sigma > 1.5$) were used. Figs. 4.7 and 4.8 illustrate the plots of parameter C and C' against the molal surface tension increment ($\sigma$), that has the dimensions of (dyne g/cm mol) respectively. The values of $\sigma$ were 1.55 for ferrous sulfate, 2.16 for ammonium sulfate, and 2.73 for sodium sulfate (Melander and Horvath, 1977). According to equation 2.20, chromatographic retention is not only governed by the electrostatic and hydrophobic effect but also by the salt mediated changes in the surface tension. Thus a straight line should result when C and C' are plotted against $\sigma$. In this experiment, straight line relationships were observed between C' vs $\sigma$ for all the five proteins studied (Fig.4.8). In the case of C vs $\sigma$ high correlations were observed for all the proteins except for ribonuclease. A similar experiment was conducted by Melander et al. (1984) with similar results.
Fig. 4.7: Graph illustrating the dependence of hydrophobic interaction parameters (C) on the molal surface tension increment of salts (ferrous sulfate, ammonium sulfate, and sodium sulfate) in the eluent.
Fig. 4.8; Graph illustrating the dependence of slope ($C'$) on the molal surface tension increment of salts (ferrous sulfate, ammonium sulfate, and sodium sulfate) in the eluent.
Fig. 4.8 also shows that the change of $C'$ per unit change in molal surface tension increment is higher for more hydrophobic proteins than for less hydrophobic proteins (myoglobin and ribonuclease).

4.2 PROTEIN-PROTEIN INTERACTIONS

4.2.1 Lysozyme-lysozyme interaction

Fig. 4.9 illustrates the retention factors of lysozyme-lysozyme interaction at four pH values. The values were obtained by affinity chromatography with stationary phases having lysozyme as the immobilized ligand and with increasing and decreasing salt gradients in the electrostatic and hydrophobic interaction modes of chromatography. The above graph shows that at pH 7.2, isocratic retention factors first decrease with increasing salt molality until a minimum is reached, after which further increases in salt molality results in increased retention factors, in accordance with equation 4.1. At sufficiently high salt concentrations, plots of log $k$ vs $m$ yield straight lines with slopes representing hydrophobic interaction parameters. This behaviour was observed at higher salt concentrations in all four pH values.

Figure 4.10 represents the plot of log $k$ vs log $m$ relationship of lysozyme-lysozyme interaction at pH 7.2 with slopes representing electrostatic interaction parameters at sufficiently low salt concentrations. A detailed discussion of the above data are given in later sections.
Fig. 4.9; Plot of the logarithmic retention factors of lysozyme-lysozyme interaction against the salt molality of ammonium sulfate in the mobile phase.
Fig. 4.10: Plot of the logarithmic retention factors of lysozyme-lysozyme interaction against the logarithmic salt molality of ammonium sulfate in the mobile phase.
4.2.2 Avidin-lysozyme interaction

Several methods of isolating and purifying lysozyme and avidin have appeared in the scientific literature (Durance, 1987). The fact that avidin was difficult to separate from lysozyme prompted the suggestion that some form of interaction between the two proteins may have occurred. Certain positive observations for this suggestion made by Durance (1987) are given below.

1. When protein fractions rich in lysozyme and avidin were separated on a carboxymethylcellulose (CMC) column, the avidin peak was well separated from the lysozyme peak, as seen in an $A_{280}$ profile. But the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of avidin fractions revealed a distinct protein band at the position typical of lysozyme.

2. SDS-PAGE of the avidin containing fractions from the primary isolation on Duolite-C-464 also indicated that lysozyme was a major contaminent of avidin.

3. Electrophoresis of egg white with added avidin showed a decreased mobility of avidin as compared to pure avidin.

In the present experiment, avidin-lysozyme interaction was studied with a lysozyme immobilized affinity column and the results are illustrated in Figures 4.11 and 4.12. The results show an interaction between the two proteins at both pH values studied. U-shaped graphs were obtained in agreement with the equation (4.1) when log k vs m was plotted. From
Fig. 4.11; Plot of the logarithmic retention factors of lysozyme-avidin interaction against the salt molality of ammonium sulfate in the mobile phase.
Fig. 4.12: Plot of the logarithmic retention factors of lysozyme-avidin interaction against the logarithmic salt molality of ammonium sulfate in the mobile phase.
these graphs it is clear that both hydrophobic and electrostatic interactions are involved in avidin-lysozyme interaction.

4.2.3 Effect of salt and pH on the hydrophobic interaction parameter

4.2.3.1 Lysozyme-lysozyme interaction

Results obtained from the simple and multiple regression analysis are given in Tables 4.4 and 4.5 respectively. These results indicate that the $r^2$ values of the linear model ($C'$) were higher than the $R^2$ values of multiple regression model (C). Therefore, in the case of lys-lys interaction, results were interpreted using the $C'$ values.

It can be seen from Table 4.5 and Fig. 4.9 that the $C'$ values are decreasing slightly with increasing pH. This suggests in view of equation 4.1, that the hydrophobic contact area between the protein and the immobilized ligand decreased with increasing pH. According to Melander et al. (1989), when hydrophobic binding sites contain ionogenic groups, C parameters can be affected by pH. But when the strength of interaction was evaluated by the lysozyme-lysozyme dissociation constant values ($K_{Dp}$) (Table 4.6), a gradual decrease with increasing pH, indicating stronger interactions was found. A similar observation has been made by Fausnaugh and Regnier (1986) when lysozyme was chromatographed on a hydrophobic interaction column at varying pH values. They also noted an increase in the strength of hydrophobic interaction...
Table 4.4; A, B and C parameters of lysozyme-lysozyme and lysozyme-avidin interactions.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys-Lys</td>
<td>7.2</td>
<td>-1.2455</td>
<td>-0.1782</td>
<td>1.1505</td>
<td>0.942</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>-1.5218</td>
<td>-0.0269</td>
<td>1.3627</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-2.2839</td>
<td>-0.3042</td>
<td>1.9145</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>-2.3957</td>
<td>0.3422</td>
<td>1.6133</td>
<td>0.985</td>
</tr>
<tr>
<td>Avi-Lys</td>
<td>7.8</td>
<td>-1.6764</td>
<td>-0.5135</td>
<td>1.4576</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>-1.0910</td>
<td>-0.0972</td>
<td>1.2057</td>
<td>0.998</td>
</tr>
</tbody>
</table>
Table 4.5: Slopes ($C'$) and intercepts of the plots of lysozyme-lysozyme and lysozyme-avidin interactions.

<table>
<thead>
<tr>
<th>pH</th>
<th>Slope ($C'$)</th>
<th>Intercept</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lys-Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>1.0566</td>
<td>-1.4912</td>
<td>0.964</td>
</tr>
<tr>
<td>6.8</td>
<td>1.3522</td>
<td>-1.5118</td>
<td>0.996</td>
</tr>
<tr>
<td>5.0</td>
<td>1.7851</td>
<td>-2.1514</td>
<td>0.998</td>
</tr>
<tr>
<td>3.2</td>
<td>1.7309</td>
<td>-2.5128</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>Avi-Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>0.9446</td>
<td>-1.1996</td>
<td>0.990</td>
</tr>
<tr>
<td>6.5</td>
<td>0.9711</td>
<td>-0.9236</td>
<td>0.954</td>
</tr>
</tbody>
</table>
Table 4.6: Dissociation equilibrium constants ($K_{MP}$) of lysozyme-lysozyme and lysozyme-avidin interactions.

<table>
<thead>
<tr>
<th>pH</th>
<th>$V_m$ (ml)</th>
<th>$V_0$ (ml)</th>
<th>$V_t$ (ml)</th>
<th>$K_{MP}$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>6.196</td>
<td>7.04</td>
<td>9.408</td>
<td>0.2602</td>
</tr>
<tr>
<td>5.0</td>
<td>6.196</td>
<td>7.04</td>
<td>12.05</td>
<td>0.1229</td>
</tr>
<tr>
<td>Lys-lys*</td>
<td>6.8</td>
<td>6.196</td>
<td>7.21</td>
<td>14.95</td>
</tr>
<tr>
<td>7.2</td>
<td>6.196</td>
<td>7.21</td>
<td>17.23</td>
<td>0.0738</td>
</tr>
<tr>
<td>Avi-lys**</td>
<td>6.5</td>
<td>8.45</td>
<td>9.858</td>
<td>16.90</td>
</tr>
<tr>
<td>7.8</td>
<td>8.45</td>
<td>9.858</td>
<td>14.45</td>
<td>0.2238</td>
</tr>
</tbody>
</table>

* $K_{MP}$ values are calculated at 1.1m salt molality
** $K_{MP}$ values are calculated at 0.9m salt molality
(i.e. an increase in intercept) with increasing pH when log k vs m was plotted. But no changes were observed in the surface area of hydrophobic interaction. According to their suggestion, changes in the strength of interaction were due to changes in the ionization state of amino acids in the contact surface area. With increasing pH, ionization state of lysozyme decreased and hence the strength of interaction increased. This could be the reason for the present observation also. But, it is difficult to give a reasonable explanation for the reduction in the surface area of hydrophobic interaction with increasing pH. It appears that hydrophobic interactions play a dominant role in lysozyme dimerization at higher pH values.

4.2.3.2 Lysozyme-avidin interaction

Fig 4.11 illustrate the log k vs m plot of avidin-lysozyme interaction and the calculated C and C' values are given in Tables 4.4 and 4.5. It can be seen from these results that the $R^2$ values of C are higher than the $r^2$ values of C'.

Results show that, the C' values are not varying much with the changing pH. On the other hand C values show a smaller reduction in the surface area of hydrophobic interaction with decreasing pH. But the strength of interaction, evaluated by $K_{MP}$ values (Table 4.6) and by the intercept of the line tangent to the hydrophobic portion of the log k vs m plot showed stronger interactions at low pH. According to this observation strength of hydrophobic interaction is increasing at low pH.
4.2.4 Effect of salt and pH on electrostatic interaction parameter

Many proteins are regarded as spherical molecules with randomly dispersed charges on the protein surface. If the charge distribution on the protein molecule is not symmetrical, it will lead to an uneven electrostatic potential, depending on the orientation of the molecule with respect to the charged groups on the molecule (Kopaciewicz, 1983). Therefore, unless the charged groups on the protein molecule is highly asymmetrical, only a fraction of the total charge on the molecule can interact with the stationary phase (Melander et al., 1989). Thus, B is dependent on the effective charge of the protein molecule and not on its net charge (Melander et al., 1989).

Values of B for a given protein were expected to be directly proportional to the surface density of ionized ligates on the stationary phase surface and the charge on the protein surface, and inversely proportional to the charge on the salt co-ion (Melander et al., 1989). Thus, in lysozyme-lysozyme and avidin-lysozyme interactions, B is a measure of surface charge and density of ionized ligates on lysozyme and avidin molecules. The net charge of a protein at its pI is zero and increasingly positive as the pH falls or increasingly negative as the pH rises. But certain studies done with ion exchange resins have indicated, that even at the iso-electric point proteins have many charged groups on their surfaces (Gorbanove et al., 1986). In the pH range of this study, both
avidin (pI=10) and lysozyme (pI=11) are positively charged.

4.2.4.1 Lysozyme-lysozyme interaction

In lysozyme-lysozyme interaction it was possible to obtain an electrostatic interaction parameter only at pH 7.2 (Table 4.7 and Fig. 4.10). Below that pH, there were no electrostatic interactions detected. Even at that pH, the slope of log k vs log m curve was very low compared to the C parameter. Zero electrostatic interactions at low pH may be due to high concentrations of positive charges on lysozyme molecules, such that they repel each other. Therefore hydrophobic interactions must play a dominant role in lys-lys interactions especially at low pH.

4.2.4.2 Avidin-lysozyme interaction

The observed dependence of B on the eluent pH is depicted in Table 4.7 and Fig. 4.12. In avidin-lysozyme interaction, parameter B increased in absolute value with increasing pH and seemed to be more highly dependent on pH than C. As Melander et al. (1989) have indicated, changes in B and C parameters could occur with changes in pH due to the large changes in anionic and cationic sites on the macromolecule. In the two pH values of this study lysozyme and avidin were more highly charged at pH 6.5 than at pH 7.8. Therefore the tendency of avidin to interact with lysozyme may be greater at pH 7.8 than at pH 6.5. This may lead to a higher B value at a higher pH.
Table 4.7: Electrostatic interaction parameters (B) of lysozyme-lysozyme and lysozyme-avidin interactions.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys-Lys</td>
<td>7.2</td>
<td>-0.1782</td>
</tr>
<tr>
<td>Avi-Lys</td>
<td>7.8</td>
<td>-0.5135</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>-0.0972</td>
</tr>
</tbody>
</table>
4.2.5 Effect of pH on dimerization of lysozyme: A comparison of $K_{MP}$ values with sedimentation equilibrium constants

Sophianopoulos and Van Holde (1964) have proposed that dimerization in solutions of lysozyme occurs in the pH range of 5–9 and polymerization occurs above pH 9. These conclusions were based on the sedimentation equilibrium studies performed with lysozyme solutions. They also discovered that dimerization was favoured when monomers lost protons and thus the procedure was pH dependent.

In the pH range of the present experiment (3.2–7.2) according to Sophianopoulos and Van-Holde, lysozyme monomers were present at pH 3.2 and dimers were present in the other three pH values. The apparent dissociation equilibrium constants calculated at the four pH values are given in Table 4.6. Lower values of $K_{MP}$ indicated stronger interactions between mobile and immobilized proteins. The results show a decrease in $K_{MP}$ values with increasing pH indicating stronger interactions between lysozyme molecules. A reason for this may be dimerization. Dimerization equilibrium constants obtained by Sophianopoulos and Van Holde (1964) at three pH values 6.51, 7.95, and 9.0 were 6.23, 3.66, and 2.9 g/100ml respectively. These results also show a decrease with increasing pH and are therefore in agreement with the present study.

The reason for the reverse trend observed in avidin-lysozyme interaction, that is an increase in $K_{MP}$ values with decreasing pH, is more difficult to explain from the present
experiment. It may be due to a change in the configuration of avidin or lysozyme. Ingraham et al. (1985) indicated that lysozyme molecules do not undergo large structural changes as they move through the column during HIC and RPC. Therefore changes in avidin may cause $K_{MP}$ values to increase with increasing pH.
5. CONCLUSIONS

The effect of salts on hydrophobic and electrostatic interaction parameters of proteins with chromatographic stationary phase and the effect of salt on protein-protein interactions were investigated in this study. Melander and Horvath's recently developed chromatographic model was used for this purpose.

Two methods of evaluating hydrophobic interaction parameters were employed, viz. a) multiple regression analysis to calculate C values according to the three parameter model, b) simple regression analysis to calculate slopes (C'), according to the linear model at higher salt concentrations (>0.3m).

Comparison of correlation coefficients obtained for C and C' values showed that C' values are suitable to evaluate experiments carried out only at higher salt concentrations (>0.3m), and C values are suitable to evaluate experiments done at both lower and higher salt concentrations and having prominent U shape curves (eg. avi-lys interaction). The results indicated that lactalbumin, lysozyme and ovalbumin are more hydrophobic than myoglobin and ribonuclease.

The observed high correlations of C and C' values with molal surface tension increments (δ) confirmed the view, that the dependence of retention in HIC is related to the salt
concentration and molal surface tension increment of salts. Furthermore, it was observed that the proteins with higher $C'$ values had higher slopes when $C'$ was plotted against $\delta$. This indicated that more hydrophobic proteins are more sensitive to molal surface tension increments than less hydrophobic proteins.

The attempt made to correlate $C$ and $C'$ values with ANS, CPA and Bigelow's average hydrophobicity indices showed no clear correlations. Omission of ovalbumin improved the correlation coefficient of $C'$ with ANS to a 5% significance level. Lack of correlation with average hydrophobicity indicated that $C$ or $C'$ are not measures of total hydrophobicity.

An attempt was made to develop a new chromatographic method to quantify both hydrophobic and electrostatic interactions in protein-protein interactions using the same model. The study done on lysozyme dimerization indicated that lysozyme-lysozyme interaction was predominantly hydrophobic in the pH range of this study. Slight electrostatic interactions were observed only at pH 7.2.

In the case of avidin-lysozyme interaction, both electrostatic and hydrophobic interactions were observed. At both pH values studied, a greater change was observed in the strength of hydrophobic interaction than in the surface area of hydrophobic interaction. Also, parameter B seemed to be more dependent on pH than parameter C. The results also indicated that there are interactions between lysozyme and
avidin in the two pH values studied. Furthermore, the affinity chromatography method developed in this research may be a useful technique for studies on lipid-protein, carbohydrate-protein, etc. interactions using proper ligand binding techniques.


