BILE ACID CONTENT OF HUMAN SERUM. II. THE BINDING
OF CHOLANIC ACIDS BY HUMAN PLASMA PROTEINS

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Although many observations indicate that bile acids and their derivatives may be bound by serum proteins, little information upon the quantitative aspects of this reaction is available. Lecomte du Noiy (1) observed that the activity of bile salts in lowering the surface tension of aqueous solutions was suppressed by the presence of serum.

The activity of human albumin in preventing the lysis of red blood cells by the bile salts was believed by E. J. Cohn to indicate the binding of these substances by albumin (2). The bile acids which are contained in the serum of patients with hepatic disease have been found in this laboratory to be largely non-dialyzable (3). These observations indicate that these substances are bound by serum proteins. This binding to proteins is a factor which may influence the serum concentration and renal clearance of the bile acids, as well as their relationship to such other serum constituents as the lipids and bilirubin. The present report is concerned with the binding of several bile acids by certain plasma protein fractions, as measured by the dialysis-equilibrium method, and with the effect of pH upon this reaction.

TABLE I
BINDING OF BILE ACIDS BY HUMAN PLASMA PROTEIN FRACTIONS

<table>
<thead>
<tr>
<th>Plasma protein fraction* [Cohn (4)]</th>
<th>mM X 10^-4 of bile acid bound by 100 mg. of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deoxycholic acid</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0.5</td>
</tr>
<tr>
<td>III</td>
<td>1.8</td>
</tr>
<tr>
<td>IV-1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Major components of these fractions are as follows: I, fibrinogen; II, γ-globulin; III, β-globulins; IV-1, α-globulins; V, albumin.

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1 Fellow of the New York Heart Association.

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acid content of the system, the quantity of bile acid bound by the protein within the sac was calculated.

The Donnan effect was considered to be of negligible magnitude and was disregarded in the calculations.

Commercial samples of deoxycholic acid, hyodeoxycholic acid and cholic acid were employed in these studies. The following compounds were kindly supplied by Dr. Erwin H. Mosbach: Taurine and glycine conjugates of deoxycholic and cholic acids; chenodeoxycholic acid; lithocholic acid; 7-hydroxy cholanic acid; 3-hydroxy 12-keto cholic acid; 3, 7-dihydroxy 12-keto cholic acid; and the formyl derivatives of deoxycholic and cholic acids. 3, 12-dihydroxy 7-keto cholic acid was donated by Dr. Norman A. Hulme of Sterling-Winthrop Research Institute. 3-hydroxy, 12-keto, Δ9-11 cholic acid was given by Dr. Karl Pfister of Merek Research Laboratories. The protein fractions of human plasma were generously donated by Dr. J. M. Ashworth of the American Red Cross through E. R. Squibb and Sons.

RESULTS

Binding of bile acids by various plasma proteins

The extent of binding of deoxycholic and cholic acids, by 100 mg. of each of 5 plasma protein fractions, is indicated by Table I. The protein, in a volume of 10 ml., was equilibrated against 12.7 x 10^-3 mM of bile acid in a volume of 50 ml. Among the plasma proteins albumin exhibits the greatest binding activity towards both of these bile acids. The uptake of deoxycholic and glycodeoxycholic acids by Fractions III and IV-1 was approximately half that shown by albumin. Fibrinogen and gamma globulin did not bind either deoxycholic or cholic acid.

Extent of binding by albumin of the series of cholic acids

The chemical structure of the bile acid was found to influence the extent to which it is bound to human serum albumin.

Ten ml. of a 1 per cent human serum albumin solution (1.4 x 10^-3 mM of albumin) was equilibrated with 50 ml. of buffer containing 12.7 x 10^-3 mM of a number of bile acids and their derivatives. Table II lists the bile acids and derivatives studied, together with the number of moles of the bile acid bound by each mole of albumin under these conditions. The extent of binding decreases as the number of hydroxyl groups on the ring system is increased. It was greatest for the bile acids with a single hydroxyl group and least for cholic acid, which has three hydroxyl groups. The position of the hydroxyl groups in the ring system has little influence upon the degree of binding. Neither conjugation of the carboxyl group with glycine or taurine, nor covering up the hydroxyl groups by the formyl radicals greatly changed the extent of binding.

The introduction of a keto group in either the 7 or 12 position of the ring system suppresses the affinity for albumin. In the only unsaturated compound tested (3-hydroxy, 12-keto, Δ9-11 cholic acid)

<table>
<thead>
<tr>
<th>Monohydroxycholanic acids</th>
<th>Structure</th>
<th>Moles of cholic acid bound by one mole of albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithocholic acid</td>
<td>3-OH cholanic acid 7-OH cholanic acid</td>
<td>6.5 6.7</td>
</tr>
<tr>
<td>Dihydroxycholanic acids</td>
<td>3, 12 di-OH cholanic acid</td>
<td>2.3</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>3, 6 di-OH cholanic acid</td>
<td>3.2</td>
</tr>
<tr>
<td>Hyodeoxycholic acid</td>
<td>3, 7 di-OH cholanic acid</td>
<td>3.0</td>
</tr>
<tr>
<td>Chenoxycholic acid</td>
<td>3, 7, 12 trihydroxycholanic acid</td>
<td>.94</td>
</tr>
<tr>
<td>Trihydroxycholanic acids</td>
<td>Cholic acid</td>
<td>3, 7, 12 tri-OH cholanyl taurine</td>
</tr>
<tr>
<td>Conjugated cholanic acids</td>
<td>Glycodeoxycholic acid</td>
<td>3, 12 di-OH cholanyl glycine</td>
</tr>
<tr>
<td></td>
<td>Taurodeoxycholic acid</td>
<td>3, 12 di-OH cholanyl taurine</td>
</tr>
<tr>
<td></td>
<td>Glycocholic acid</td>
<td>3, 7, 12 tri-OH cholanyl glycine</td>
</tr>
<tr>
<td></td>
<td>Taurocholic acid</td>
<td>3, 7, 12 tri-OH cholanyl taurine</td>
</tr>
<tr>
<td>Keto cholanic acids</td>
<td>—</td>
<td>3-OH, 12-keto cholanic acid</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>3, 7 di-OH, 12-keto cholanic acid</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>3, 12 di-OH, 7-keto cholanic acid</td>
</tr>
<tr>
<td></td>
<td>(Unsaturated)</td>
<td>3-OH, 12-keto, Δ9-11 cholanic acid</td>
</tr>
<tr>
<td>Formylated cholanic acids</td>
<td>Diformyldeoxycholic acid</td>
<td>Diformyl 3, 12 di-OH cholanic acid</td>
</tr>
<tr>
<td></td>
<td>Triformylcholic acid</td>
<td>Triformyl 3, 7, 12 tri-OH cholanic acid</td>
</tr>
</tbody>
</table>

* With larger amounts of albumin, it is possible to demonstrate slight binding of these two cholic acid derivatives by albumin.
lenic acid), the introduction of a double bond into the ring system increased the extent of binding with albumin.

Effect of pH on binding reaction

The effect of hydrogen ion concentration upon the interaction with albumin was studied, in the case of deoxycholic acid, over a pH range of 5.9 to 11.6 (Figure 1). The reaction is indifferent to hydrogen ion concentration until the pH of 9.0 is reached. Above pH 9.0 the affinity for albumin decreases rapidly, and is nearly absent above pH 11.0. Denaturation of albumin by alkaline pH was excluded by the finding that the albumin solutions, after the experiment had been completed, exhibited the usual uptake of deoxycholate at pH 7.6.

Calculation of binding constants

It is apparent from Table II that each molecule of albumin can bind more than one molecule of cholic acid. The number of molecules of cholic acid bound by one molecule of albumin is determined by the concentration of unbound cholanic acid. The relationship between bound and unbound cholanic acid involves two parameters: the dissociation constant of the albumin-bile acid complex (K) and the maximum binding capacity of the albumin molecule (n).

If it is assumed that in a molecule the size of albumin, the reactivity of a binding site is not markedly affected by the state of other binding sites, then the reaction can be treated as a simple bi-molecular reaction between the binding site and the cholanic acid.

The equilibrium conditions of this reaction may be formulated as follows (8):

\[
\frac{1}{r} = \frac{K}{n} \frac{1}{(A)} + \frac{1}{n}
\]

where \( r \) is the ratio of moles of bound cholic acid to moles of albumin; \( A \) is molar concentration of unbound cholanic acid; \( n \) is maximum moles of cholanic acid which can be bound by one mole of albumin; and \( K \) is dissociation constant of the albumin-cholanic acid complex. It is apparent that a plot of \( 1/r \) as a function of \( 1/A \) should assume a linear form, the vertical intercept representing \( 1/n \) and the slope representing \( K/n \). By this method, the constants \( K \) and \( n \) may be calculated for each bile acid.

Such data have been obtained for deoxycholic acid and cholic acid (Figure 2). Solutions of the sodium salt of each bile acid in buffer, were prepared with a bile acid concentration of \( 1.27 \times 10^{-4}, 2.54 \times 10^{-4}, 5.08 \times 10^{-4}, \) and \( 12.70 \times 10^{-4} \) mM.
per ml. Fifty ml. of each solution were equilibrated with 10 ml. of a 1 per cent solution of albumin in buffer. Measurement of unbound bile acid concentration in the outer solution after equilibration, and calculation of quantity of bile acid bound by the albumin within the sac, provided the data for the curves in Figure 1. From the intercept and slope of these curves, it may be calculated that for deoxycholic acid, \( K = 7.4 \times 10^{-4} \) and \( n = 12 \), for cholic acid, \( K = 6.5 \times 10^{-4} \) and \( n = 4 \).

**DISCUSSION**

The binding of the cholanic acid series of compounds by serum albumin might have been predicted from knowledge of the interaction of serum albumin with long chain fatty acids and with a variety of other organic anions (9). Serum albumin has been considered to be unique among the plasma protein fractions in its ability to bind organic anions (10). The present data confirm the preeminence of albumin in this regard, but indicate that the lipoprotein-containing Fractions III and IV-1 also interact with the cholanic acids, although to a lesser extent than does albumin.

The pH effect, namely the suppression of binding above pH 9, is compatible with the existence of an electrostatic bond between the positively charged \( \epsilon \)-amino group of lysine (pK 9.3), and the negatively charged carboxylate group of the cholanic acid, as the primary force responsible for the binding. This finding parallels that of Klotz and Walker in the binding of methyl orange by bovine serum albumin (11).

However, the existence of secondary forces between albumin and the bile acid is indicated by the variation in extent of binding among closely related cholanic acids (Table II). The data indicate that the polarization of the cholanic acid ring system by the successive introduction of hydroxyl groups suppresses the affinity for albumin.

There are three times as many sites in albumin available for binding deoxycholic acid, a dihydroxy compound, as are available for binding cholic acid, which possesses 3 hydroxy groups. These observations are consistent with the postulated role of van der Waal forces acting upon the non-polar region of the smaller molecule, in determining the affinity of the substance for albumin (10). The marked reduction of affinity for albumin which results from the presence of a keto group in the ring system, likewise appears to be caused by changes in the polarity of the ring system.

The cyclopentano-perhydrophenanthrene ring system of the bile acids is also found in such biologically important substances as cholesterol, steroid hormones, and cardiac glycosides, which differ from one another in the structure of the ring system and in the nature of substituent groups upon the ring. Study of how the interaction of cholanic acids with albumin is modified by changes in the structure of the cholanic acids may yield information of interest. The testing of additional cholanic acid derivatives will provide further information on the relationship between molecular structure and affinity for the various plasma proteins.

**SUMMARY**

The binding of bile acids and their derivatives by the protein fractions of human plasma has been studied by the dialysis-equilibrium method. Albumin exhibits the greatest binding activity to-
wards these compounds. The lipoprotein-containing globulins, Cohn Fractions III and IV-1, bind approximately half as much deoxycholic acid and cholic acid as does albumin. γ-globulin and fibrinogen do not interact with the bile acids.

The affinity for albumin is reduced by the introduction of polar groups into the steroid nucleus. Thus the extent of binding decreases in the order monohydroxy > dihydroxy > trihydroxy cholanic acid. Binding of keto-cholanic acids to albumin could not be detected.

The effect of pH upon the binding reaction suggests that the primary attraction between albumin and the cholanic acids is an electrostatic bond between the positively-charged lysine side chains of the former and the negatively-charged carboxyl groups of the latter.

The binding constants of albumin with two bile acids were calculated.

REFERENCES

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