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Binding of Molybdenum(VI) to Soyabean Protein by Physico-chemical Methods

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The interaction of molybdenum(VI) has been studied with soyabean protein using polarographic (at pH 5.57) and equilibrium dialysis (at pH 5.57, 7.50 and 9.30) methods. The intrinsic association constants and the number of binding sites have been calculated from Scatchard plots. The effect pH and temperatures on these constants were studied by equilibrium dialysis method. The number of sites available for binding with molybdate ions is much less than the actual number of cationic groups, which are 14, 11 and 10 at pH values 5.57, 7.50 and 9.30, respectively by equilibrium dialysis at 25 ºC. These nearly similar values were found at pH 5.57 by polarographic method at 25 ºC. The values of different thermodynamic parameters have been reported. The large positive entropy change of binding coupled with the small enthalpy change of binding have been interpreted to mean that the major contribution to the free energy of binding comes from the release of the solvent molecule from the molybdate-soyabean complex.

Key Words: Soyabean, Molybdenum.

INTRODUCTION

Malik and Arora[1,2] have made a quantitative study on the binding of the molybdate ion with fibrillar and globular proteins employing different physico-chemical methods. Several workers have used animal proteins for the binding V(V) and Mo(VI) anions by physico-chemical methods[3-14]. But no such studies are reported on vegetable protein like soyabean protein (SBP). Inspite of the several biological[15,16] and bio-chemical[17-22] functions of molybenum, its binding as molybdenum(VI) anion to SBP, a vegetable protein characterized with respect to its hydrogen ion equilibria[23], has never been reported. Soyabean protein has been reported to contain 94 carboxyl, 18 imidazole, 46 ε-amino and 41 guanidino groups *i.e.*, 105 total cationic groups per 10^5 g of the protein. In this paper the binding between SBP and molybdate(VI) has been studied using polarographic and dialysis equilibrium methods. The effect of pH and SBP concentration on the binding has been discussed and a probable mechanism of combination has been suggested.

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EXPERIMENTAL

Sodium molybdate (E. Merck) was dissolved in doubly distilled water and estimated gravimetrically by oxine method. Sodium acetate and acetic acid (BDH) were used for the preparation of acetate buffer of pH 5.57, while phosphate and carbonate buffers of higher pH were prepared from reagent grade chemicals. Potassium chloride (BDH) solution was used for the adjustment of ionic strength of solutions.

Protein solution: Soyabean protein (SBP) was extracted from soyabean powder (BDH) by alkali extraction followed by the gradual addition of HCl to lower the pH to the isoelectric point[24]. It was dissolved in dilute alkali solution and centrifused to obtain the clean solution. The concentration of protein solution was determined by colorimetric method. It was stored in a refrigerator and purified toluene was added to check its surface denaturation.

Polarographic measurements: Diffusion current measurements were made on a Toshniwal polagraph in conjunction with an Osaw galvanometer in the external circuit. The polarographic cell as recommended by Tanford[25] was used. Purified nitrogen was passed through the reaction mixtures to displace the dissolved oxygen and triply distilled mercury was used for the dropping mercury electrode (dme). The polarographic cell was immersed in a water thermostate at 25 ºC. The capillary used had flow rate of about 2.2 mg/s with a drop time of 3.5 s. The rate of flow of mercury drops was determined by the Lingane's method. Since, the capillary characteristics (m, t) have a marked effect on the diffusion current (which is directly proportional to $m^{2/3}t^{1/3}$), these factors were, therefore controlled carefully through out these experiments. The following mixtures were prepared for the polarographic determinations: (i) Different quantities of SBP (2.0 to 25 g/L) were taken in different pyrex boiling tubes and 10×10^{-4} M Mo(VI) solution was added to each of them. The total volume was made 10.0 mL by adding acetate buffer of pH 5.57, double distilled water and KCl solution to have an ionic strength at 0.15 M. The results are expressed in the form of protein concentration *vs.* $id/(id)_o$, where $(id)_o$ and id are the diffusion currents of molybdenum(VI) in the absence and presence of the soyabean protein. (ii) A fixed quantity of SBP (5.0 g/L) was taken in different boiling tubes and varying quantities of Mo(VI) (1.0 to 50×10^{-4} M) were added in a total volume of 10.0 mL made so by buffer, distilled water and KCl. (iii) Different quantities of Mo(VI) (1.0 to 50×10^{-4} M) were taken as in (ii) in the absence of protein.

Dialysis equilibrium experiments: In the dialysis equilibrium method a solution of known protein concentration (5.0 g/L) at the desired ionic strength (0.15 M) in KCl (supporting electrolyte) (in order to inhibit the Donnan equilibrium) was placed inside a dialysis tubing of cellophane which had been previously rinsed and finally soaked in a KCl solution of the required ionic strength. The dialysis cellophane tubing was inserted in a glass stoppered pyrex boiling tube having the outside solution of supporting electrolyte (KCl) and Mo(VI) solution. The requisite pH of the inside and outside solutions was maintained with the help of buffers of pH 5.57, 7.50 and 9.30, respectively. Several identical sets of mixtures having fixed 'inside' protein

and varying 'outside' Mo(VI) (1.0 to 50 × 10^{-4} M) solutions (total volume 5.0 mL inside and outside) were adjusted. The tubes were shaken on a shaker at 25 ºC for a time just sufficient to attain the equilibrium. Similar sets were arranged at 10 ºC for pH 5.57, 7.50 and 9.30. The outside Mo(VI) solution was then separated and analyzed for its molybdenum content spectrophotometrically using thioglycollic acid as the complexing reagent[26]. In a solution when no precipitation occurred, the average number of moles of Mo(VI) bound per mole of SBP (m.w. 10^5 g), V_M, may be computed directly from the relation, $V_M = (Co-C_F)/[T]$, where Co and C_F are the total and free molar concentrations of Mo(VI) and [T] is the total molar concentration of protein (5.0 g/L). Control studies showed that Mo(VI) binding by the dialysis tubing was essentially negligible, but a small loss of SBP always occurred when the tubing was tied off and the initial volume of protein was always less than the outside solution.

RESULTS AND DISCUSSION

Polarographic measurements: An ampholyte like protein would normally affect both the half-wave potential (E½) and diffusion current (id) of the reduction wave of the metal ion[25]. In the present investigation no displacement in E½ of the Mo(VI) species in the presence of soyabean protein was observed. Tanford's method[27], based on the variation of the diffusion currents ratio $[id/(id)_o]$ could be applied satisfactory at pH 5.57 which includes ionized carboxyls and protonated ε-amino, imidazole and guanidino groups of the SBP. The significant reduction in the diffusion current pH 5.57 as determined experimentally [for sets (i) and (ii)] by plotting the curves $id/(id)_o$ *vs.* concentration of protein at fixed Mo(VI) or concentration of Mo (VI) at fixed protein] and provided enough information concerning the linking of Mo(VI) to SBP. Similar kind of reduction in the diffusion current of the dyes and molybdate anions in the presence of proteins was reported by Malik *et al.*[2,28] and Arora *et al.*[3,5-9] in the case of molybdate and vanadate species. Hence the decrease in the diffusion current of molybdenum species in the presence of increasing quantity of soyabean protein must be attributed to interaction. These conclusions are in agreement to Tanford[25], who believed that when any reducible substance was bound to a protein there is a reduction in the diffusion current and this decrease may be used to calculate the linking constants.

If id and $(id)_o$ are the diffusion currents (current heights) of Mo(VI) species in the presence and absence of protein, respectively and C_o, C_F and C_b the molar concentrations of total, free and bound Mo(VI) respectively, then the molar concentration of bound molybdenum to protein can be determined by the following equations:

$$C_o = C_F + C_b, \quad \text{and} \quad C_b = \frac{[C_o - \{id/(id)_o\}.C_o]}{1-k}$$

where k (the fraction coefficient) is the limiting (constant) value of $id/(id)_o$ when so much protein has been added that all the Mo(VI) is protein bound. The value of k is obtained by an extrapolation method.

$$\frac{id}{(id)_o} = k; \text{ (at a limit } C_F \rightarrow 0)$$

At present the value of k was found to be 0.40 at pH 5.57. It is seen that the addition of gradually rising quantity of soyabean protein results in the decrease of diffusion current of Mo(VI) till it attains a limiting value (k). Constancy in the diffusion current indicates complete linking of Mo(VI) to soyabean protein (SBP). The polarographic data were analyzed by means of Scatchard's equation[29] in the form $V_M/C_F = nK - KV_M$ the value of V_M (average mole of Mo(VI) bound per 10^5 g of SBP) is determined by the relation, $V_M = Cb/[T]$, where [T] and Cb have their usual meanings. n is the average maximal number of binding sites on SBP with the same association constant K. If all the binding sites are equivalent and independent, a plot of V_M/C_F as a function of V_M should give a straight line such that the intercept on the V_M/C_F axis is Kn, as V_M approaches zero as a limit and the intercept on the V_M axis is n, as V_M/C_F approaches zero as a limit. Deviation from the linear nature takes place when binding occurs at more than one set of sites with different association constants[29,30]. The contribution of electrostatic factors to the interaction[31] or the change in ionic species of reacting species may also cause deviations[32].

The results obtained are shown in Fig. 1. A linear relationship does not exist between V_M/C_F and V_M. Infact, the Satchard's plot of the data shows two straight line regions which indicates the existence of more than one class of Mo(VI) linking sites[33]. The intercept of the upper part with the absecissa in Fig. 1 gives a value of $n_1 = 6$, for the number of linking sites in the first class; corresponding the slope K_1, yields the linking constant, $K_1 = 0.86 \times 10^4$. To determine the number of reactive sites in the second class and their linking constants, one plots V'_M/C_F *vs.* V'_M where $V'_M = V_M$ - number of primary reactive sites, for the results at pH 5.57. This method substracts out the contribution of the n_1 to the experimental data. This computation is valid when the two classes of reactive sites does not affect binding at the other. From Fig. 2 we have $n_2 = 2.5$ and $K_2 = 0.39 \times 10^4$ for the second class of Mo(VI) lining sites. Again, the departure of points above $V_M = 2.5$ is either owing to the consequence of the change in the ionic species of Mo(VI)[34] or due to the participation of the third class of reactive sites in the combination. It is not surprising since the dissociation of a weak acid (here molybdic acid) is pH and concentration dependent and is thus a subject of the electrostatic effects such that is operative in ampholytes at varying pH. Imagining that there are additional third class of binding sites then from Fig. 3, the rest $n_3 = 4$ sites may correspond to the most weaker sites with $K_3 = 0.07 \times 10^4$. These values of different classes of sites $n_1 = 6$, $n_2 = 2.5$, $n_3 = 4$ and their associates constants being $K_1 = 0.86 \times 10^4$, $K_2 = 0.39 \times 10^4$ and $K_3 = 0.07 \times 10^4$, respectively. A fewer number of Mo(VI) binding sites determined from diffusion current measurements clearly indicates that only a few groups participate in Mo(VI)-SBP combination, since the actual number of positive sites are 105 for 10^5 g of SBP. The reason for the involvement of lesser sites may be a conformational one.

Soyabean protein, being a globular protein, possess a significant structure brought about by hydrophobic interactions, hydrogen bonding and other intersegement cohesive forces. However, since the folded core of a protein is known to be inaccessible to water, a rough estimate of 40-60 % of the total sites may be available to interact with anions[35]. The reduced reactivity in native proteins has been attributed to the unavailability of most of the groups concerned due to the folding of the protein molecule[36]. However, from the work of certain workers[37-40] the lack of reactivity can more readily be attributed to hydrogen bonding between neighbouring polar side groups of the amino acid residues. Further information about the nature of the linking was collected from dialysis equilibrium technique, where it was possible to work at pH-values other than pH 5.57.

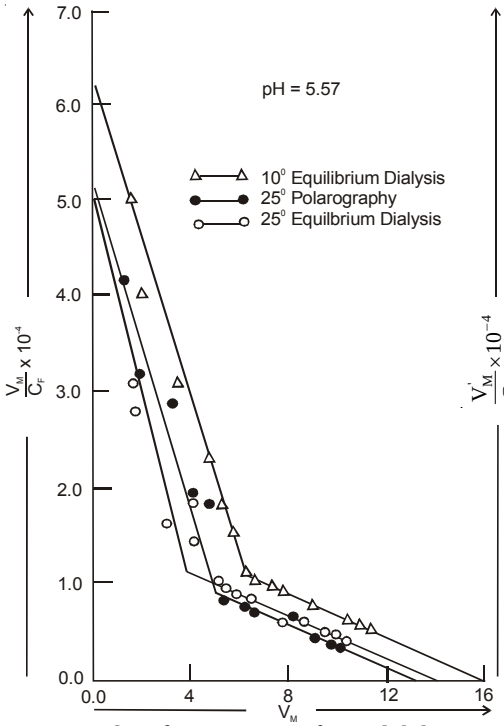

Fig. 1. Plot of V_M/C_F *vs.* V_M for molybdate-soyabean system

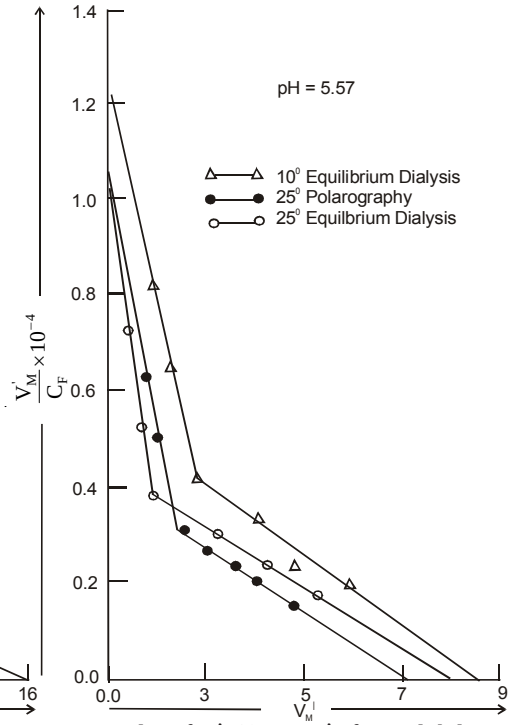

Fig. 2. Plot of V'_M/C_F *vs.* V'_M for molybdate-soyabean system

Dialysis equilibrium results: Equilibrium between a small ion and a macromolecule separated by a cellophane membrane is a suitable way of preparation of accurate isotherms. This technique is not affected from the presence of other competitive ions, since it involves a direct determination of a small molecule by suitable methods. If the dialysis membrane does not interact with any of the ligand and if the Donnan equilibrium is negligible, the value of V_M can be calculated easily. The results collected at different pH-values (5.57, 7.50 and 9.30) and at two temperatures

(10 and 25 ºC) are shown graphically in different ways (Figs. 1 to 5) as in the case of polarographic method. The association constants and binding sites determined from Scatchard's plots are complied in Tables 1 and 2.

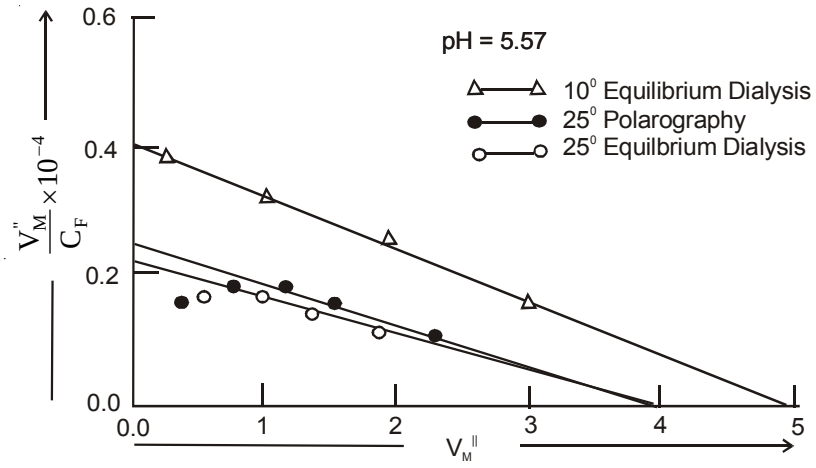

Fig. 3. Plot of V''_M/C_F *vs.* V''_M for molybdate-soyabean system

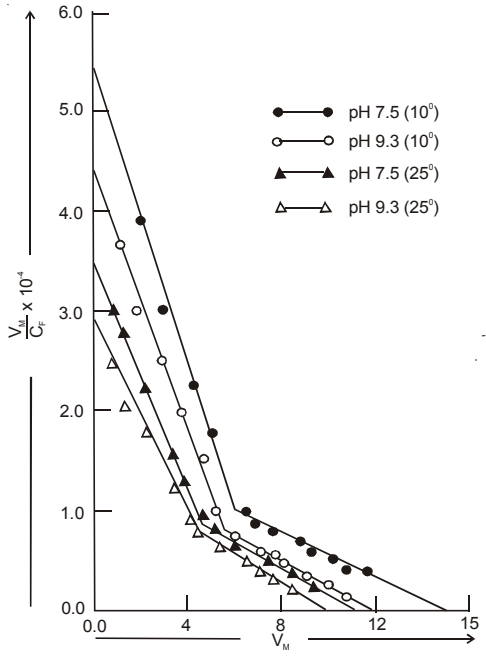

Fig. 4. Plot of V_M/C_F *vs.* V_M for molybdate-soyabean system by equilibrium dialysis technique

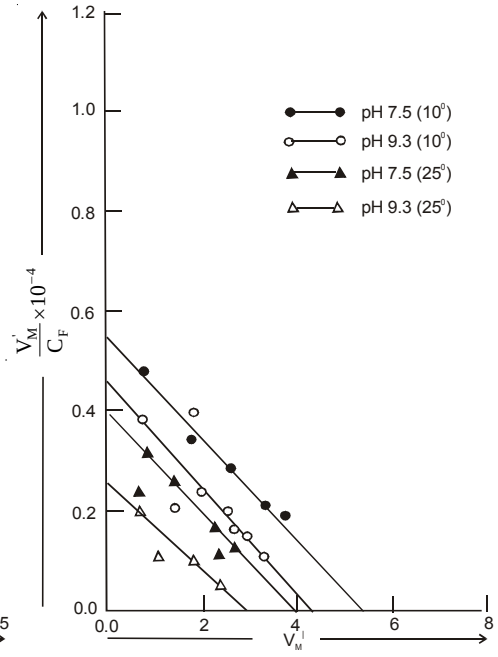

Fig. 5. Plot of V'_M/C_F *vs.* V'_M for molybdate-soyabean system by equilibrium dialysis technique

TABLE-1
VALUE OF BINDING CONSTANTS FOR MOLYBDENUM(VI) WITH SBP FROM EQUILIBRIUM DIALYSIS (ED) AND POLAROGRAPHIC (POL) METHODS

pH	Technique	n	n_1	n_2	n_3	log K	log K_1	log K_2	log K_3
Temperature = 10 ºC									
5.57	ED	16	7	3.7	5	3.574	3.954	3.477	2.819
5.57	POL	-	-	-	-	-	-	-	-
7.50	ED	15	8	5.0	-	3.563	3.837	3.041	-
9.30	ED	12	7	4.0	-	3.522	3.808	3.041	-
Temperature = 25 ºC									
5.57	ED	14	6.0	3.0	5	3.535	3.919	3.602	2.803
5.57	POL	13	6.0	2.5	4	3.854	3.934	3.591	2.845
7.50	ED	11	6.4	4.0	-	3.522	3.732	3.000	-
9.30	ED	10	6.2	3.0	-	3.477	3.681	2.903	-

TABLE-2
BINDING CONSTANTS AND THERMODYNAMIC PARAMETERS OF MOLYBDENUM (VI)-SBP SYSTEM

pH	Technique	n	log K	ΔG^o K cal mol^{-1}	ΔH^o K cal mol^{-1}	ΔS^o cal deg^{-1} mol^{-1}
Temperature = 10 ºC						
5.57	ED	16	3.574	-4.649	-1.008	+12.85
5.57	POL	-	-	-	-	-
7.50	ED	15	3.563	-4.635	-1.060	+12.63
9.30	ED	12	3.522	-4.581	-1.163	+12.07
Temperature = 25 ºC						
5.57	ED	14	3.535	-4.842	-1.009	+12.86
5.57	POL	13	3.584	-4.910	-	-
7.50	ED	11	3.522	-4.797	-1.161	+12.20
9.30	ED	10	3.477	-4.763	-1.166	+12.07

ED = Equilibrium dialysis; POL = Polarography

Although these constants at pH 5.57 compare favourably by both the methods, yet the values from polarography was found to be somewhat different from those obtained by the equilibrium dialysis. This slight difference is understandable in view of the fact that equilibrium dialysis generally has been accepted as a reliable and accurate method for the determination of binding between small molecules and proteins. This can also be explained by the fact that in equilibrium dialysis more time is allowed for equilibrium while in polarography observations were made immediately after preparing the protein-molybdate mixtures.

From pH 5.57 to 9.30 a non-linear relationship was found to exist between V_M/C_F and V_M plots and the extrapolation to V_M/C_F (nK) and the V_M axis (n) are shown in Figs. 1 and 4. The binding constants of SBP-Mo(VI) at different pH values are

given in Table-1. The fact that the relationship is non-linear over the entire range of Mo(VI) concentration studied indicated that the second class of binding sites if it existed at pH 9.30 must have a much smaller association constant than that observed for the primary sites (Table-1). A comparision of the binding constants at different pH-values indicated that protein-Mo(VI) combination is highly pH dependent. It is considered likely that free positive sites (protonated lysyl, histidyl and arginyl) on the SBP molecule may be the primary sites (n_1). It may also be concluded that the difference in pK values of the molybdic acid and the reactive positive sites may account, at least in part, for the difference in the binding behaviour.

The different thermodynamic constants *viz.*, free energy change (ΔG^o), enthalpy change (ΔH^o) and entropy change (ΔS^o) for the binding have been determined by the usual mathematical thermodynamic equations (expression) and are given in Table-2. The sequence of free energy values are in agreement with diminishing positive charge and rising negative charge on SBP molecule. The values of ΔH^o are found to be -1.008, -1.060 and -1.163 kcal/mol at pH value 5.57, 7.50 and 9.30, respectively which suggested a stronger interaction at higher pH than at lower pH. The relation between linking and pH indicated that the net charge on SBP molecule has a great influence on anion-protein combination. In a similar way any change of temperature causes alternation in the linking capacity which may be ascribed owning to structural changes in the protein molecule.

Conclusion

From the results obtained in this study various possible hypothesis can be proposed for the Mo(VI)-SBP interaction. The pH-dependent linkage constants suggested that protonated sites are the main sites for binding the anionic Mo(VI) species. Progressive SBP deprotonation ($>N^+H_2 \rightleftharpoons >NH + H^+$, $-N^+H_3 \rightleftharpoons -NH_2 + H^+$, *etc.*) and increased negative charge ($-COOH \rightleftharpoons -COO^- + H^+$) would cause such type of pH- dependent results. The persistence of binding at pH 9.30 may be attributed to the guandinum $(H_2N-\overset{+}{C}(=NH_2)-NH_2)$ groups of the arginyl residues which have a higher value of ionization constant (pK > 12.0). The total number of linkage sites (n) at two temperatures show that all the positive groups are not equally available for combination. The smaller number of linkage sites thus does not allow us to determine which groups (arginine, lysine or imidazole) are involved in binding with molybdenum species. These observations are in agreement with those earlier reported by Malik *et al.*[2] and Arora *et al.*[3-9] in the interaction of metal oxoanion with globular and fibrillar proteins. Similar conclusion were drawn by Craig *et al.*[41] in the interaction of ovalbumin with chloroaurate ions below the isoelectric point of this protein.

The pH dependent results between Mo(VI) and soyabean protein could thus be explained by assuming an interaction with the positively charged nitrogen groups. The complex formation between Mo(VI) and histidine[42] as well as guanidine[43] further

goes to support present results in lower pH range. Towards higher pH (above iso-electric point) the decreased binding may be due to deprotonation of the imidazolium and amino groups while guanidinium groups remained protonated (pK > 12). However, owing to the complexity of the macromolecular structure it was difficult to found out the exact groups which were involved in Mo(VI)-SBP interaction.

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