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A Study on the Interaction Between Steroids and Proteins

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The binding of cholesterol and its stearate ester was studied with soluble ovalbumin and Transfusion gelatin using partition and dialysis equilibrium methods. The intrinsic association constants and binding sites were found to be pH and temperature dependent. The linear nature of binding plots suggests the involvement of single one class of sites in the interaction. The binding was found to be maximum in the physiological pH range while lesser at pH 11.5, which was ascribed to protein denaturation. Approximately similar values of log K at all pH values suggested the involment of identical sites in steroid-protein interaction.

Key Words: Soluble ovalbumin, Transfusion gelatin.

INTRODUCTION

Several studies on the lipid-protein interactions have been carried out to emphasize the structure of membrane proteins[1-7]. Cholesterol, a very weak amphiphile, has been shown to be present in bile as mixed micelles with bile salts and phospholipids[8,9], and its interaction with a few well characterized proteins has been reported due to great physiological significance of these combinations[10-13]. A few workers have applied the equilibrium dialysis method to study the binding of corstisol to proteins[14]. In steroid-protein interactions it has been reported that the introduction of oxo or hydroxyl groups in various position of steroids weakens the interaction and that introduction of methyl groups has the opposite effect[15,16]. Pearlman and Crey[17] reported the binding of testosterone to human serum albumin by equilibrium dialysis method. Although the significance of cholesterol and its esters in biological membranes is well established[18-22], but their interaction with soluble ovalbumin (OAS)[23,24], a more thermodynamically stable form than ovalbumin (OA) towards denaturating agents and transfusion gelatin (TG) (used as plasma expander) still not investigated. The present paper deals with the binding constants of cholesterol and its stearate ester with OAS and TG under varying condition of pH and temperatures employing equilibrium dialysis and equilibration of the two

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type of phases *i.e.* an organic phase and an aqueous solution of protein in buffers as done by Goodman[25] in the binding of long-chain fatty acids (*n*-heptane) and aqueous solution of human serum albumin (HSA).

EXPERIMENTAL

Transfusion gelatin (conc. 6 %, m.w. 75000) was obtained from National Chemical Laboratory, Poona, India. Ovalbumin (*m.w.* 45000) was purchased from V.P. Chest Institute, University of Delhi, India. Soluble ovalbumin (OAS) was prepared from ovalbumin by the method of Smith and Back[23,24] by heating a 5 % aqueous solution of ovalbumin, adjusted to pH 9.90 at room temperature, at 55 ºC for 16 h. The small amount of protein precipitated at pH 4.7 is removed by centrifugation. The clear solution was used as soluble ovalbumin. Its concentration was determined by the biuret method using bovine serum albumin (BSA) as the standard protein. Cholesterol and cholesteryl stearate were BDH products which were further purified by recrystallization from benzene. Their stock solutions of desired strength were prepared in dimethyl formamide. Buffers of different pH-values were prepared from reagent grade chemicals. Potassium chloride solution was prepared for the maintenance of the ionic strength of the reaction mixtures.

Partitioning: Substrates were equilibrated in a two phase system consisting of protein solution and an immiscible organic solvent (toluene). The experiments were arranged as follows: (i) a series of substrate solutions were prepared in toluene and equilibrated with different buffers of pH values 3.50 to 11.50 by shaking for a period of 72 h, a time just sufficient to attain the equilibrium. The mixtures were centrifuged and the clear organic phase was analyzed for the steroids colorimetrically[26], (ii) varying concentrations of cholesterol or its ester in 5.0 mL of toluene were taken in different stoppered conical flasks and the same volume of 0.6 % protein in different buffers, previously staurated with purified toluene, was added. The solutions were than shaken for 72 h to attain the equilibrium at 25 ºC. The solutions were centrifuged and the organic phase was analyzed as in (i).

Dialysis: In dialysis equilibrium experiment, a solution of known protein concentration 5.0 mL of 0.6 % was placed inside in visking cellophane bag at the desired ionic strength. The bags were inserted in a glass stoppered tube containing the outside solution of 5 mL known concentration of steroid. The desired pH of the inside and outside solution was maintained with the help of suitable buffers (pH 3.5, 5.5, 7.5, 9.5 and 11.5) at 10 and 25 ºC.

In each experiment duplicate samples containing 5.0 mL of 0.6 % protein solution were dialyzed against the equal volume of steroid solution

of indicated strength and pH 3.5, 5.5, 7.5, 9.5 and 11.5 at 10 and 25 ºC temperature. After the equilibrium was reached, dialysis bags were removed and the outside solutions were estimated as described above in experiments under partitioning.

Calculation of thermodynamic parameter: The standard free energy change (ΔF º) of the combining sites was calculated by the following relation:

$$\Delta F^{\circ} = -2.303\ RT \log K$$

where R is universal gas constant (1.99 cal mol^{-1} K^{-1}), T is the absolute temperature and log K is the logarithm intrinsic association constants at different pH values for the steroid-protein combination. The enthalpy change (ΔHº) and entropy change (ΔSº) of steroid-protein combination. The enthalpy change (ΔHº) and entropy change (ΔSº) of steroid-protein interaction were calculated by the following expressions:

$$\Delta H^{\circ} = \frac{2.303 R T_1 T_2}{\Delta T^{\circ}} \log \frac{K_2}{K_1}$$

$$\Delta S^{\circ} = \frac{\Delta H^{\circ} - \Delta F^{\circ}}{\Delta T^{\circ}}$$

where, K_2 and K_1 represent the intrinsic association constants at two different temperatures respectively and ΔT° is the difference of the two absolute temperatures T_2 and T_1, respectively. The nature of ΔF º, ΔHº and ΔSº is inductive of the energetics of steroid-protein bonding forces.

RESULTS AND DISCUSSION

The amount of steroid bound to protein (S_B) was determined by subtracting the unbound steroid (S_F) from the total initial concentration (S_T) after the equilibrium was reached. The moles of steroid bound per mole of each protein (r) were determined by the usual relation, $r = S_B/[P]$, where [P] is the total molarity of the protein. Fig. 1 shows the data on the binding of cholesterol by TG at different pH values. It has been presented as r, the number of steroid molecule bound per protein molecule, *vs.* the log free equilibrium of steroid concentration. These plots show progressive rise in binding (r) with increasing free equilibrium concentration of the steroid. The value of r also increased regularly from pH 3.50 to 9.50 and then decreased at pH 11.50. However, in the case of ester the value of r at each pH value was smaller than cholesterol. If the total number of binding sites (n) is known, the value of log S_F at which $r = n/2$ immediately gives the value of log K, but since in the present case no saturation limit was attained, the value of log S_F and subsequently the actual number of binding sites can not be calculated from the logarithmic plots.

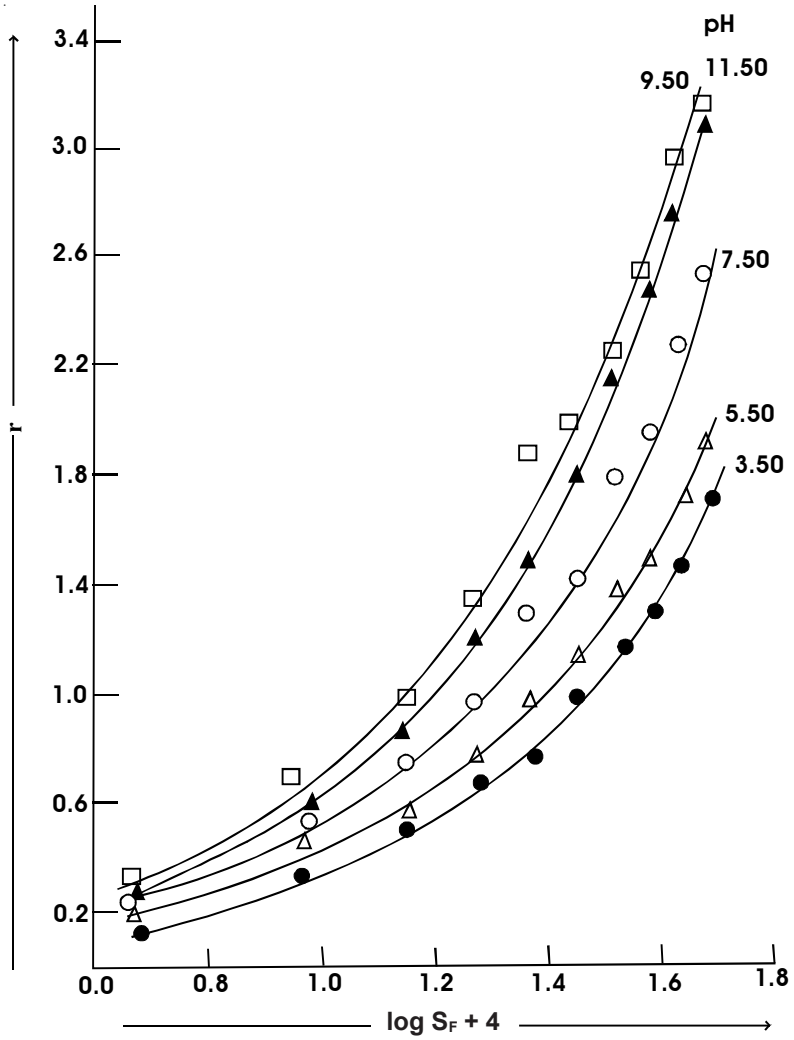

Fig. 1. Plots of r (mole cholesterol bound per mole protein) *vs.* log S_F (logrithm of free cholesterol) for transfusion gelatin-cholesterol system at 25ºC and at different pH-values

The value of association constant (K) and maximal number of binding sites (n) for the steroid-protein combination were calculated by fitting the experimental data according to reciprocal plot[24] in the form:

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{Kn} \times \frac{1}{S_F}$$

A linear plot of 1/r *vs.* $1/S_F$ (Figs. 2 and 3) indicated the binding at a single set of equivalent sites. The intercept on the ordinate axis and slopes of the curves gave the value of 1/n and 1/Kn, respectively and these values

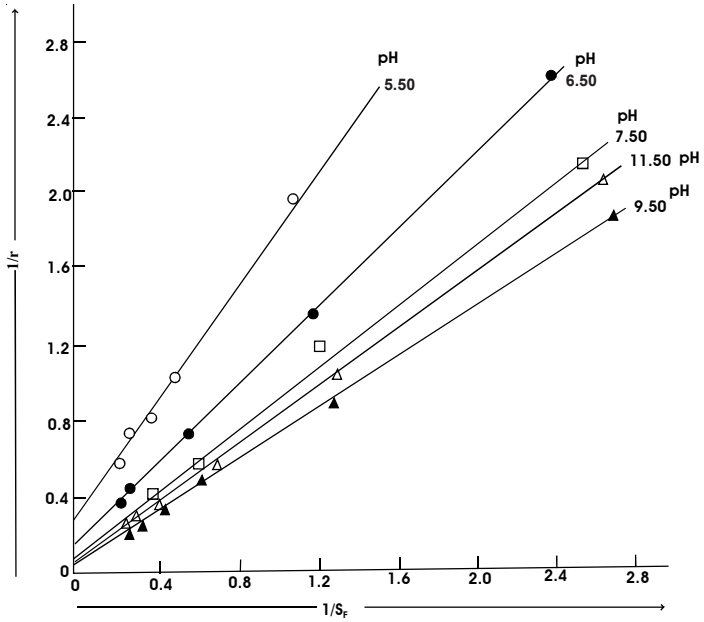

Fig. 2. Plots of 1/r *vs.* $1/S_F$ for cholesterol-OAS system at different-pH values

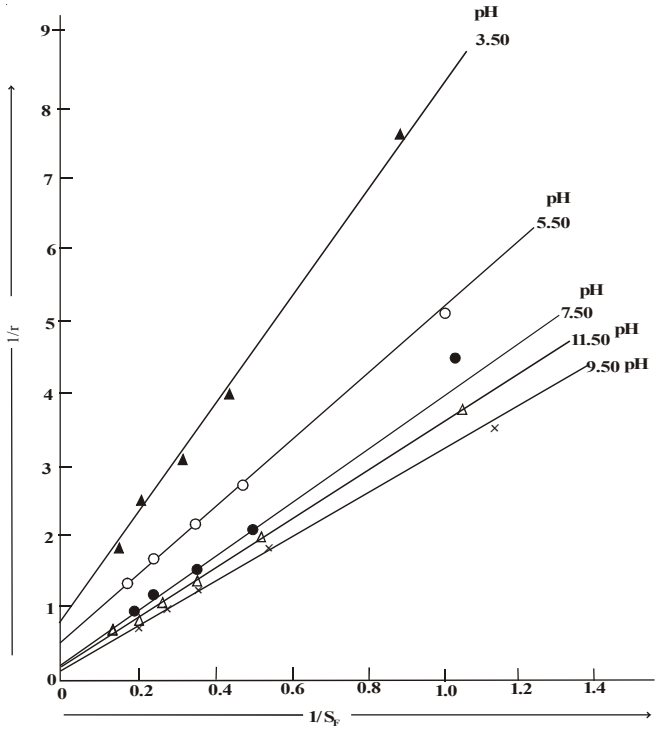

Fig. 3. Plot of 1/rs *vs.* $1/S_F$ for cholesteryl stearate-transfusion gelatin system at different pH values

are summarized in Tables 1 and 2. The result indicated that all the combining sites are identical, although their number is less for TG than OAS. This difference could be interpreted on the basis of molecular shape of the respective proteins, OAS being spherical has large surface area than TG which possess a cylindrical structure. The results of dialysis equilibrium technique at 10 ºC are complied in Table-3. The value is found to be higher at 10 ºC than at 25 ºC which may be attributed to the conformational changes in the structure of protein molecules. The themodynamic parameters (ΔF º, ΔHº and ΔSº) summarized in Tables 1-4 also revealed pH dependence like r, log K and n, and support the fact that with rising temperature there is decrease in the inter-and intramolecular attractive forces. Approximately similar values of log K at all pH values (Tables 1 and 2) indicated that a single class of site is reacting with cholesterol and its ester at all pH values and at the two temperatures. The appearance of the different number of sites (n) is, therefore, not responsible for the enhanced binding which must then be due to increased availability of the same class of the sites[27]. The different values of r, as the pH and temperature changes, are indicative of the above point in view.

TABLE-1
BINDING DATA FOR CHOLESTEROL-PROTEINS BY EQUILIBRIUM DIALYSIS METHOD AT DIFFEENT pH VALUES, TEMP. = 25 ± 0.1ºC AND μ = 0.15 M

TG + Cholesterol				OAS + Cholesterol			
pH	log K	n	ΔF º Kcal/mol	pH	log K	n	ΔF º Kcal/mol
3.50	1.937	3	-2.6484	5.50	1.972	5	-2.7083
5.50	1.944	4	-2.6668	6.50	1.977	6	-2.7129
7.50	1.958	5	-2.6852	7.50	2.034	10	-2.8004
9.50	1.967	7	-2.7129	9.50	2.114	12	-2.8985
11.50	1.950	6	-2.6760	11.50	2.079	11	-2.8534

TABLE-2
BINDING DATA FOR CHOLESTERYL-STEARATE BY EQUILIBRIUM DIALYSIS METHOD AT DIFFEENT pH VALUES, TEMP. = 25 ± 0.1ºC AND μ = 0.15 M

TG + Cholesteryl-stearate				OAS + Cholesteryl-stearate			
pH	log K	n	ΔF º Kcal/mol	pH	log K	n	ΔF º Kcal/mol
3.50	1.843	1.2	-2.5432	5.50	1.944	2.5	-2.7083
5.50	1.856	1.8	-2.5487	6.50	1.954	3.0	-2.7129
7.50	1.857	3.3	-2.5487	7.50	1.977	4.0	-2.8004
9.50	1.875	5.0	-2.5736	9.50	2.041	6.0	-2.8985
11.50	1.863	4.0	-2.5570	11.50	2.021	5.0	-2.8534

TABLE-3
BINDING DATA FOR CHOLESTEROL-PROTEINS BY EQUILIBRIUM DIALYSIS METHOD AT DIFFEENT pH VALUES, TEMP. = 10 ± 0.1ºC AND μ = 0.15 M

TG + Cholesterol				OAS + Cholesterol			
pH	log K	n	ΔF º Kcal/mol	pH	log K	n	ΔF º Kcal/mol
3.50	1.944	3.5	-2.5340	5.50	2.000	6.0	-2.6069
5.50	1.977	5.0	-2.5770	7.50	2.079	12.0	-2.7079
7.50	2.000	8.0	-2.6069	9.50	2.130	15.0	-2.7964
9.50	2.033	10.0	-2.6500	11.50	2.114	14.0	-2.7564
11.50	2.021	9.0	-2.6343	–	–	–	-2.7542

TABLE-4
THERMODYNAMIC CONSTANTS FOR THE BINDING OF CHOLESTEROL TO TRANSFUSION GELATIN AND SOLUBLE OVALBUMIN

TG + Cholesterol			OAS + Cholesterol		
pH	ΔHº Cal/mol	ΔSº Cal/deg/mol	pH	ΔHº Cal/mol	ΔSº Cal/deg/mol
3.50	-180.1	+9.29	5.50	-411.6	+8.34
5.50	-848.9	+6.15	7.50	-720.4	+6.60
7.50	-1080.0	+5.30	9.50	-1158.0	+6.50
9.50	-1827.0	+3.30	11.50	-900.5	+5.50
11.50	-1627.0	+2.80			

The molecule of cholestrol possesses a hydroxyl group (-OH) and alkyl side chains, both of which can be involved in the interaction with polar ($-COO^-$, $-NH_3^+$, $>NH_2^+$) and a polar groups (alkyl side chains) on the protein surface. Hydrogen bond formation between -OH and ionized carboxyls at low pH can be suggested as was postulated by Malik *et al.*[28-30] in the interaction of silicic acid with fibrillar and globular proteins. The -OH group of cholesterol is capable of hydrogen bonding to the glycerol ester oxygen of phospholipids[31,32] which is analogous to aliphatic -OH groups of proline and hydroxyproline amino acid residues of proteins. Hydrogen bonding between steroids and proteins has also been reported in the existing literature[33]. If the ionized carboxyls were the steroid binding sites then a larger value of r would be expected at pH 5.50 where all these groups remains deprotonated. In fact values of r were found to increases up to pH 9.50, thereby suggesting the involvement of some other groups as well in the binding process. The involvement of the amino groups was ruled out from the binding data of acetylated proteins[12]. Furthermore, the iodination or ketonization of tyrosine residues caused a decreased steroid

binding affinity[12]. Hence, it was realized that tyrosine residues played a significant role in the steroid-protein interaction. OAS and TG markedly differ in their tyrosine contents, having about 11 and 2.5 mol of each protein, respecively.

The lesser values of the binding data of cholesterol ester lend further support the involvement of the -OH group from cholesterole in the interaction. Binding of the ester can be attributed through hydrophobic bond formation. The hydrophobic bonding between cholesterole and membrane proteins has been reported in the existing literature[1,2]. The involvement of van der Walls forces in interaction in the steroid-serum albumin has also been reported[34]. It can be expected that the hydrocarbon part of steroid would associate with a polar groups of proteins present in the neighbourhood of the binding sites. The difference in binding behaviour of TG and OAS could be due to their fibrillar and corpuscular nature as well as to their different molecular weights and widely different number of reactive sites present in them. From the pH dependence of binding, we speculate about the involvement of phenolic groups of tyrosyl residues in the interaction. Klotz *et al.*[35] have also implicated the role of phenolic groups while Tanford[36] reported the formation of weak bonds with some tyrosine amino acid residues of the serum albumin molecule.

The present binding data also find support by the work of Levedahl *et al.*[37] who observed a progressive rise in equilibrium constant between pH 2.50 to 11.0. The log K was found to be a linear function of pH and an abrupt decrease above pH 11.0 was attributed to irreversible denaturation of proteins in the higher pH range. Thus, the type of relationship between binding data and pH indicated that the net charge on the protein had little or no influence on the binding. According to Westphal and Ashley[13] the binding between neutral steroids and proteins may be explained as mediated by hydrogen bond and van der Walls forces. The low negative free energy values of bonds are in line with the above forces in interaction. The rising values of enthalpies with increasing pH also supported the formation of weak bonds between steroids and the proteins under investigation. The decreasing positive entropy values with rising pH is an indication of the disordering of protein structure, presumably, owing to the degradation and denaturation of polypeptide chains.

The pH and temperature dependence of the binding data (Tables 1-4) revelaed some alterations in the environmental conditions of the interacting system. A similar type of temperature dependence was reported by Arora *et al.*[38] in the binding of catechin to bovine serum albumin while the pH and temperature dependence of binding sites in cholesterol-protein interaction is in line with the vanadate-trypsin[39] and beryllium-TG interaction[40]. The free energy of binding is mostly due to changes in entropy with

the apparent enthalpy contribution being small usually in the range of 0.18 to 1.627 kcal/mol for cholestrol-TG interaction while for cholestrol-OAS interaction it lies in the range of 0.4116 to 0.9000 kcal/mol between pH 3.50 to 11.50. The entropy changes are in the range of 2.80 to 9.29 cal/mol/deg within which the entropy values of cholesterol-TG interaction and for cholestrol-OAS interaction the entropy changes are in range of 5.50 to 8.34 cal/mol/deg. The thermodynamics of this combination is comparable to that of other ligand-protein interactions[41,42]. The positive value of entropy probably indicates that the transfer of steroid molecule from the solvent to protein is accompanied by the release of water of hydration from macromolecule and the ions of buffer and that the configuration of folded molecule changes into the unfolded one[43]. The diminished enthalpy at lower pH values may be attributed to the greater solubilization of steroid by the unionized swollen protein molecules and therefore, lesser release of water of hydration[44]. In all the low values of enthalpy and entropy for cholesterol-protein interaction suggested the involvement of weak bonding forces in the binding process[45].

From the results, it is apparent that phenolic or aliphatic hydroxyl groups as well as alkyl side chains of amino acid residues are responsible for interaction. It is also clear that steroid binding is a maximum near physiological pH. This study also throws some light on the significance of tyrosine in the formation of lipoproteins of living systems[10,11]. Chapman *et al.*[45] have also postulated that the increasing solubilizing effect of rising protein concentration on cholesterol may be due to trapping of cholesterol in regions of high protein density, which appears to be a consequence of the large hydrophobic forces. The lesser values of thermodynamic parameters *viz.*, $\Delta F°$, $\Delta H°$ and $\Delta S°$, are in line with the weaker forces like, hydrophobic and hydrogen bonding in the present system. It will be interesting to study other physiologically active steroids with proteins of known biological activity and structure.

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