INTRODUCTION

The study of the combination of metallic cations with protein has for years been one of the central problems of biochemistry. In accordance with this is the large number of papers dealing with this phenomenon. The article of Gurd and Wilcox (1) gives a masterly review of the literature up to 1956. Investigation of the interaction of metal ions with a protein may by expected to supplement the study of hydrogen ion equilibria as a way of evaluating the reactivity of individual groups of clusters of groups in the protein (2, 3). In general, various measures of reactivity constitute the most direct approach to defining in part the structural characteristics of the protein in solution (1).

The study of metal ion interaction bears like wise on the preparation of isomorphous crystalline protein derivatives for x-ray diffraction analysis (4). These interactions are also of importance in protein fraction (5), elucidation of structure (6) and in discussing the mechanism of the metabolic processes (7-10).

Among the different methods that have been applied for characterising the metal protein complexes, the pH-metric method has been proved to be a rapid technique for this purpose (11-18). This study has, however, got its limitations in determining the binding of metal cations to proteins in view of the difficulties encountered in the choice of the suitable electrode in realising the condition, where accurate measurement may be made. Malik and Coworkers (19-20) gave enough evidence that the hydrogen ion titration curves of proteins in absence and presence of metal ions may be caused to calculate the binding data and the relevant intrinsic association constant of metal-
protein interactions.

The knowledge of metal complexes of simple organic molecule was first given by Bjerrum’s work on metal amine formation in aqueous solution (21). This method was further extended to metal complexes of amino acids, peptides and finally to metal-protein complexes. The basic concept on which hydrogen ion titration data may be utilized to elucidate the nature of the binding of ions other than hydrogen ions was, however, put forward by Tanford (22). Generally the metal ions form complexes with the substance which combine with the hydrogen ions. The reaction therefore, becomes a competitive one and may be shown as follows:

\[ \text{M}^{2+} + \text{NH}_3 \rightleftharpoons \text{M(NH}_3)_2^{2+} \rightleftharpoons \text{M(NH}_3)_2^{2+} \]  

The equilibrium in such a reaction may be measured indirectly by observing the displacement of the corresponding hydrogen ion equilibrium:

\[ \text{NH}_4^+ \rightleftharpoons \text{NH}_3 + \text{H}^+ \]  

A competition between metal ion and hydrogen ion for a common site necessitates that the metal can combine only with the basic functional groups. The uptake of metal ions will shift the hydrogen ion equilibria in the direction of the basic side of the functional groups. It is, thus, possible to obtain the binding data directly from the difference in hydrogen ion titration curves of protein in presence and absence of metal ions.

Arora and coworkers have studied the interaction between mercuric ions and ovalbumin by pH-metric and equilibrium dialysis methods (23). The binding of copper and cadmium ions with ovalbumin has been measured indirectly by observing the displacement of H+ from acidic groups on the ovalbumin (24). A calcium ion-selective electrode to measure free Ca\(^{2+}\) activity was used to study the binding of calcium ions to soyaprotein as function of pH (25). A few workers
have reported the zinc dependent folding of a zinc finger peptide, a necessary prerequisite for the protein to bind DNA (26-28). A literature survey indicates that so far, some work has been done with soluble ovalbumin (29-33), but no copper, lead, palladium and zinc metals binding studies are available on soluble ovalbumin by pH-measurement. Soluble ovalbumin is well characterized with respect to its ionisable group (34). The present chapter reports on the pH-metric investigation of the binding of copper, lead, palladium and zinc ion to soluble ovalbumin. The binding affinity has been compared with the help of intrinsic association constant and free energy of the metal protein complexes formed in the aqueous solutions.

**EXPERIMENTAL**

**Reagents**: A solution of soluble ovalbumin (Sigma Product) was prepared with ovalbumin by the method of Smith and Back (as described in Chapter I) and its concentration determined by drying a known aliquot in an air oven at 105°C 0.05 M. Solution of metal, Cu(II), Pd(II), Pb(II) and Zn(II) ions were prepared by dissolving chemically pure samples (A.R.) of the cupric chloride, palladium chloride, lead nitrate and zinc sulphate salts in double distilled water, and their metal contents were estimated by the usual methods. 1.0 M hydrochloric acid and 1.0 M potassium hydroxide were prepared from their B.D.H. samples (1.5 M) Potassium Chloride (BDH) solution was used for the maintenance of ionic strength of the reaction mixtures as 0.15 M.

**TECHNIQUES**:

**pH-measurements**: The pH-measurements were carried out with a Systronic pH-meter using a wide range glass electrode. The instrument was standarized against 0.05 M potassium hydrogen phthalate (pH 4.0) and 0.05 M borax (pH 9.2) for acidic and basic ranges, respectively. Purified nitrogen was passed through the reaction mixtures slowly for about 10-15 minutes to ensure inert atmosphere. All the pH
measurements were carried out at 25°C temperature.

**Procedure**: The following sets of solution were prepared for the pH measurements:

(i) 1.5 ml of 5% soluble ovalbumin was taken in different pyrex boiling tubes along with 15 ml of 1.5 M KCl and different volume of 0.1 M HCl or 0.1 M KOH were added. The total volume was made 15.0 ml by adding requisite amount of double distilled water.

(ii) 1.5 ml of 5% soluble ovalbumin 0.3 ml of 0.05 M Cu(II), Pd(II), Pb(II) and Zn(II) separately were mixed in different tubes along with 1.5 ml of the supporting electrolyte of 1.5 M KCl to maintain the ionic strength 0.15 M.

(iii) Water and metal ions were similarly arranged in the absence of protein.

Two similar sets were prepared, one for acid titration and other for alkali titration. The pH value of all the mixtures were determined after 24 hours of mixing.

**RESULTS AND DISCUSSION**

pH-titration curves of proteins with acid or base are usually plotted in the form of the number of hydrogen ions bound or removed from a mole of protein vs the pH value, according to the reaction:

\[ \text{PH}_i + rH^+ \rightleftharpoons \text{PH}_{i+r} \]  (i)

\[ \text{PH}_i - rH^+ \rightleftharpoons \text{PH}_{i-r} \]  (ii)

Where \( \text{PH}_i \) represents a species of the protein at the commencement of the titration, and \( r \) is the number of hydrogen bound or removed per mole of the protein.

The experimental basis for the determination of \( r \) is the difference between the number of moles (per mole of protein) of strong acid or base required to bring the pH of the protein solution from the initial
value to a given value, and the number of moles needed to effect the same pH range in the same value of solvent at the same ionic strength when the protein is absent. In practice, the protein is titrated and from the amount of acid or base required to effect a given pH change the value of \( r \) is calculated making certain assumptions.

If we consider a solution of hydrochloric acid of concentration \( C_1 \) mole per litre and the concentration of hydrogen ions also as \( C_1 \) mole per litre, then the pH of solution is given by \( \text{pH}_1 = -\log f_1 C_1 \), where \( f_1 \) is the hydrogen ion activity coefficient in this solution. If the same solution contains gm per litre of isoionic protein, the hydrogen ion concentration will be \( C_2 \), since \( (C_1 - C_2)/g \) moles of hydrogen ions will have combined with each gram of protein and the pH will be \( \text{pH}_2 = -\log f_2 C_2 \). Then

\[
\log \frac{C_2}{C_1} = \text{pH}_1 - \text{pH}_2 + \log \frac{f_1}{f_2}
\]

The assumption of Cohn and Berggren, discussed by Edsall and Cohn was \( f_1 = f_2 \), so that the last term disappeared. For this assumption to be true, the ionic strength of the two solutions should be the same. This is equivalent to the assumption that the isoionic protein molecule, which bears equal number of opposite charge, makes no contribution to the ionic strength, while the additional charges born by the protein molecule as a result of ion binding contribute as if they were separated small ions. The equation can be manipulated to give:

\[
\frac{C_1 - C_2}{g} = \frac{C_1}{g} [1 - \text{antilog} (\text{pH}_1 - \text{pH}_2)], \text{ for the bound hydrogen ions}
\]

\[
\frac{C_1 - C_2}{g} = \frac{C_1}{g} [1 - \text{antilog} (\text{pH}_2 - \text{pH}_1)], \text{ for the bound hydroxyl ions}
\]

The total number of hydrogen ion bound in the acid segment of the titration curve is equal to the total number of cationic groups in
that protein, which is taken as equal to the number of hydrogen ions
dissociated (r) at the isoinic pH of the protein. In the acidic range the
hydrogen ions bound are subtracted from the total number of cationic
group to get the value of r at each specified pH value. In alkaline range
the total number of hydrogen ions bound is added to the number of
hydroxyl ions bound which is equal to r at that specified pH value. In a
similar way the value of r is calculated in the presence of cation, if this
value of r is greater than former, the difference is equal to the value of
\( V_m \) i.e. moles of metal cation bound per mole of protein of the two
curves (r vs pH) coincides, it shows no interaction between cation and
the reactive groups of the protein. The results of the present
investigation are summarized in Tables 1 to 10.

The difference in pH with and without protein provided a way
for determining bound hydrogen and hydroxyl ions with protein
applying the equation of Cohen and Edsall (35). Figure 1 shows the
plots of hydrogen ions dissociated (r) in presence and absence of metal
ions against pH. Such plots were constructed by Tandord’s method
(36). It could be seen that the hydrogen ion titration curves of protein
in presence and absence of metal ions are markedly different. Assuming
that the metal ions compete with the hydrogen ions dissociated for
the common sites, the following conclusions have been made (i) the
amount of hydrogen ions dissociated (r) by protein is greater in the
presence of metal ions than with protein alone. This would be the
case if replacement of hydrogen ions is looked from the protonated
sites, (ii) the extent with which any metal ion displaces the hydrogen-
ion equilibria of protien towards the basic side of the functional groups
may be taken as the measure of the degree of metal-protein interaction.
Further, Gurd and Murray (37) postulated one-to-one binding in
preference to intramolecular cross-linking, hence the number of
hydrogen ions displaced per protein molecule as determined from
titrination curve gives directly the binding data \( V_m \), the number of active

(75)
sites covered by metal ions. Such data are given in Table 10.

It was found that all the metals interacted with carboxyl and imidazole groups of soluble ovalbumin. Figure 2 shows that the value of $V_m$ progressively rises with rise in pH, this indicated that as the negative charge on protein increases, the electrostatic attraction between metal ions and deprotonated sites on protein increase. A sudden jump in each plot in Figure 2 indicates the change in the mode of interaction, i.e., upto pH 5.50 the binding behaviour is different than that at pH 6.50 and 7.00. It could be observed that the changes which occur during the titration of metal-protein mixtures are mostly in the pH range 3.00 to 5.50, where carboxyl groups are expected to lose their protein (38), it may be concluded that the carboxyl groups from aspartyl and glutamyl residues offer the main sites for binding of metal ions.

The protein contains 51 carboxyl groups which started ionizing from pH 3.50, however, a lesser value of $V_m$ could be explained by the fact that although all carboxyl groups were fully deprotonated at pH 5.50, though the positive locii produced a repulsive electrostatic effect on metal ions. The increase in $V_m$ beyond pH 5.50 is either due to the involvement of imidazole from histidyl residues or due to on set of metal-hydroxides. Since there was no metal-hydroxide precipitation in metal-protein mixtures, hence the histidyl imidazoles participated in metal-protein interactions.

The value of intrinsic association constant $\log (K)$ for metal-protein interaction was calculated with the help of Scatchard’s equation (39).

$$K = \frac{V_m}{(n \cdot V_{H^+} - V_m) \cdot C_F}$$

where $K$ is the intrinsic association constant, $V_m$ is the number of moles of metal ion associate per mole of protein, $V_{H^+}$ is the number of hydrogen ion bound at that pH, $C_F$ is the free equilibrium metal
concentration and \( n \) is the total sites on protein molecule.

These values of \( \log K \) for metal-carboxyl and metal-imidazole systems are complied in Table 10. The \( \log K \) values compare favourably with the values obtained for other proteins and model compounds (13). It may be noticed that the \( \log K \) values for metal-imidazole system of protein are, however, somewhat less as compared to the first association constants of metal-free imidazole systems. This could be attributed to the fact that histidyl residues in soluble ovalbumin are stearically hindered by the presence of large size substituents.

The free energy change of the combination at 25°C was calculated by the relation: \( \Delta G^0 = -2.303 \cdot RT \log K \), and are tabulated in Table 10. The order of respectively of these metals with carboxyls was found as \( \text{Cu(II)} > \text{Pd(II)} > \text{Zn(II)} > \text{Pb(II)} \), while for metal-imidazole as, \( \text{Cu(II)} > \text{Pd(II)} > \text{Zn(II)} > \text{Pb(II)} \). The binding results show that stronger combination took place with imidazole of histidyl residues than carboxyl from aspartyl and glutamyl residues. This study provided the potentiality for the imidazole groups as the stronger binding sites for combination with metal ions on the soluble ovalbumin molecule. The order of reactivity of the different metal ions with soluble ovalbumin may be correlated with the periodic properties of the metal under investigation. It may be concluded that soluble ovalbumin molecule behaved like the simple ligands in their interaction with a variety of transition metal ions.

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(77)
Table 1

Concentration of soluble ovalbumin = 5 gm/l
Ionic strength = 0.15
Total volume = 15 ml
Temperature = 25°C

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Table 2

Concentration of soluble ovalbumin = 5 gm/l
Ionic strength = 0.15
Total volume = 15 ml
Temperature = 25°C

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Table 3:

Concentration of soluble ovalbumin = 5 gm/l

Ionic strength = 0.15

Total volume = 15 ml

Temperature = 25°C

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(80)
Table 4

Concentration of soluble ovalbumin = 5 gm/l
Ionic strength = 0.15
Total volume = 15 ml
Temperature = 25°C

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Table 6

Concentration of soluble ovalbumin = 5 gm/l
Concentration of Cupric Chloride = 1 x 10^{-3} M
Ionic strength = 0.15
Total volume = 15 ml
Temperature = 25°C

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(83)
**Table 7**

**Concentration of soluble ovalbumin** = 5 gm/l
**Concentration of Lead Nitrate** = 1 x 10^{-3} M
**Ionic strength** = 0.15
**Total volume** = 15 ml
**Temperature** = 25°C

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### Table 9

Concentration of soluble ovalbumin = 5 gm/l  
Concentration of Zinc Sulphate = 1 x 10^{-3} M  
Ionic strength = 0.15  
Total volume = 15 ml  
Temperature = 25°C

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