The Binding of Thyroxine by Serum Albumin as Measured by Fluorescence Quenching*

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SUMMARY

The binding of thyroxine by bovine and human serum albumin has been studied by measurements of the quenching of the ultraviolet fluorescence of the protein. The results obtained by this method have been compared with those of equilibrium dialysis measurements. Both approaches agree in assigning to serum albumin a single binding site of high affinity and three or more weak sites. The binding constant for the combination of albumin with a single molecule of thyroxine is 1.6 x 10^6 ± 25% at pH 7.4. At pH 7.4 the binding constant for triiodothyronine is about one-sixth that for thyroxine.

Three serum proteins bind thyroxine and presumably assist in its transport within the body (1). The binding proteins are an α-globulin (thyroxine binding globulin), a prealbumin (thyroxine-binding prealbumin), and serum albumin. Serum albumin appears to be of minor importance in the physiological transport of thyroxine, since its affinity for thyroxine is much lower than that of the other two proteins. However, it is the only one of the three readily available in a state of sufficient purity for quantitative physical studies of the interaction.

The binding of thyroxine by human and bovine serum albumin has been the subject of several recent investigations, which diverge somewhat in their conclusions (2-4). The experimental approaches were of two kinds. Sterling and Tabachnik (2) and Sterling, Rosen, and Tabachnik (4) utilized equilibrium dialysis with the use of thyroxine labeled with trace amounts of 131I. The binding data, which were analyzed according to the method of Scatchard (5), were consistent with the presence of four equivalent sites, each with an intrinsic binding constant of 1.0 x 10^6 and five or more weak sites (3).

The present investigation introduces an alternative technique and partially resolves these discrepancies. Human and bovine serum albumin contain 1 and 2 tryptophan residues, respectively. The wave length of maximum fluorescence intensity of tryptophan (340 mp) overlaps extensively with the absorption band of thyroxine (λmax 325 mp). According to the theory of Förster, tryptophan fluorescence should be quenched by a radiationless exchange mechanism and should provide a means for monitoring the binding of thyroxine (8). A similar method was used by Velick, Parker, and Eisen in their studies of hapten-antibody interactions (10).

EXPERIMENTAL PROCEDURE

Fluorescence Measurements—The ultraviolet fluorescence intensity was measured with an Aminco spectrofluorometer equipped with an Osram ultraviolet source. Temperature was maintained at 26 ± 0.2°C by the use of a hollow cell holder through which water from a constant temperature bath was circulated. The wave lengths of activation and emission of fluorescence were 290 mp and 350 mp, respectively.

Fluctuations in lamp intensity and photomultiplier response necessitated the use of a control solution with which measurements of fluorescence intensity at varying thyroxine levels could be compared. In a typical experiment, 2 ml of an albumin solution were placed in each of two 1-cm² quartz cuvettes. Thyroxine was added to one of these with continuous magnetic stirring. The other solution, to which no thyroxine was added, served as the control. Rapid alternate measurements of emitted intensity were made upon the solution and control. Five determinations were made for each thyroxine level.

The observed relative intensities were corrected for dilution of the albumin and for attenuation of the exciting and emitted beams due to absorption by thyroxine. The latter correction was made by multiplying the uncorrected relative intensities by the antilog of the average of the excess optical densities due to thyroxine at 290 mp and 350 mp. The correction usually ranged up to 5%.
Equilibrium Dialysis (11)—The method employed was similar to that of Sterling and Tabachnik (2) and Sterling et al. (4), with several modifications. To eliminate a nondialyzable contaminant that preliminary experiments suggested to be present, thyroxine-131I was dialyzed against buffer for 24 hours at 3°C. The material that passed through the Visking membrane was added to nonradioactive thyroxine, and the mixture was used for binding experiments. The relationship between counts per min and total thyroxine concentration was established by parallel determinations of radioactivity and optical density at 325 mμ. A molar extinction coefficient of 6.18 × 10^4 was used for thyroxine at pH 11 (12).

In the binding measurements, 2-ml portions of 0.1% albumin, placed within Visking tubing, were dialyzed, with shaking, against 10 or 15 ml of buffer (0.1 M phosphate-3 × 10^{-4} M ethylenediaminetetraacetate, pH 7.40). The results reported here were obtained at room temperature (24 ± 2°C). The ethylenediaminetetraacetate was added in accordance with a previous suggestion (2, 4). In each case, two parallel sets of measurements were made in which the added thyroxine was placed initially inside or outside the sac. After 48 hours of dialysis, the sacs were opened and the radioactivities of the inside and outside solutions determined with a well-type γ-scintillation counter. From the known relationship between radioactivity and thyroxine concentration, the latter quantity was computed for both solutions.

In a preliminary experiment, the equilibration of labeled thyroxine in the absence of albumin was examined under otherwise similar conditions. Two levels of thyroxine were used which spanned the range of concentrations employed in a typical equilibrium dialysis experiment. After 48 hours the radioactivity was the same (within 5%) for inside and outside solutions, whether the thyroxine had been placed initially inside or outside the sac. This result excluded any intrinsic barriers to the passage of thyroxine through the membrane.

Correction for the iodide contaminant in the radioactive thyroxine is required, particularly when most of the radioactive thyroxine is bound to albumin. Aliquots (approximately 0.1 ml) of each inside and outside solution were analyzed by paper electrophoresis, in 0.1 M barbital, pH 8.6. Under these conditions thyroxine moves relatively slowly compared to iodide, so that there is little difficulty in separation. After completion of electrophoresis, the paper strips were dried and the iodide bands were located by staining with palladium chloride. To raise the iodide content to a level sufficient to permit visible staining, nonradioactive iodide was added to each solution prior to electrophoresis. Two segments of each dried paper strip were cut out and analyzed for radioactivity. One of these corresponded to the iodide band and the other, which contained the thyroxine, corresponded to the part of the strip lying between the iodide band and the origin. The ratio of the counts arising from the thyroxine band to the sum of the two was taken as the fraction (fT) of the over-all radioactivity originating from the thyroxine. The radioactivity of each inside or outside solution was multiplied by fT and the corrected radioactivity (in counts per min) was converted to thyroxine concentration. The addition of carrier iodide and precipitation as PdI minimized any loss of iodide during drying.

The procedure used for equilibrium dialysis experiments with triiodothyronine was entirely analogous to that used for thyroxine. The concentration of stock solutions was computed from the absorbance at 320 mμ, assuming a molar absorbance of 4.66 × 10^4 at pH 10 (13).

Ionization of Thyroxine and Triiodothyronine—The pH dependence of the ionization of thyroxine and triiodothyronine in the presence of excess albumin was determined from the absorbance at 325 and at 320 mμ, respectively. To correct for any increase in absorbance arising from the ionization of albumin thyroxines, a control solution containing albumin alone was titrated to a series of pH values between 4 and 10 and the (interpolated) absorbance at a given pH was subtracted from those of the solutions containing thyroxine or triiodothyronine. Absorbances were measured with a Beckman DU spectrophotometer.

Materials—Crystalline bovine serum albumin was purchased from Armour. Most of the determinations upon human serum albumin were made upon a fat-free preparation obtained from Dr. D. S. Goodman. A second preparation of crystalline human serum albumin was purchased from Pentex and used for several determinations. All albumin samples were examined for the presence of thyroxine-binding globulin and thyroxine-binding prealbumin (1) by reverse flow electrophoresis after addition of radioactive thyroxine to a 4% solution of albumin. No thyroxine-binding globulin or thyroxine-binding prealbumin was detected by autoradiography. Assuming that 1% of the total radioactivity could be visualized in the thyroxine-binding globulin and thyroxine-binding prealbumin areas of the electrophoresis patterns, it is estimated that this procedure could detect such contaminants in the amount of about 1 part in 5 million parts of albumin, or less than 1:1000 of their concentrations in serum. These estimates are probably correct within an order of magnitude.

Nonradioactive sodium L-thyroxine was purchased from Glaxo, Ltd., and was recrystallized from 0.5% aqueous Na2CO3 to yield the pentahydrate. Paper chromatography revealed a single ninhydrin-positive spot.

Sodium L-triiodothyronine, purchased from Calbiochem, showed thyroxine at a concentration about one-tenth that of triiodothyronine (based on visual inspection of paper chromatograms stained with ninhydrin). The original triiodothyronine was mixed with a tracer quantity of thyroxine-131I and purified by column chromatography according to the procedure of C. Lewallen.¹ The purified triiodothyronine, used for the experiments reported here, contained less than 2% of the original radioactivity. Of the residual radioactivity, half migrated with the triiodothyronine component on paper chromatography in butanol-dioxane-ammonia and presumably represented triiodothyronine-131I contamination of thyroxine-131I. Thus, the thyroxine content of the purified triiodothyronine preparation was probably less than 0.1%.

Radioactive thyroxine and triiodothyronine (20 mC per mg in 50% propylene glycol) were purchased from Abbott Laboratories.

Visking tubing (3 inch) was boiled briefly in three changes of glass-redistilled water prior to use to eliminate the leaching during dialysis of ultraviolet-absorbing material.

Theory—The curves representing the dependence of fluorescence quenching upon the molarity of added thyroxine may be analyzed to yield binding constants by a method anal-

¹To be published.
ogous to that of Velick, Parker, and Eisen (10). For this purpose it was necessary to relate the observed degrees of quenching directly to the number of moles of thyroxine bound per albumin molecule (r). This was accomplished by determining the curve of relative intensity against the thyroxine to albumin ratio for concentrations of albumin sufficiently high so that essentially all thyroxine was bound (see under “Results”).

The observed relative intensity (I) is given by

\[ I = y_0 I_0 + y_1 I_1 + \sum_{j>1} y_j I_j \]  

(1)

where \( y_0 \) is the mole fraction of albumin which has no bound thyroxine; \( I_0 \) is the relative fluorescence intensity corresponding to such uncombined albumin; \( y_1 \) and \( I_1 \) are the corresponding quantities for the species with 1 mole of bound thyroxine; and \( y_j \) and \( I_j \) are the analogous parameters for the species with \( j \) moles of bound thyroxine, where \( j \) is equal to, or greater than, 2.

It is convenient to introduce the reduced quantities \( i_1 \) and \( i_j \), which are defined by

\[ i_1 = \frac{I_1}{I_0}; \quad i_j = \frac{I_j}{I_0} \]  

(2)

Equation 1 may be rewritten

\[ i = y_0 + y_1 i_1 + \sum_{j>1} y_j i_j \]  

(3)

If \( \bar{i} \) is defined as the average value of \( i \) for the higher species \((j > 1)\),

\[ \bar{i} = \frac{\sum y_j i_j}{\sum y_j} - \frac{\sum y_j i_j}{(1 - y_0 - y_1)} \]  

(4)

then

\[ i = y_0 + y_1 i_1 + (1 - y_0 - y_1) \bar{i} \]  

(5)

Defining \( r \) as the average number of thyroxines bound per albumin, we also have

\[ r = y_1 + r(1 - y_0 - y_1) \]  

(6)

where \( \bar{r} \) is the average value of \( r \) for the higher species and is defined by

\[ \bar{r} = \frac{\sum r_j y_j}{\sum r_j y_j} - \frac{\sum r_j y_j}{(1 - y_0 - y_1)} \]  

(7)

Combining Equations 5 and 6 and solving for \( y_0 \),

\[ y_0 = \frac{\bar{i}(1 - r) + i_1(r - r) + \bar{r}(r - 1)}{1 - \bar{i} - \bar{r} + r_0} \]  

(8)

If the over-all equilibrium constants corresponding to the processes

\[ A + T \rightleftharpoons AT \]
\[ AT + T \rightleftharpoons AT_2 \]
\[ AT_{j-1} + T \rightleftharpoons AT_j \]  

(9)

are defined by

\[ K_1 = \frac{[AT]}{[A][T]} \]
\[ K_2 = \frac{[AT_2]}{[AT][T]} \]
\[ \vdots \]
\[ K_j = \frac{[AT_j]}{[AT_{j-1}][T]} \]  

(10)

where \([A], [T], [AT]\), etc., are the molar concentrations of free albumin, free thyroxine, and the 1:1 complex, then

\[ y_0 = \frac{[A] + [AT] + [AT_2] + \ldots}{[A] + [AT][T] + K_1 [A][T]^2 + \ldots} \]  

(11)

and

\[ \frac{1}{y_0} = 1 + K_1[T] + K_2 [AT]^2 + \ldots \]

Thus \( K_1 \) is obtainable from the initial slope of the curve of \( 1/y_0 \) with respect to \([T]\) and \( K_2 \) is obtainable from the initial slope of \([1/y_0] - 1 - K_1[T]/[T]\) with respect to \([T]\).

The quantity \( i_1 \) may be determined from the initial slope of the curve of \( i \) as a function of \( r \). From Equations 3, 5, 10, and 11 we have

\[ i = (1 + i_1 K_1 \cdots K_j[T]^j)/(1 + K_1 \cdots K_j[T]^j) \]  

(12)

The quantity \( i \) may be expanded in a MacLaurin series in terms of \( r \):

\[ i = 1 - (I - i_1) r + \ldots \]  

(13)

The limiting slope of \( i \) as a function of \( r \) is given by

\[ \left( \frac{di}{dr} \right)_{r=0} = i_1 \]  

(14)

The accuracy of the estimation of \( r \) by this means is of course dependent upon the curvature of the plot of \( i \) with respect to \( r \).

**Equilibrium Dialysis**—The equilibrium dialysis data were analyzed according to the equation of Scatchard in its most general form (5, 10).

\[ \frac{r_j}{[T]} = n_j k_j - n_j k_j \]  

(15)

where \( n_j \) denotes the number of sites of type \( j \), each of which corresponds to a microscopic binding constant \( k_j \), and \( r_j \) is the number of moles of thyroxine bound by sites of this class per mole of albumin. Each \( k_j \) is the equilibrium constant for binding by a single site of type \( j \). The \( k_j \) values are related to the constant defined in the preceding section by

\[ \sum n_j k_j = K_1 \]  

(16)

If the sites are all equivalent, Equation 15 may be written in simplified form in terms of a single intrinsic binding constant \( k \) (10).

\[ \frac{r}{[T]} = nk - nk \]  

(17)

In practice, linearity of \( r/[T] \) as a function of \( r \) may be regarded as evidence for a single class of binding sites. If pronounced curvature is present, the data may be fitted by an appropriate choice of constants with Equation 15.
FIG. 1 (upper left). Relative fluorescence intensity of bovine serum albumin as a function of the mole ratio of thyroxine to albumin (0.1 M PO₄, pH 7.4, 3 × 10⁻⁴ M ethylenediaminetetraacetate). ○, 2.2 g per liter of albumin; ●, 3.5 g per liter; △, 0.073 g per liter; □, 0.52 g per liter.

FIG. 2 (upper right). Relative fluorescence intensity of human serum albumin as a function of the mole ratio of thyroxine to albumin. The other conditions are the same as for Fig. 1. ○, 3.0 g per liter of albumin; ●, 2.0 g per liter; △, 0.099 g per liter.

FIG. 3 (lower left). The relative fluorescence intensity of bovine serum albumin (0.1 g per liter) as a function of the molarity of free thyroxine, computed from Equation 18. The other conditions are the same as for Fig. 1.

FIG. 4 (lower right). The data of Fig. 3 plotted according to Equation 11 with a line drawn through the points by inspection. For comparison, two theoretical curves are included for four equivalent sites with two different values of $K_i$. By "equivalent" it is meant that the intrinsic binding constants ($k_1, \ldots, k_i$) of the four sites are identical. However, the constants $K_1, \ldots, K_4$, as defined by Equation 10, are given by $K_1 = 4k$; $K_2 = \frac{1}{2}k$; $K_3 = \frac{3}{2}k$; $K_4 = \frac{1}{4}k$ (11), where $k$ is the intrinsic constant for each site.
RESULTS

Fluorescence—The stepwise addition of thyroxine to bovine or human serum albumin in 0.1 M [PO]7+ pH 7.4, resulted in a progressive quenching of the ultraviolet fluorescence at 350 nm. The fluorescence of γ-globulin, a protein which does not bind thyroxine, is not quenched by thyroxine added under similar conditions (14). The residual intensity of albumin fluorescence approached at very high thyroxine levels (Fig. 1) did not, however, correspond to quantitative quenching. The limiting extents of quenching were 85% and 90% for bovine and human serum albumin, respectively.

At concentrations of albumin of 2 g per liter or greater, the curves of relative intensity of fluorescence as a function of thyroxine to albumin ratios ([T]:[A]) were superimposable for values of [T]:[A] less than 1.0 and were independent of albumin concentration (Figs. 1 and 2). For the purposes of data analysis it was assumed that the binding of thyroxine was quantitative in this range of high albumin concentration and that the mole ratio of total thyroxine to albumin was equal to the average number of moles of thyroxine bound (r) per mole of albumin. In this manner, working curves of r as a function of relative intensity were obtained for human and bovine serum albumin. From these working curves r could be determined from the relative intensity (I/I0 or i) for a thyroxine-albumin mixture of any composition.

For the determination of binding constants it was necessary to work at much lower albumin concentrations (0.05 to 0.1 g per liter) in order to obtain an appreciable degree of dissociation (Fig. 3). The quantity r was obtained as a function of [T]. Measurements were not extended to values of r greater than 0.8, as the presence of curvature in the working curves at higher values of r renders the value of r very sensitive to slight errors in i.

The mole fraction of uncombined albumin, y0, was computed from Equation 8. In the case of human serum albumin, i, and...
FIG. 8 (upper left). Equilibrium dialysis data for human serum albumin, plotted according to the equation of Scatchard. Data are combined for three separate runs. A theoretical curve is included for the case of one strong and three weak sites. It is computed from Equation 15 with the use of the cited values of intrinsic constants. The solvent is the same as for Fig. 1.

FIG. 9 (upper right). Equilibrium dialysis data for bovine serum albumin, plotted according to the equation of Scatchard. Two theoretical curves are included for comparison. The solvent is the same as for Fig. 1.

FIG. 10 (lower left). Values of $K_1$ as a function of pH for thyroxine and triiodothyronine, as determined by equilibrium dialysis at very low concentrations of adsorbate. The buffer is 0.1 M phosphate. The concentration of human serum albumin is 0.15 g per liter. The molarities of thyroxine and triiodothyronine are $10^{-2}$. $\bigcirc$, thyroxine; $\square$, triiodothyronine. Data from three runs are combined in each case.

FIG. 11 (lower right). Ionization of thyroxine and triiodothyronine in the presence of a large excess of bovine serum albumin, as monitored by the absorbance at 325 nm for thyroxine and at 320 nm for triiodothyronine. The solvent is 0.1 M phosphate. The absorbances are corrected for dilution and for absorption by albumin. $\bigcirc$, 0.83% bovine serum albumin, $6.3 \times 10^{-5}$ M thyroxine; $\bullet$, 0.83% bovine serum albumin, $3.1 \times 10^{-5}$ M triiodothyronine.
are almost identical ($i_1 = 0.07; i_2 = 0.08$) and Equation 8 may, with negligible error, be replaced by

$$y_0 = \frac{i - i_1}{1 - i_1}$$  \hspace{1cm} \text{(19)}$$

In the case of bovine serum albumin, $i_1 = 0.27$ and $i_2 = 0.15$. If $r$ is taken as 2.0 for values of $r$ of 0.8 or less, the difference between $y_0$ as computed from Equation 8 and Equation 19 is less than 10% for $r = 0.8$ and is negligible for $r < 0.5$.

Values of $1/y_0$ were plotted as a function of $[T]$, and $K_1$ and $K_2$ were estimated according to Equation 11. For both human and bovine albumin, $1/y_0$ was linear with respect to $[T]$ up to $r = 0.8$ (Fig. 4). Thus, $K_2$ was too small to be determined accurately by this method. The upper limit of its magnitude is about one-fifth that of $K_1$. The value of $K_1$ was $1.6 \times 10^6 \pm 25\%$ for both human and bovine albumin.

The low magnitude of $K_1$ in comparison with $K_2$ is only explicable if a single strong binding site is present with a value of $k_1$ close to $1.6 \times 10^6$. Other sites present must be of much lower affinity.

Fluorescence quenching was also utilized to estimate the value of $K_1$ for the human serum albumin triiodothyronine system. The procedure was entirely analogous to that employed in the case of thyroxine. A working curve of $r$ as a function of relative intensity was obtained with the use of a 1% albumin concentration (Fig. 3). This empirical relationship was used to compute $y_0$ and $[T]$ from quenching data at an albumin concentration of 0.1 g per liter (Fig. 4). A value of $K_1$ of $3.8 \times 10^6 \pm 30\%$ was obtained at pH 8.6 (average of three runs) with Equations 18 and 19.

The pH dependence of fluorescence quenching was also examined. The intensities of fluorescence of separate aliquots of human serum albumin that had been titrated to a series of pH values were determined in the presence and absence of a fixed concentration of thyroxine or triiodothyronine. The relative intensities are shown in Fig. 7 as a function of pH. A major decrease in quenching by thyroxine occurs with decreasing pH between pH 7 and pH 5, in harmony with the drop in association constant in this range (see below). In the case of triiodothyronine, the fall in quenching occurs at a higher pH (between pH 8 and pH 7) (Fig. 8). The pH variation of thyroxine is centered about the pK of its phenolic group, which is 6.2 (12), and probably reflects the decrease in binding constant accompanying the titration of the phenolic hydroxyl. In the case of triiodothyronine, the data are consistent with its pK of 8.5 (13).

Equilibrium Dialysis—Curves of $r/[T]$ as a function of $r$ showed a pronounced curvature convex to the abscissa for both human and bovine albumin. It is impossible to fit curves of this form by assuming a single class of sites. They could, however, be fitted on the assumption of a single strong site and three weak sites (Figs. 8 and 9).

For human albumin $K_1$ was $1.6 \times 10^6 \pm 25\%$, in good agreement with the figure from fluorescence quenching. The complete curve could be fitted adequately on the basis of a single strong site of $k_1 = 1.3 \times 10^6$ and three weak sites with $k_2 = 0.06 \times 10^6 \pm 50\%$. The wide error for $k_2$ stems from the severe scatter of the data (Fig. 8).

In the case of bovine serum albumin (Fig. 9), the value of $K_1$ ($2.0 \times 10^6 \pm 25\%$) obtained was distinctly higher than that found by the fluorescence technique. The values of $k_1$ and $k_2$ were $1.9 \times 10^6 \pm 25\%$ and $0.06 \times 10^6 \pm 50\%$, respectively.

Because of the limited range of the accessible values of $r$, the figure of three for the number of weak sites is essentially a lower limit. The existence of other sites of still lower binding affinity could not be ruled out.

In one set of experiments the equilibrium dialysis of $\text{I}^3$ labeled thyroxine and triiodothyronine with human albumin (0.1 g per liter) was examined at very low concentrations ($10^{-9}$ M) of the former. Under these conditions only a very small mole fraction (less than $10^{-3}$) of the albumin can combine with adsorbate, so that it becomes feasible to ignore the contribution of higher species. Moreover, the concentration of free albumin may be effectively replaced, for computational purposes, by the total albumin concentration ($[A]$) to which it is very nearly equivalent. Thus

$$K_1 = \frac{[A]_T}{[A]_f} \cong \frac{1}{[A]_0} \frac{[T}_{bound]}{[T]_{free}}$$

The ratio $[T]_{bound}/[T]_{free}$ was determined in the usual manner from measurements of radioactivity inside and outside the sac. This approach provides a means for the direct determination of $K_1$, thereby complementing the extrapolation method. The values obtained at a series of pH values are shown in Fig. 10.

As previously noted (2, 4) the apparent binding of thyroxine was greater for the series in which thyroxine was initially placed within the sac than for those in which it was placed outside. The mean values of $K_1$ (Fig. 10) are reasonably consistent with those obtained by direct extrapolation at pH 7.4. The binding constant shows a major drop in magnitude between pH 8 and pH 6, but remains large even at pH 5.5.

The binding constants for triiodothyronine are smaller than those for thyroxine by a factor of 2 to 4 at pH 8.6 and by a factor of 6 at pH 7.4. Also the zone of pH variation for triiodothyronine is displaced to higher values of pH.

Ionization of Bound Thyroxine and Triiodothyronine—The ionization of the phenolic hydroxyls of thyroxine and triiodothyronine results in a shift in absorption spectra to longer wave lengths, with the development of new maxima at 325 m$\mu$ and 322 m$\mu$, respectively (12, 13).

Fig. 11 shows the pH dependence of absorbance at 325 m$\mu$ for thyroxine, and at 320 m$\mu$ for triiodothyronine, in the presence of a very large excess of human serum albumin, so that binding is essentially complete at all pH values. The midpoint of the ionization profile of bound thyroxine is pH 5.8. Thus the pK of albumin-bound thyroxine is significantly more acidic than that of free thyroxine (pK 6.2). In the case of triiodothyronine, the limiting absorbance at alkaline pH is difficult to determine precisely because of an important contribution from the ionized tyrosines of serum albumin at pH values above 9.5. However, the apparent midpoint for bound triiodothyronine is in the pH range 8.5 to 8.8, which is close to the pK of 8.5 of free triiodothyronine (13). The acid displacement of the pK of bound thyroxine is consistent with a preferential binding of the ionized form. It is apparent, however, that the nonionized form is also bound, since substantial binding of thyroxine is present at low pH values where thyroxine is not ionized (Figs. 8 and 10).

**DISCUSSION**

The principal conclusion of this investigation is that the thyroxine-binding sites of bovine and human serum albumin are of at least two kinds. The data are best accounted for in terms
of a single site of high affinity and three, or possibly more, weaker sites.

While this general picture is in accord with that developed by Tritsch et al. (3), some quantitative differences exist. The figure obtained in this study for the binding constant of the strong site is somewhat smaller. A smaller estimate for the number of weak sites was also found in the present study, but this estimate should be regarded as essentially a lower limit.

While the values of $K_1$ obtained by fluorescence quenching and by equilibrium dialysis are in good agreement for human serum albumin, an appreciable difference occurs in the case of bovine serum albumin. No obvious explanation can be presented for this internal discrepancy.

The presence of a small amount of thyroxine-binding globulin or prealbumin would influence the equilibrium dialysis results more than the fluorescence quenching. The latter would be affected only if the quantity of contaminant was sufficient to distort the shape of the Scatchard plot appreciably. However, at the lowest levels of free thyroxine to which the equilibrium dialysis studies were extended, the presence of a very small amount (0.1 to 0.5%) of an impurity possessing high affinity for thyroxine could distort the shape of the Scatchard plot appreciably and result in an erroneously high value of $K_1$. A contamination of this degree can be ruled out since no thyroxine-binding component other than albumin was detected by electrophoresis.

The utility of the fluorescence quenching method in the study of the binding of thyroxine by protein carriers seems well established. Its advantages are particularly evident in the determination of $K_1$ by measurements at low levels of thyroxine. Fluorescence measurements require much less time than equilibrium dialysis and a complete determination can be made with only about 5 mg of protein. The precision is comparable to that obtainable by equilibrium dialysis. The fluorescence approach may well prove to be the method of choice, particularly when the binding protein is available in limited quantities.

The quantitative differences between the results of this investigation and those of the two earlier studies require explanation. An inspection of the data of the two groups reveals that the divergence between them arises primarily at low levels of thyroxine, where the number of moles bound per mole of albumin is less than 1. The curves of Sterling and Tabachnik and Sterling et al. show very few points in this range, and the authors mention that considerable difficulty exists in obtaining reproducible data at low thyroxine levels (2, 4). By correcting for contaminants in labeled thyroxine, we were able to use the equilibrium dialysis method at low thyroxine levels. The data of Tritsch et al. include a large number of points corresponding to less than 2 moles of thyroxine bound per albumin (3). These points produce a pronounced upward curvature of the Scatchard plot. Similar behavior was observed in the present investigation. For values of $r$ greater than 1 the equilibrium dialysis data presented here do not differ greatly from those of Sterling and Tabachnik (2) and Sterling et al. (4) under comparable conditions.

The reason for the minor quantitative difference between the value of $K_1$ found in this study and that reported by Tritsch et al. (3) is uncertain. The assay system of the latter group is rather complicated and the potential sources of error are numerous. However, it is not possible at present to pinpoint the probable origin of the difference. In any event the general conclusions reached in the two investigations are similar.

Free thyroxine, in human plasma, experimentally determined at 37°, is in the range of 3 to 6 $\times 10^{-11}$ m (15, 16). If 5 to 20% of total thyroxine in plasma is bound to albumin, the $K_1 = 1.6 \times 10^6$ for albumin at 26° yields a calculated free thyroxine of 0.5 to 2.0 $\times 10^{-11}$ m. This difference cannot be accounted for by the effect of free fatty acids in plasma (17), but may, at least in part, be due to temperature dependence of protein-thyroxine interactions (4).

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