Thyroxine-Protein Interactions

I. BINDING OF THYROXINE TO HUMAN SERUM ALBUMIN AND MODIFIED ALBUMINS*

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In previous reports (1, 2), it was suggested that the binding of thyroxine to human serum albumin resulted primarily from an electrostatic interaction between anionic portions of the thyroxine molecule and cationic sites on the protein. From the reduction in thyroxine binding exhibited by acetylated albumin preparations, it seemed likely that part of the reactive cationic sites were made up of e-amino groups of lysyl residues in the protein (2). The results of an investigation of the effect of guanidination and acetylation of albumin on thyroxine binding are described in this paper. The data provide further support for the involvement of lysyl e-amino groups in the binding reaction.

EXPERIMENTAL PROCEDURE

Materials

Human Serum Albumin—The human serum albumin used in these experiments was obtained from the American Red Cross through the courtesy of Dr. James H. Pert. The albumin was human Fraction V (batch No. 1972) which had been prepared from pooled human plasma by the method of Cohn et al. (3) and was shipped as a lyophilized powder. During the fractionation by the Cohn procedure, the temperature was carefully maintained at -5° except for the initial 15 minutes of ethanol addition when the temperature rose to +2°. Neither stabilizers, such as N-acetyltryptophan and sodium caprylate, nor heavy metals had been added to the protein.

Before its use in the binding experiments, the HSA was either dialyzed against glass-distilled water or deionized by passage through an Amberlite MB-1 resin (4) and then dialyzed. The HSA was estimated to be over 98% albumin and was free of other thyroxine-binding serum proteins as shown by electrophoresis on paper with Veronal and the glycine-acetate buffer of other thyroxine-binding serum proteins as shown by electrophoresis on paper with Veronal and the glycine-acetate buffer of other thyroxine-binding serum proteins as shown by electrophoresis on paper with Veronal and the glycine-acetate buffer.

Acetylated Albumin—The HSA was acetylated with acetic anhydride by the method of Fraenkel-Conrat, Bean, and Lineweaver (6). Excess acetic anhydride was removed by dialysis.

Acetylated HSA was allowed to react with 0-methylisourea hydrochloride at pH 8 to 10 during the addition of acetic anhydride as described previously (7).

Guanidinated Albumin—HSA in 20% concentration was allowed to react with 0.5 n 0-methylisourea hydrochloride at pH 10.5 or pH 11 for 4 days at 2° (8). The reaction was terminated by adjusting the solution to pH 8, and excess reagent was removed by dialysis. The 87% guanidinated HSA (G87) was allowed to react at pH 10.5. The other guanidinated preparations, 93% guanidinated HSA (G93) and 90% guanidinated HSA (G90), were reacted at pH 11.

Acetylated HSA was allowed to react with 0-methylisourea hydrochloride at pH 11 for 4 days to form acetylated-guanidinated albumin which was 30% acetylated and 55% guanidinated (Ac30G55).

Guanidinated HSA (G93) was allowed to react with acetic anhydride at pH 8 to 10 to form guanidinated-acetylated albumin (G93Ac-1).

All protein solutions were stored at -20°.

A molecular weight of 69,000 was assumed for human serum albumin. The molecular weights of the acetylated and guanidinated albumins were calculated from the extent of modification.

Radioactive Compounds—[31]Labeled L-thyroxine and [131]I-labeled L-triiodothyronine were obtained from Abbott Laboratories. The radioactive compounds contained less than 1% inorganic 131I-iodide as a contaminant as shown by chromatography on paper with butanol-dioxan-ammonia solvent (4:1:5 by volume). [31]I-Thyroxine solutions of known concentration were made up by adding a small amount of the radioactive compound to carrier thyroxine solutions. Stock solutions containing 100 μg per ml or 500 μg per ml of thyroxine were made up in 0.02 N NaOH. Solutions of 1 mg per ml and 2 mg per ml of thyroxine were made up in 0.04 N and 0.08 N NaOH, respectively. All thyroxine solutions were stored in plastic containers at 4°.

O-Methylisourea—The O-methylisourea hydrochloride used to prepare guanidinated albumin was obtained from Lederle Laboratories through the courtesy of Dr. J. J. Denton.

O-Methylisourea hydrogen sulfate was synthesized by the method of Fearing and Fox (9) and was used to prepare homoaarginine from the copper salt of lysine (10). The O-methylisourea hydrogen sulfate and the homoarginine were prepared by Nicholas A. Giorgio, Jr. A small quantity of homoaarginine was also kindly supplied by Dr. Emil Smith.

Nonradioactive L-Thyroxine and L-Triiodothyronine—The

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1 This information was kindly supplied by J. Perrotta of E. R. Squibb & Sons.

2 The abbreviation used is: HSA, human serum albumin.
iodothyronine compounds were purchased from the Aldrich Chemical Company, Inc.

**Methods**

**Binding Experiments**—Equilibrium dialysis was used to study binding. Eight-inch lengths of Visking cellulose tubing (8\(\frac{8}{8}\) inch) were soaked in 0.1 M HNO₃ for 18 to 24 hours and then in 0.01 M HNO₃ for 3 days to remove metal ion impurities (11). The tubing was finally stored in deionized water at 4°C. Before use, the tubing was tied off at one end and rinsed with glass-distilled water. No marble was added to the inside of the bag in these experiments. The dialysis was performed in plastic tubes as described previously (1). Dialysis was done in a total volume of 10 ml with 5-ml volumes used inside and outside the bag. Protein concentrations of 0.050 g/100 ml or 0.10 g/100 ml were used. In most of the experiments, the thyroxine was added to the protein side (inside) of the bags. Ethylenediaminetetraacetic acid, 6.8 \(\times\) \(10^{-4}\) M, was also added to each tube (1). At 30°, equilibrium was reached after 6 hours with horizontal shaking. Samples for counting were usually taken after 16 to 18 hours. The fraction of thyroxine bound was determined by counting aliquots from inside and outside the bag.

\[
\text{Fraction bound} = \frac{\text{c.p.m. inside} - \text{c.p.m. outside}}{\text{total c.p.m.}}
\]

There was no detectable loss of thyroxine resulting from adsorption to the bags or to the walls of the plastic tubes when concentrations below 2.8 \(\times\) \(10^{-4}\) M were studied at pH 7.4. Above this concentration, losses of 10 to 15% were observed, and suitable corrections were applied. Experiments were discarded in which low recoveries (below 85%) of thyroxine were obtained. Although thyroxine has a limited solubility at pH 7.4, it was possible to study concentrations of thyroxine as high as 5.6 \(\times\) \(10^{-4}\) M (\(\phi\) values above 3, Fig. 1) by dividing the initial addition of thyroxine so that a total of 200 \(\mu\)g were added to the outside 5-ml solution and amounts up to 300 \(\mu\)g were added to the inside (protein side) of the bag. When these higher thyroxine concentrations were studied, samples for counting were taken at 6 hours. No detectable decomposition of thyroxine took place during dialysis as shown by chromatography on paper followed by a scan of the radioactivity on the chromatogram.

The following buffers were used: potassium phosphate, pH 4.7 to 8.0, \(\Gamma/2\), 0.15, 0.2 \(\mu\)M sodium borate-0.05 \(\mu\)M NaCl, pH 8.4 to 9.3; 0.2 \(\mu\)M sodium borate, pH 9.6 to 10.0; sodium carbonate, pH 10.4 to 10.5, \(\Gamma/2\), 0.1; sodium phosphate, pH 11.5, \(\Gamma/2\), 0.15.

After dialysis, the hydrogen ion activities of the solutions inside and outside the bags were measured with the Beckman model GS pH meter.

**Measurement of Extent of Acetylation**—The extent of acetylation was estimated from the determination of free amino groups by the ninhydrin method of Harding and MacLean (12). The latter method can be used to determine \(\epsilon\)-amino groups as shown by Ehrenpreiss, Maurer, and Sri Ram (13).

**Measurement of Extent of Guanidination**—The extent of guanidination was determined by direct analysis for homoaarginine after hydrolysis of the modified albumins. A Beckman/Spinco model 120 amino acid analyzer was used. Homoaarginine emerged about 30 ml after arginine on the 15-cm ion exchange column with the pH 5.28 citrate buffer system.

**Measurement of Protein Concentration**—Protein concentrations were measured spectrophotometrically at 280 nm with the use of an extinction coefficient of 5.3 for a 1% solution of human serum albumin (14). The buer reagent of Gornall, Bardawill, and David (15) was used to measure the protein concentrations of the highly modified proteins, Ao14 and G03Sae1.

**Measurement of Radioactivity**—\(\text{I}^{131}\) Labeled thyroxine solutions were counted in a Nuclear-Chicago Auto-Gamma well-type scintillation counter.

The distribution of radioactivity on paper chromatograms and electrophoretic strips was determined with a 4-\(\pi\) model SP-4 windowless paper strip scanner purchased from the Savant Company, Hicksville, Long Island, New York.

**Equations Used in This Study**—The reversible binding of a small molecule, \(A\), to a protein at a single set of sites, \(v\), each with the same average apparent intrinsic association constant, \(k'\), and neglecting corrections for electrostatic interactions among bound ions (16), can be represented by the equation (17-20)

\[
\frac{\nu}{\nu + k' [A]} = \frac{nk'}{1 + k' [A]}
\]

where \(\nu\) is the average number of moles of \(A\) bound per mole protein and \([A]\) is the molar concentration of free \(A\).

Equation 1 has been rearranged by Scatchard (21) to give

\[
\frac{\nu}{A} = \frac{nk'}{k' [A]}
\]

A plot of \(\nu/A\) against \(\nu\) should give a straight line with slope \(-k'\); the intercept on the \(\nu/A\) axis is \(nk'\), as \(\nu\) approaches 0 as a limit, and the intercept on the \(\nu\) axis is \(n\) as \(\nu/A\) approaches 0 as a limit.

If there are several sets of sites, \(m\), each with different intrinsic association constants, then the intercept on the \(\nu/A\) axis is \(\Sigma\):

\[
\frac{\nu}{A} = \frac{nk'}{k' [A]}
\]
**Table I**

**Binding of L-Thyroxine to Human Serum Albumin at pH 7.4**

<table>
<thead>
<tr>
<th>Total thyroxine added</th>
<th>No. of experiments</th>
<th>$\bar{v}$</th>
<th>$\bar{v}/A \times 10^{-3}$</th>
</tr>
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<tbody>
<tr>
<td>0.0224</td>
<td>6</td>
<td>0.257</td>
<td>6.85 ± 0.26*</td>
</tr>
<tr>
<td>0.0560</td>
<td>8</td>
<td>0.623</td>
<td>5.70 ± 0.28</td>
</tr>
<tr>
<td>0.0648</td>
<td>6</td>
<td>0.001</td>
<td>4.75 ± 0.31</td>
</tr>
<tr>
<td>0.112</td>
<td>5</td>
<td>1.18</td>
<td>4.48 ± 0.34</td>
</tr>
<tr>
<td>0.139</td>
<td>6</td>
<td>1.43</td>
<td>4.04 ± 0.17</td>
</tr>
<tr>
<td>0.168</td>
<td>6</td>
<td>1.69</td>
<td>3.64 ± 0.19</td>
</tr>
<tr>
<td>0.224</td>
<td>6</td>
<td>2.20</td>
<td>3.40 ± 0.32</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation.

**Results**

**Effect of pH on Binding of Thyroxine to HSA**

The observed values for the binding by deionized HSA at pH 7.4 are given in Table I. The values given in Table I and eight additional experimental points have been plotted in Fig. 1. The solid line in Fig. 1 is a theoretical curve calculated from Equation 4 by assuming the existence of two sets of sites, $n_1 = 2$, $k_1' = 2.75 \times 10^5 M^{-1}$, and $n_2 = 6$, $k_2' = 2.5 \times 10^4 M^{-1}$.

At pH 7.4, it was difficult to obtain reliable values for $\bar{v}$ beyond those shown in Fig. 1 because thyroxine precipitates at the higher concentrations required for the experiments. A second binding site with a lower value for $k_2'$ is definitely observed at pH 9.3 (Fig. 2, Table II). At pH 9.3, higher concentrations of thyroxine could be studied owing to its increased solubility.

**Effect of pH on the Binding of Thyroxine**

The effect of pH on thyroxine binding is shown in Fig. 2. Constants for the interaction at pH 7.4, 9.3, and 10.4 are given in Table II. Fig. 2 shows that there is little change in binding from pH 6.8 to 9.3 at low concentrations of thyroxine. At pH 6.0 and 4.7, a reduction in binding is observed, and the affinity of HSA for thyroxine is also decreased when the pH is raised to 10.4 and 11.5.

**Table II**

<table>
<thead>
<tr>
<th>pH</th>
<th>$\Sigma n_k' \times 10^{-3}$</th>
<th>$n_1$</th>
<th>$k_1' \times 10^{15}$</th>
<th>$n_2$</th>
<th>$k_2' \times 10^{15}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>7.0</td>
<td>2</td>
<td>2.75</td>
<td>6</td>
<td>0.25*</td>
</tr>
<tr>
<td>9.3</td>
<td>7.2</td>
<td>2</td>
<td>3.3</td>
<td>6</td>
<td>0.1*</td>
</tr>
<tr>
<td>10.4</td>
<td>1.8</td>
<td>1</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These binding constants were used to construct the theoretical curves for HSA shown in Figs. 1 and 2.
A plot of $\Sigma n k'$ against pH is shown in Fig. 3. It can be seen that thyroxine binding reaches a maximum at about pH 6.8, remains constant until pH 9.3, and then decreases above pH 9.3. It should be noted that the phenolic hydroxyl group of thyroxine has a $pK'$ of 6.73 (22).

**Binding of Thyroxine to Modified Albumins**

**Binding of Acetylated HSA**—The effect of acetylation of HSA on the interaction with thyroxine at pH 7.4 is shown in Fig. 4. The native HSA studied in Fig. 4 had been dialyzed but not deionized prior to use. Blocking free amino groups in albumin by acetylation with acetic anhydride causes a reduction of about 70% in thyroxine binding at pH 7.4. At pH 8.0 and 8.4 (Fig. 4), thyroxine binding to acetylated HSA is reduced further, to about 10% of the value observed for native HSA (Fig. 2).

**Binding of Guanidinated HSA**—The interaction of thyroxine with guanidinated HSA at pH 7.4 is shown in Fig. 4. Although the curve obtained for the interaction is not identical with that observed for native HSA, the results show that, by contrast to the effect of acetylation, extensive guanidination of albumin does not lead to a loss of affinity for thyroxine.

As shown in Fig. 4, exposure of HSA to pH 11 at 2°C for 4 days (the conditions for guanidination) produces no change in binding behavior.

The effect of pH on the binding of guanidinated HSA is shown in Fig. 5. At pH 8.4 (Fig. 5), the interaction of thyroxine with guanidinated HSA (G93) resembles the binding exhibited by native HSA at pH 7.4 (Fig. 1). From pH 9.0 to pH 10.4, the data are best fitted by assuming the existence of a third binding site for guanidinated HSA (Fig. 5, Table III) with little change in the estimated value for $k'$ ($3.4 \times 10^6$ M$^{-1}$).

**Table III**

<table>
<thead>
<tr>
<th>Guanidinated albumin</th>
<th>pH</th>
<th>$\Sigma n k' \times 10^3$</th>
<th>$n_1$</th>
<th>$k' \times 10^3$</th>
<th>$n_2$</th>
<th>$k' \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G93</td>
<td>9.3</td>
<td>10.8</td>
<td>3</td>
<td>3.4</td>
<td>6</td>
<td>0.1*</td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>9.6</td>
<td>2.8</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G90</td>
<td>10.5</td>
<td>7.7</td>
<td>2.4</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These binding constants were used to construct the theoretical curve shown in Fig. 5.

**Binding of Thyroxine to Native HSA and Guanidinated HSA at pH 10.5**—In guanidinated albumin (8, 23), lysyl $\varepsilon$-amino groups, pK' about 9.8 (24), have been converted to homoarginyl guanidinium groups, pK' > 12 (24). If positively charged $\varepsilon$-amino groups of lysyl residues in native albumin participate in the binding of thyroxine, then guanidinated albumin should be capable of binding at significantly higher pH than native albumin. This is found to be the case as shown in Fig. 6 in which the binding of native HSA is compared with the binding of guanidinated HSA (G90) at pH 7.4 and pH 10.5. In Fig. 6, $P$ has been plotted against the logarithm of the free thyroxine concentration. The latter is identical with the term $[A]$ in Equations 1 to 4. It can be seen from Fig. 6 that there is a marked decrease in thyroxine binding to native HSA at the higher pH compared to a small drop in the binding observed for guanidinated HSA. The difference in binding behavior between native HSA and guanidinated HSA at pH 10.5 is shown in a more striking way by the data given in Table IV.
At pH 11.5 (Fig. 5), guanidinated HSA (G97) also exhibits a much greater affinity for thyroxine than native HSA (Fig. 2).

**Binding of Acetylated-Guanidinated Albumin at pH 7.4**—Guanidinated albumin (G93) was treated with acetic anhydride to produce the derivative G93Ac-1. Acetylation of G93 did not inhibit thyroxine binding at pH 7.4 as shown in Fig. 7. This indicates that other groups that may be acetylated, such as the hydroxyl groups of serine of threonine, do not take part in the binding reaction.

**Binding of Guanidinated and Acetylated Albumin at pH 7.4**—Acetylated album (Ac30) was guanidinated to produce Ac30G55. Guanidination did not increase binding of previously acetylated HSA as can be seen from Fig. 7.

**Effect of Methyl Orange on Thyroxine Binding**—The anionic dye, methyl orange, appears to be bound to cationic sites on serum albumin as shown by Klotz and Urquhart (25). It was of interest to note the effect of methyl orange on thyroxine binding to HSA. As can be seen in Fig. 8, methyl orange displaces thyroxine from binding sites on albumin at pH 7.4.

**Interaction of Triiodothyronine with Human Serum Albumin**

The phenolic hydroxyl group of triiodothyronine has a pK' of 8.45 (22), so that at pH 7.4 this group is only about 8% ionized. At pH 7.4, the phenolic hydroxyl group of thyroxine (pK' = 6.73 (22)) is about 80% ionized. It was of interest to observe the effect of pH on the binding of triiodothyronine to note if any relationship exists between the ionization of the phenolic group and binding. The effect of pH on triiodothyronine binding is shown in Fig. 9 and a plot of $\Sigma \nu k'$ against pH is given in Fig. 3. As shown in Fig. 3, triiodothyronine binding reaches a maximum at a pH corresponding to the pK' of its phenolic hydroxyl group (pK' = 8.45). From Fig. 3, it can also be seen that tetraiodothyronine (thyroxine) is bound more tightly than triiodothyronine over the whole pH range investigated.

**Binding of Triiodothyronine to Acetylated HSA**—Acetylation of HSA has only a small effect on the interaction with triiodothyronine at pH 7.4 (Fig. 10) compared to the marked reduc-
tion observed in the binding of thyroxine (Fig. 4). There is a decrease in triiodothyronine binding to acetylated HSA at pH 8.0 and 8.4 (Fig. 10), but the reduction (as related to the binding by native HSA at pH 7.4) is not as large as that observed for thyroxine under these conditions (Fig. 4).

Effect of Methyl Orange on Triiodothyronine Binding—In contrast to the effect on thyroxine binding (Fig. 8), methyl orange has little effect on the binding of triiodothyronine at pH 7.4 (Fig. 11). However, at pH 9.0, when the phenolic hydroxyl group of triiodothyronine is about 80% ionized, there is a definite decrease in binding in the presence of methyl orange (Fig. 11).

DISCUSSION

The results of the present investigation indicate that positively charged lysyl ε-amino groups are directly involved in the binding of thyroxine to albumin. This postulate is based on the following experimental data: (a) acetylation of ε-amino groups leads to an appreciable reduction in thyroxine binding (Fig. 4); (b) guanidination of albumin, which converts positively charged ε-amino groups to positively charged guanidinium groups, produces no loss in binding affinity (Figs. 4 and 5); (c) thyroxine binding to native albumin is markedly reduced at pH 10.5 (Fig. 6); (d) guanidinated albumin exhibits high binding affinity for thyroxine at pH 10.5 (Fig. 6); and (e) methyl orange displaces thyroxine from binding sites on albumin (Fig. 8).

At pH 10.5, the lysyl ε-amino groups (pK' about 9.8 (24)) have lost a major portion of their positive charges, while the homoarginyl guanidinium groups (pK' > 12 (24)) are predominantly in the positively charged form. Although the reduction in thyroxine binding at pH 10.5 appears to be mainly attributable to the loss in positive charges on cationic binding sites on albumin, other factors may also contribute to the reduced binding. One of these could arise from an increase in the net negative charge on albumin at pH 10.5 leading to electrostatic repulsive forces between the protein and the negatively charged thyroxine molecule.

The ability of the anionic dye, methyl orange, to displace thyroxine from binding sites on albumin (see Fig. 8) provides further support for the view that, at pH 7.4, thyroxine is bound as an anion to cationic loci on albumin. Klotz (17, 26) and Klotz and Urquhart (25) have presented substantial evidence that indicates that methyl orange is bound to cationic sites on albumin, and that lysyl ε-amino groups are intimately involved in the binding process.

The observation that the binding of both thyroxine and triiodothyronine reaches a maximum at a pH corresponding to the pK' of their respective phenolic groups (see Fig. 3) indicates that the anionic phenolate group of thyroxine is a probable point of attachment to cationic binding sites on albumin at or above pH 6.73 (the pK' of the phenolic hydroxyl group of thyroxine). From the present data, it is difficult to assess the influence that the two other charged groups in thyroxine (the cationic amino group and the anionic carboxylate ion) may have on binding. It is likely that other forces, not involving the interaction of charged groups, contribute to the binding as shown by the fact that thyroxine is more tightly bound than triiodothyronine under conditions of pH in which there is little difference in the distribution of charge on both compounds (for example, at pH 4.7 and above pH 9.3 (Fig. 3)).

The contrast in behavior between thyroxine and triiodothyronine in relation to the interaction with acetylated serum albumin, and the effect of methyl orange on binding to native albumin, can be partially explained on the basis of the difference in pK' of their phenolic hydroxyl groups. As the ionization of the phenolic group of triiodothyronine is increased, its binding behavior becomes qualitatively similar to the binding exhibited by thyroxine (see Figs. 4, 8, 10, and 11).

The constants given here for thyroxine binding to human serum albumin at the first set of sites (k' = 2.75 × 10^6 M^−1, n = 2) differ from those reported by Tritsch et al. (27). These authors used a different method to investigate thyroxine binding and gave a value of 2.5 × 10^6 M^−1 for k', with n = 1 (27).

The serum albumin used in this study exhibits a greater affinity for thyroxine than the albumin that had been used in earlier
Physiological Significance of Thyroxine Binding to Albumin—
Thyroxine circulating in blood is almost completely bound by protein in reversible noncovalent linkage (29–31). At present, it appears that there are three principal thyroxine-binding protein fractions in human blood serum. These are thyroxine-binding α-globulin, prealbumin, and albumin (29–31). The approximate percentage distribution of total circulating thyroxine among these carriers of the hormone in human serum as given by Ingbar and Freinkel (31) is: thyroxine-binding α-globulin, 45%; prealbumin, 40%; albumin, 15%. It should be emphasized that these are only rough estimates and that they were obtained from experiments with the use of electrophoresis on paper at pH 8.6. In other investigations of serum with electrophoresis in agar gel at pH 7.4, thyroxine binding to albumin has also been shown (32, 33). From this, it would appear that albumin functions as a carrier of thyroxine in serum and is part of the system that serves to transport the thyroid hormone to the tissues.

Albumin is the only thyroxine-binding protein in serum that has been obtained in large amount in purified form. It has been difficult to prepare the other thyroxine-binding proteins in sufficient quantities for binding studies since they are present in very low concentrations. For example, a concentration of $2.6 \times 10^{-7} \text{M}$ (1 mg/100 ml) has been estimated for thyroxine-binding α-globulin in normal human serum by Robbins and Rall (29, 30). A rough approximation can be made of the relative binding constant for the globulin-thyroxine interaction with a knowledge of the $\Sigma n_i k_i$ value (7 × 10$^8$) for the thyroxine-albumin complex at pH 7.4 (Table II) and other factors which include: the concentration of thyroxine-binding α-globulin ($2.6 \times 10^{-7} \text{M}$); the concentration of albumin in serum ($8 \times 10^{-4} \text{M}$); and the ratio of globulin-bound-thyroxine to albumin-bound-thyroxine (3:1) observed on electrophoresis (31). A calculated value of $4.8 \times 10^8$ is derived for the relative binding constant for the globulin-thyroxine complex. This value is comparable to a binding constant of $7.9 \times 10^8$ calculated earlier by Robbins and Rall (29, 30) for the interaction of thyroxine-binding α-globulin with thyroxine.

**SUMMARY**

At pH 7.4 and 30°C, the experimental data obtained for the binding of thyroxine to human serum albumin can be accurately described by assuming the existence of two sets (or groups) of binding sites. A maximum of 2 moles of thyroxine are bound at the first set of sites ($n_1 = 2$), and a maximum of 6 moles of thyroxine are estimated to be bound at the second set of sites ($n_2 = 6$). Relatively strong binding takes place at the first group of sites, with a value of $2.75 \times 10^9 \text{M}^{-1}$ for the association constant, $k_1'$. A lower affinity for thyroxine is represented by the second group of sites, with a value of $2.5 \times 10^8 \text{M}^{-1}$ for $k_2'$.

Binding studies with acetylated and quinmated albumins indicate that positively charged ε-amino groups of lysine residues in albumin are involved in the binding of thyroxine at physiological pH.

From studies on the pH dependence of binding, it appears that the anionic phenolate group of thyroxine is a point of attachment to binding sites on albumin.

The data are consistent with the postulate that a contribution to the forces involved in thyroxine binding at pH 7.4 arises from the electrostatic interaction of the anionic phenolate group of thyroxine with cationic lysyl ε-amino groups in albumin.

**Acknowledgment**—The technical assistance of N. A. Giorgio, Jr., is gratefully acknowledged.

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Thyroxine-Protein Interactions: I. BINDING OF THYROXINE TO HUMAN SERUM ALBUMIN AND MODIFIED ALBUMINS
Milton Tabachnick


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