Thyroxine Binding by Human Scrum Albumin*

GEORGE L. TRITSCH, CHARLES E. RATHKE,| NORMA E. TRITSCH, AND CATHERINE M. WEISS

From the Roswell Park Memorial Institute, Buffalo 8, New York

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Thyroxine and triiodothyronine are transported in the blood as noncovalent complexes with certain serum proteins. This has been shown by many investigators and was the subject of an extensive recent review by Robbins and Rall (1). The binding proteins are an α-globulin (called thyroxine-binding globulin), a prealbumin, and serum albumin. The first appears to be the most significant binder of the thyroxine normally present under physiological conditions. Of these three, only albumin is presently available in a state of reasonable purity, and for this reason we have studied the binding of thyroxine by this protein. The present paper presents our findings with regard to the intensity and the optimal conditions for this interaction.

The measurements of the binding are based on the observation by Klebanoff (2) that thyroxine has a stimulatory effect on the oxidation of reduced diphosphopyridine nucleotide by hydrogen peroxide in the presence of peroxidase. This stimulatory effect is obliterated by albumin, and it will be shown that this is a quantitative measure of thyroxine binding by albumin. This is in agreement with the preliminary observation by Klebanoff that plasma proteins can inhibit the thyroxine-potentiated oxidation of diphosphopyridine nucleotide by the peroxidase system, although an ultrafiltrable inhibitor was found to be present as well.

EXPERIMENTAL PROCEDURE

Methods—The rate of oxidation of DPNH to DPN was determined by the decrease in optical density of a 1 cm depth of the reaction mixture at 340 mA in a Beckman model D spectrophotometer at room temperature. The blank contained only the buffer used in the reaction cell. Volumes of 0.1 ml or less were measured in calibrated micropipets of the Lindeström-Lang-Holter type. Thyroxine was dissolved in 0.155 M sodium hydroxide, and microliter quantities of this solution (0.010 M in thyroxine) were added to the various buffered solutions. The effect of this amount of base on the pH was negligible.

The pH was measured in a Beckman model G pH meter. Unless the effect of pH was under study, all experiments were performed at pH 7.35. Appropriate mixtures of only NaH₂PO₄ and Na₂HPO₄ were used to prepare buffers between pH 5.4 and 8.6. Phosphate was selected because it is a good buffer in the physiological range and because phosphate has been shown to cause minimal interference in the binding of small molecules by albumin (3). At pH 5.37 and below, acetate buffers were used. With the exception of those instances in which the effect of ionic strength was under investigation, the reaction mixtures were of 0.155 ionic strength, so as to approximate the ionic strength of physiological fluids. Buffer solution of ionic strength 0.233 (2.0 ml) was placed in the spectrophotometer cuvette. The other reactants, except for the minute amount of thyroxine in NaOH as noted above, were dissolved in water and added to the buffer. The final volume was brought to 3.0 ml with water to give an ionic strength of 0.155.

Protein concentration was determined by the biuret method (4), which was calibrated by means of Kjeldahl determinations. Albumin, in the presence of other proteins, was measured by the method of Bracken and Klotz (5).

Materiale—Horse-radish peroxidase (lot No. P 60B-213; 68 purpurogallin units per mg), DPNH, and the sodium salt of L-thyroxine were purchased from the Sigma Chemical Company. The human serum albumin was the crystallized Pentex product (lot No. 45F04). It was shown by electrophoresis at pH 8.6 to be free from globulins, and showed the expected heterogeneity on electrophoresis at pH 4.7. A molecular weight of 69,000 was assumed for the albumin.

The crystallized bovine serum albumin and the Cohn fractions were purchased from Pentex, Inc. The normal human plasma was obtained through the courtesy of Dr. Elias Cohen and the Roswell Park Memorial Institute blood bank. Polyvinylpyrrolidone was purchased from the Oxford Laboratories.

RESULTS AND CONCLUSIONS

Conditions for Binding—Fig. 1 describes an experiment designed to demonstrate the binding of L-thyroxine to human albumin. The addition of 0.05 μmole of L-thyroxine to the peroxidase system caused an immediate decrease in optical density at a rate of 0.320 optical density unit per minute (solid circles, Fig. 1). When 0.04 μmole of albumin was added to such a reaction mixture before all the DPNH was oxidized, (open circles, Fig. 1), the rate of reaction immediately decreased to 0.019 optical density unit per minute. This slope was identical with the one observed when the same amounts of L-thyroxine and albumin were mixed beforehand and added to the reaction mixture (solid squares, Fig. 1). These results indicate that the binding of thyroxine by albumin is instantaneous, and that the order of addition of thyroxine and albumin is of no consequence. The fact that the slopes were identical also indicated that the concentration of unbound thyroxine was the same in both cases. As will be shown below, the slope of the curve is a direct measure of the amount of unbound L-thyroxine. In the presence of
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Fig. 1. Effect of human albumin on the thyroxine-potentiated oxidation of DPNH. All reaction mixtures contained 2.0 ml of phosphate buffer, pH 7.35, ionic strength 0.233; 10 μmoles of H₂O₂ in 0.1 ml of water; and 0.40 μmole of DPNH in 0.1 ml of water and enough water (added before the peroxidase) to make the total volume 3.0 ml after the last addition. The timer was started, and readings were taken at 30-second intervals. At 2 minutes, 0.1 mg of horse-radish peroxidase in 0.1 ml of water was added to each cuvette. At 4 minutes, the following materials were added: ■, 0.05 μmole of thyroxine in 0.005 ml of 0.005 M NaOH; ■■, a mixture of 0.05 μmole of thyroxine in 0.005 ml of 0.155 M NaOH plus 0.049 μmole of albumin in 0.1 ml of water; ○, 0.05 μmole of thyroxine in 0.005 ml of 0.155 M NaOH followed by 0.049 μmole of albumin in 0.1 ml of water at 6½ minutes; □□, no further additions.

Quantitative Determination of L-Thyroxine Binding at pH 7.35

It was shown by Klebanoff that the increase of the rate of the peroxidase reaction was directly proportional to the L-thyroxine concentration. We have repeated his experiment at pH 7.35 and an ionic strength of 0.155 and have also observed a linear relationship (Fig. 2). Thus, Fig. 2B may be used as a calibration curve for the determination of the concentration of free L-thyroxine in solutions of pH 7.35 phosphate buffer, ionic strength of 0.155. The observations illustrated in Fig. 1 were therefore extended as shown in Fig. 3. Here, various amounts of human albumin were added to L-thyroxine in the assay mixture, and the decrease in the rate of the reaction was observed. The picture obtained was similar to Fig. 2A in which the effect of varying the amount of L-thyroxine alone was observed. Since the rate of the reaction is proportional to the concentration of free L-thyroxine, Fig. 2B allows the determination of the concentration of unbound L-thyroxine in each of the reaction mixtures used. Fig. 4 shows the data plotted according to the

Fig. 2. Effect of thyroxine concentration on the DPNH oxidation at pH 7.35. A, the result when different quantities (the numbers next to the curves indicate micromoles of L-thyroxine added) of thyroxine are added to the mixtures of buffer, H₂O₂, DPNH, and peroxidase indicated in Fig. 1. B, the data from A yield a linear calibration curve.

Fig. 3. Effect of variation in the amount of thyroxine and human albumin on the thyroxine-potentiated oxidation of DPNH at pH 7.35. Quantities of buffer, H₂O₂, DPNH, and peroxidase, are the same as in Fig. 1. The numbers next to the curves refer to the following mixtures of micromoles of thyroxine and albumin: 1, 0.025 + 0.0432; 2, 0.050 + 0.0432; 3, 0.050 + 0.0231; 4, 0.100 + 0.0432; 5, 0.125 + 0.0432; 6, 0.150 + 0.0432; 7, 0.175 + 0.0432; 8, 0.200 + 0.0432.
The molar concentrations of thyroxine bound at a constant initial concentration of reactants were plotted against pH, and the points obtained are shown in Fig. 5.

**l-Thyroxine Binding at pH 4.55**—From an inspection of Fig. 5 it is apparent that at the normal pH of serum (pH 7.3 to 7.4), l-thyroxine binding by human albumin is close to the maximal value that is attained at approximately pH 8. It is also evident that at pH 5 and below, only approximately half as much l-thyroxine is bound by the albumin. Thus, it was of interest to repeat the experiments illustrated in Fig. 4 at a pH below 5. The binding was therefore studied in a pH 4.55 acetate buffer of an ionic strength of 0.155, and the results were plotted (solid squares) in Fig. 4. From the results it is evident that the straight line shown in Fig. 4 accounts for the experimental data, and that $n$ equals 2 and $k$ is equal to $0.08 \times 10^6$ M$^{-1}$. This is of the same order of magnitude as $k_2$ obtained at pH 7.35 above. Since the assay system is much less sensitive to thyroxine at pH 4.55 than at pH 7.35, there is probably more error in these binding data than in the ones obtained at pH 7.35.

**Effect of Ionic Strength**—Mixtures of NaH$_2$PO$_4$ and Na$_2$HP0$_4$ of pH 7.35 but differing in ionic strength were prepared, and the ability of 0.05 pmole of human albumin to inhibit the potentiation of the DPNH oxidation by 0.1 pmole of l-thyroxine was determined at several ionic strengths (Fig. 6, solid circles). A calibration curve was constructed at each ionic strength to determine the effect of ionic strength on the ability of 0.1 pmole of L-thyroxine in the absence of albumin to potentiate the rate of DPNH oxidation (Fig. 6, open circles). From the calibration curve at the appropriate ionic strength, the percentage of the 0.1 pmole of L-thyroxine bound by the 0.05 pmole of albumin was determined. Fig. 6 indicates that L-thyroxine alone is best able to potentiate the oxidation of DPNH by the peroxidase system at low or high ionic strengths. Thus, ionic forces are of great importance in that reaction. The ability of albumin to bind L-thyroxine, with the peroxidase system as a measurement of binding, is sensitive only to low ionic strength. Although the efficacy of L-thyroxine alone to act as a catalyst was quite sensi
Since the purification of the specific thyroxine-binding globulin has not yet been achieved, it was not possible to compare the behavior of this protein with that of albumin. However, fractions of human serum proteins which have been shown to be rich in thyroxine-binding globulin (8) are available (Cohn Fraction IV-4). The ability of this fraction to bind L-thyroxine, as measured by the peroxidase assay, could be accounted for completely by the 27%, by weight, of albumin present in this fraction. Normal human plasma that was found to be 0.5 mM in albumin (3.45 g of albumin per 100 ml) behaved as though a solution of pure albumin of this concentration was used. When the results of the experiments with the plasma were plotted as in Fig. 4 (for these calculations, the plasma was treated as though it were a 0.5 mM albumin solution), the points fell on a curve essentially identical to the one in Fig. 4. Somewhat greater scattering of the data was observed with this sensitive method of plotting. Similar results were obtained with the Cohn fraction. Thus, by the use of the peroxidase assay system, thyroxine binding by the thyroxine-binding globulin which is present in plasma and in the Cohn fraction cannot be detected. The implications of this finding will be discussed below.

Human albumin, to which different amounts of L-thyroxine were added, was subjected to equilibrium dialysis with methyl orange at pH 7.35. The binding of methyl orange by the thyroxine-albumin mixtures (1 to 4 moles of L-thyroxine per mole of albumin) was identical to that of albumin without thyroxine. This indicated that L-thyroxine and methyl orange are bound at different sites and do not compete for the same sites.

Human albumin was acetylated with acetic anhydride (9) until six and one-half amino groups remained unacetylated as compared to 56 free amino groups in the native protein. These values were obtained from Van Slyke amino nitrogen determinations. The ability of this acetylated protein to bind L-thyroxine at pH 7.35 was determined, and the results were plotted (open squares) in Fig. 4. From the results it is evident that acetylation destroys some, but not all, of the thyroxine-binding sites on the albumin molecule. Acetylated albumin behaves at pH 7.35 as does the native protein at pH 4.55.

**DISCUSSION**

The objective of this study was to determine the nature of the site and the forces involved in the binding of thyroxine to serum albumin. There are many studies with similar general objectives in the literature (10), but the particular experimental approach herein employed has not been applied in published binding studies. In the present study, the concentration of bound thyroxine was not measured experimentally. It was calculated from a knowledge of the total amount of thyroxine added and the amount remaining unbound in the experiment. It was also assumed that the extent to which albumin can inhibit the thyroxine-potentiated peroxidase reaction is a measure of thyroxine binding by albumin.

Great caution must be exercised in making use of the data herein presented to explain thyroxine binding under physiological conditions: In a typical assay experiment, the total protein concentration was 0.117 g per 100 ml, and the iodine level (measured as butanol-extractable iodine) was 865 μg of I₂ per 100 ml. The former is 1/10 of the normal concentration found in serum and the latter is 200 times the normal level.

The finding that plasma behaved as a thyroxine binder, as though it contained only albumin, requires elaboration. The capacity of the thyroxine-binding globulin is, on the average, 0.2 μg of thyroxine per ml of plasma (1). In our experiments, 0.1 ml of plasma was used. The thyroxine-binding globulin in this amount of plasma can thus bind no more than 0.000025 μmole of thyroxine. This is too small an amount to be detected when 0.05 μmole of thyroxine is added, and the bulk of this is bound by the albumin. Even though it has been shown (1) that the association constant for the thyroxine-binding globulin is approximately 3000 times (7.9 × 10⁹) as great as the one obtained in this work for the strong binding site on albumin, the amount of thyroxine that can be bound by the low concentration of the thyroxine-binding globulin in plasma is too small to be detected in the presence of the large amount of albumin by the assay employed. Therefore, no conclusions can be drawn from the data presented as to the manner in which the thyroxine-binding globulin binds thyroxine.

The study of the pH dependence of thyroxine binding indicates that some of the binding sites are affected by the hydrogen ion concentration, and that the amount of thyroxine bound by a given quantity of albumin increases as the pH changes from 6 to 8. Thus, the removal of protons favors thyroxine binding.

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However, binding still occurs below pH 5. Fig. 4 shows that at pH 4.55 1 molecule of albumin can bind 2 thyroxine molecules. At pH 7.35, a total of 6 thyroxine molecules can be bound: The first one is held relatively firmly, and the remaining 5 are held with forces comparable to the ones that are operative at pH 4.55. This suggests that the assumptions made to calculate the curved line in Fig. 4 do not explain the total picture. The sites that function at pH 4.55 presumably also function at pH 7.35. Five sites with a $k_z$ of $0.06 \times 10^6 \text{M}^{-1}$ were assumed in the calculation of the curve in Fig. 4. There must therefore be three sites with a similar $k$ that are not functional at pH 4.55. This presents a possible picture at pH 7.35 of one strong pH-dependent site, three pH-dependent weak sites, and two pH-independent weak sites. All these sites would have to be taken into account to calculate a theoretical curve to explain the variation of $r$ with pH. This would involve the fitting of so many parameters that the results would not not convincing. Therefore, no attempt was made to calculate a theoretical curve to account for the data presented in Fig. 5, and, as a result, no conclusions are being drawn from the experimental data as to what chemical groups are involved in the binding process. The fact that acetylation destroys the single strong pH-dependent binding site (Fig. 4) eliminates imidazole groups as being part of this binding site since acetyl imidazole compounds are rapidly hydrolyzed (11) even in neutral solution.

**SUMMARY**

The ability of human serum albumin to bind thyroxine has been measured by determining the inhibition by albumin of the thyroxine-accelerated oxidation of reduced diphosphopyridine nucleotide by hydrogen peroxide in the presence of horse-radish peroxidase. A maximum of approximately 6 molecules of thyroxine can be bound per molecule of albumin. The data presented suggest three different kinds of thyroxine binding sites on the albumin molecule.

The first site holds 1 thyroxine molecule approximately 40 times as tightly as the other sites bind the remainder. The data may be interpreted to indicate a strong pH-dependent binding site ($k = 2.5 \times 10^6 \text{M}^{-1}$), three pH-dependent weak sites, and two pH-independent weak sites. A study of the pH dependence of the binding revealed that binding decreases but is not abolished as the pH is lowered from 7.35 to 5. Studies with acetylated albumin eliminated the imidazole group as the ionizable group at the pH-dependent strong site.

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