EQUILIBRIUM DIALYSIS STUDIES OF THE BINDING OF THYROXINE
BY HUMAN SERUM ALBUMIN *

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In previous work from this laboratory (1) studies on equilibrium dialysis were carried out to
determine the binding constants for the interaction of human serum albumin with thyroxine
and its analogs. The classical technique of equilibriu m dialysis as described by Scatchard and
others (2–6) was employed.

At present it seems clear that thyroxine in hu-
man serum migrates to at least three different sites
during electrophoresis at pH 8.6 (7–10). The
three sites most frequently reported have been the
thyroxine-binding α-globulin (TBG), albumin,
and the “prealbumin” region. The thyroxine-
binding α-globulin has generally been considered
of major physiological importance, the others be-
ing regarded as secondary carriers. Albumin is
the only one of the thyroxine carriers of serum
readily available in pure form and with a known
molecular weight.

The findings of the previous work (1) ap-
peared compatible with the hypothesis that the
anionic phenolate groups of thyroxine and triiodo-
thyronine interact with free cationic groups on the
protein molecule. In the present report the ob-
servations have been extended to studies with
variations in temperature and pH, as well as to
experiments with mercaptalbumin, isoctane-ex-
tracted albumin, and acetylated albumin prepara-
tions. The present findings provide further sup-
port for the concept of an interaction of the
anionic phenolate groups of thyroxine and four
cationic groups on the protein molecule. More-

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over, the evidence suggests that the four primary
binding sites may be ε-amino groups of lysine resi-
dues. The binding constants for the albumin-thy-
roxine interaction have been employed to calcu-
late the theoretical free thyroxine concentration in
human serum.

MATERIALS AND METHODS

Most of the albumin used was supplied by the Ameri-
can Red Cross; these amorphous albumin solutions had
been stabilized by the addition of 0.02 M sodium caprylate
and 0.02 M sodium acetyltryptophanate. Consequently,
all Red Cross albumin preparations were dialedyzed against
several changes of isonic saline solution and then de-
ionized by passage through Amberlite MB-1 resin, ac-

genric to the method of Dintzis (11). Such treatment
has produced isionic albumin, freed of added stabilizers
(11). Bound fatty acids (12) may be removed to an
extent of approximately 1 mole of fatty acid remaining
per mole of albumin (6, 13) after passage through the
mixed-bed resin.

Upon electrophoretic analysis the deionized amorphous
albumin preparations were found to be 98 per cent pure.
Addition of minute tracer amounts of 14C-labeled thyrox-
ine, followed by electrophoresis at pH 8.6 in Tris-maleate
buffer, demonstrated that the albumin solutions were free
of detectable thyroxine-binding α-globulin or prealbumin.

Crystalline human mercaptalbumin, which had been
passed through the mixed-bed resin, Amberlite MB-1, was
supplied as a dry powder by the Protein Foundation,
Jamaica Plain, Mass., though the courtesy of Drs. J. L.
Onclay and R. B. Pennell.

For studies of albumin free of bound fatty acid, the
amorphous Red Cross albumin, which had been deionized,
was subjected to isoctane extraction by the method of
Goodman (13).

For studies of the mechanism of binding, acetylated
human serum albumin preparations were made. Acetyla-
tion was carried out after the procedure of Fraenkel-
Conrat, Bean and Lineweaver (14). The acetic anhy-
dride was added gradually over a 60-minute period to a
5 per cent protein solution in half-saturated sodium ace-
tate, the reaction mixture being held in an ice bath. The
acetylated protein was precipitated at pH 4.0 and col-
lected by centrifugation (15), then dialyzed against three
successive daily changes of deionized water at 4°C. The degree of acetylation was controlled by the amount of acetic anhydride added to each protein solution. The extent of acetylation was subsequently measured by the ninhydrin reaction.

All protein solutions were stored frozen. Repeated freezing and thawing had no detectable effect on the thyroxine-albumin interaction. The protein concentrations were measured by the biuret reaction as described by Gornall, Bardawill and David (16), employing as standards protein solutions prepared from weighed amorphous proteins, or checked by micro-Kjeldahl determinations, or both. Protein concentrations were also determined spectrophotometrically at 280 µM, with an extinction coefficient of 5.3 for a 1 per cent solution of human serum albumin (17).

I131-labeled L-thyroxine was obtained from E. R. Squibb and Co. or from Abbott Laboratories. Nonradioactive L-thyroxine was purchased from Mann Research Co., New York, N. Y. The radioactive tracer was added to solutions of carrier thyroxine which had been diluted from a stock solution prepared by dissolving a weighed amount of the material. The tracer, in the presence of adequate amounts of carrier, was stable at 4°C for 3 weeks or more upon chromatographic analysis, whereas in dilute solution the carrier-free tracer underwent decomposition.

Each radioactive shipment was checked for purity by paper chromatography on duplicate 56-cm strips of Whatman 3MM paper, previously prepared with application of nonradioactive carriers at the origin. Descending paper chromatography was carried out with a butanol: dioxane: ammonia system (4:1:5, vol/vol) for 16 hours; the dried paper strips were then scanned with a Nuclear-Chicago Actigraph IIIB, model C-100 B. After scanning, the position of carriers was obtained by color development upon spraying with palladium chloride for iodide and 4-aminoantipyrene for thyroxine and analogs (18). In the studies illustrated, no radioactive peaks other than thyroxine were observed, except for small traces of inorganic iodide (less than 2 per cent). In a few instances shipments with iodide contamination in excess of 2 per cent were employed, with appropriate corrections, based on the observation that binding of iodide by albumin was small in comparison with thyroxine binding. Paper chromatography repeated after dialysis revealed no alteration of thyroxine under the experimental conditions.

The following buffers were employed for dialysis: potassium phosphate, pH 7.0 and pH 7.4, 0.15 ionic strength; Tris-sodium chloride, pH 7.4 and pH 8.6 (0.05 M Tris-0.1 M NaCl); sodium borate, pH 8.6, 0.15 ionic strength; sodium glycinate, pH 9.8, 0.2 M glycinate; potassium phosphate, pH 10.5, 10.9, and 11.2 with 0.15 ionic strength.

The phosphate buffers were isotonic (ionic strength, 0.15) and the Tris-sodium chloride buffers were very close to this ionic strength. The sodium glycinate buffer was somewhat hypertonic.

Equilibrium dialysis procedure. Dialysis bags were made from Visking sausage casing (30/32 inch). The dialysis membranes were specially cleaned by soaking them first in 0.1 M nitric acid for 16 to 24 hours, and then for 2 to 3 days in 0.01 M nitric acid, followed by storage in deionized water at 4°C (1). Prior to use the bags were washed repeatedly with deionized or glass-distilled water and dried for 6 to 16 hours at room temperature. To insure optimal mixing, a marble was placed within the bag before addition of protein solution. Five ml of protein solution with buffer was added inside the bag and 5 ml of isotonic buffer solution was added to the outside. Dialysis was carried out in 50 ml plastic centrifuge tubes (28 × 102 mm); adsorption of thyroxine in dilute solution by glassware was obviated by the use of plastic (cellulose nitrate) tubes. The tubes were placed in a water-bath shaker with temperature at 38° or 30°C and in a few instances at 5°C.

Recovery of 131I-thyroxine ranged from 95 to 105 per cent, even in control runs in the absence of protein, indicating no significant binding by the dialysis bags or by the walls of the plastic tubes.

Owing to the limited solubility of thyroxine at pH 7.4, it was necessary to work with low thyroxine concentrations, and it was not feasible to vary the thyroxine concentrations more than tenfold. Higher concentrations resulted in visible precipitation. In most of the studies the concentration of thyroxine ranged from 2.6 × 10-6 M to 2.6 × 10-8 M, expressed as the final concentration in the 5-ml protein solution. As shown below, this tenfold variation of thyroxine concentration proved adequate for studies of the primary binding sites. With the increased solubility of thyroxine in more alkaline solutions, a greater range of concentrations was possible. At pH 9.8 the concentration of thyroxine was varied 90-fold without difficulty. The albumin concentration usually employed within the dialysis bag was 0.1 g per 100 ml (1.45 × 10-3 M). The use of higher concentrations of albumin, such as 0.2 g per 100 ml, restricted the possible ratios of thyroxine to protein because of the limited thyroxine solubility. Variations in protein concentration had no appreciable effect upon the results obtained. Therefore the more dilute albumin solutions were used in the majority of the experiments, since they permitted a larger number of experimental points.

EDTA at a final concentration of 3.4 × 10-6 M was also added (1). Presumably the EDTA prevents thyroxine in dilute solution on the nonprotein side of the bag from forming complexes with minute traces of metal ions (19, 20). When dialysis studies were run without EDTA, proper equilibrium was not obtained in tubes to which the thyroxine was added outside the bag.

To achieve equilibrium, a period of 4 hours was adequate at 38°C, but overnight (16 hours) shaking in the water bath was usually employed and was always used at temperatures below 38°C. Appreciable bacterial growth was not observed. In control tubes with no protein inside the bag, radioactivity was essentially the same in both compartments after 4 or 16 hours of shaking at 38°C. With the dilute protein solutions employed, no significant volume change was observed, although similar
Fraction Bound = \frac{\text{cpm inside} - \text{cpm outside}}{\text{total cpm}}

\[ \frac{\nabla}{A} = \frac{\text{moles } T_4 \text{ bound}}{\text{mole albumin}} \]

\[ A = \text{concentration (activity) of free } T_4 \]

\[ n = \frac{\text{maximum moles } T_4 \text{ bound}}{\text{mole albumin}} \]

\[ k = \text{apparent association constant} \]

**Fig. 1.** Equilibrium dialysis study of thyroxine binding by human serum albumin (Scatchard plot).

Studies with undiluted serum, described separately (21), resulted in a volume change of 0.5 ml owing to osmosis under the conditions described.

In all studies a series of graded increments of thyroxine (carrier thyroxine mixed with tracer) was employed with the same protein solution. No change in pH occurred during dialysis, as measured with the Beckman model G pH meter. After equilibration, aliquots were taken from both the inside and outside of the dialysis bag and counted in a Packard Auto-Gamma well-type scintillation counter. The fraction of thyroxine bound was obtained from the quotient: (cpm inside - cpm outside)/total cpm.

The Scatchard approach (2-4) was used to obtain the binding constants for the interaction of thyroxine and albumin. Figure 1 illustrates the Scatchard equation and defines the symbols. From the protein concentration employed, the thyroxine concentration added, and the "fraction bound," the value for \( \tilde{p} \) was computed for each of the tubes of the study. The value for \( A \), the free thyroxine concentration, was obtained from counts per minute outside by employing the known thyroxine concentration added. The values of \( \tilde{p}/A \) plotted against \( \tilde{p} \) give a straight line when all the binding sites are equivalent and independent (2-6). As illustrated in the idealized Scatchard plot in Figure 1, the dotted line extrapolations to the axes are employed to obtain binding constants. The intercept on the \( \tilde{p}/A \) axis is \( n \), as \( \tilde{p}/A \) approaches zero as a limit, and the intercept on the \( \tilde{p}/A \) axis is \( kn \) as \( \tilde{p} \) approaches zero as a limit. The quotient \( kn/n \) gives \( k \), the apparent association constant, an expression that is the reciprocal of the dissociation constant. The Scatchard equation may be derived from the mass law (2-5). When binding takes place at more than one set of sites with different association constants, deviations from linearity are observed. Curvature may also occur in the binding of di- or trivalent ions by proteins, since the addition of successive ions to each of a class of essentially identical sites may produce differences in apparent association constants because of appreciable alteration of the net charge of the protein-ion complex (2-6).

The binding of each concentration of thyroxine was always determined by setting up tubes in duplicate. In one tube the labeled compound was added to the protein solution inside the dialysis bag; in the other tube it was added to the protein-free solution outside. If both of the duplicate tubes reach equilibrium there should be the same fraction of 1\(^{131}\)-thyroxine bound. Therefore, \( \tilde{p} \) and \( A \) should be the same in both cases where the interaction is a true equilibrium. Figure 2 shows that reasonable correspondence between duplicates is actually obtained, and that a linear relationship exists between \( \tilde{p}/A \) and \( \tilde{p} \) for the binding of thyroxine by crystalline human mercurialalbumin at pH 8.6 and 30°C. This experiment was typical of all acceptable studies under the varying experimental conditions; reasonable equilibration was indicated by the close correspondence of "inside" and "outside" points. In the presence of EDTA the values of paired tubes usually agreed within 5 per cent.

Inspection of Figure 2 reveals that all the triangles are above the line and all the circles below it. This was typical of all studies except that occasionally some of the inside and outside points corresponded so closely as to be
indistinguishable. The line was determined by points representing the mean values of the inside and outside points. Figures subsequent to Figure 2 illustrate the mean values.

In the absence of EDTA the points obtained from tubes to which thyroxine was added to the nonprotein side of the bag were quite far below a line drawn through the points obtained from inside additions, as illustrated previously (1). Therefore, EDTA was necessary for equilibration only where thyroxine was added outside the bag but was employed in all studies nevertheless for uniformity of conditions.

RESULTS AND COMMENTS

1. The binding of thyroxine by crystalline human mercaptalbumin. The findings in a typical equilibrium dialysis study are illustrated in Figure 2. The binding data were analyzed by means of the Scatchard equation as described in Figure 1 and Methods. Satisfactory correspondence between the duplicates (triangles and circles in the figure) is evident upon inspection; such satisfactory agreement was obtained in the presence of EDTA. A linear relationship exists between $\bar{V}/A$ and $\bar{V}$. The extrapolations to the $\bar{V}/A$ axis, $kn$, and the $\bar{V}$ axis, $n$, are shown by the dotted lines. The intercepts gave a value of 4.43 for $n$; the $kn$ value obtained was 665,000. The quotient 665,000/4.43 equals 150,000, the value for $k$, the association constant. An association constant of this magnitude represents a somewhat firmer binding than that of many small molecules known to be bound to albumin, such as methyl orange (5) and tryptophan (6), but much less firm binding than the interaction of human serum albumin with the free fatty acids of serum (12).

The results of albumin-thyroxine interactions at pH 8.6 are quite similar to the findings at pH 7.4, as shown below; however, this is not the case when the small molecule is triiodothyronine, as is subsequently discussed. At pH 8.6 the same results were obtained with Tris-sodium chloride and with sodium borate buffers.

2. The binding of thyroxine by human serum albumin at pH 7.4 and 38°C. The interaction of L-thyroxine with amorphous preparations of serum albumin was studied at pH 7.4 in two buffer systems: 1) potassium phosphate buffer, ionic strength 0.15; and 2) Tris-sodium chloride (0.05 M Tris-0.1 M NaCl). The two buffer systems gave similar results at the same pH. A typical experiment with dialyzed and deionized amorphous albumin is illustrated in Figure 3. The value for $kn$ is 420,000, $n$ is 4.2, and $k$ is therefore 100,000. It will be observed that the two points near the ordinate are not close to the line. Such scattering, above or below the line, was frequent in most experiments for the points representing the smallest amounts of thyroxine. It is evident on mathematical grounds that less accurate

![Figure 2](image-url)  
**FIG. 2. Equilibrium dialysis study of L-thyroxine binding by crystalline human mercaptalbumin.** 30°C, pH 8.6, 0.05 M Tris-0.1 M NaCl; $\triangle$ = thyroxine added to protein solution inside bag; $\bigcirc$ = thyroxine added to nonprotein solution outside bag.

![Figure 3](image-url)  
**FIG. 3. Equilibrium dialysis study of L-thyroxine binding by human serum albumin.** 38°C, pH 7.4, phosphate buffer, 0.15 ionic strength. The points represent the mean values of "inside" and "outside" additions for each amount of thyroxine.
data can be obtained where the "fraction bound" is high (in excess of 90 per cent), since the numerator represents the difference between a large and a small number. The most accurate data were invariably obtained for points in the intermediate portion of the curve. At pH 7.4 the limited solubility of thyroxine made it impossible to obtain points for higher concentrations of thyroxine than are illustrated. Inspection of the dotted line extrapolations to the axes reveal that the extrapolation to the abscissa is longer than that to the ordinate. Consequently the values for $n$ may be subject to an error of about 10 per cent and may be somewhat less accurate than the values for $k$, owing to the shorter extrapolation required. In ten equilibrium dialysis experiments with amorphous albumin in phosphate buffer at pH 7.4, the mean value for $n$ was 4.0 ± 0.3 (standard deviation), and the mean value for $k$ was 107,000 ± 12,000. In eight experiments at pH 8.6 with Tris-sodium chloride buffer, the mean value for $n$ was 4.1 ± 0.2 and the mean value for $k$ was 110,000 ± 15,000 (cf Table I).

It is evident that the results at pH 7.4 and 8.6 do not differ appreciably. The results selected for illustration showed the least scatter of experimental points and are considered the most accurate. Since the number of binding sites should be an integer, it may be inferred from the data that the albumin molecule has a primary class of four thyroxine binding sites with an apparent association constant of approximately 100,000. No immediate explanation is apparent for the difference in association constants of 150,000 for crystalline human mercaptalbumin and 100,000 for amorphous human albumin, which differ in that each of the mercaptalbumin molecules possesses a free sulfhydryl group. The amorphous albumin preparations from the American Red Cross may be presumed to consist partly of mercaptalbumin, although the proportion was not determined in the individual lots. These amorphous preparations had been stabilized prior to acquisition by the addition of 0.02 M sodium caprylate (octanoate) and 0.02 M sodium acetyltryptophanate. This concentration corresponds to 5.5 molecules of caprylate and acetyltryptophanate per albumin molecule in the original solution. The binding of caprylate to albumin is relatively weak (22), and much of it was probably removed by the dialysis against isotonic saline prior to use. Moreover, passage through the mixed-bed resin removes these preservatives as well as much of the bound fatty acid (11–13). Since both the crystalline mercaptalbumin and the amorphous albumin preparations had been passed through Amberlite MB-1, one cannot assume that differences in the amount of fatty acids bound by the two proteins could explain the discrepancy between the association constants. However, in the absence of data on bound fatty acids, this possibility remains to be excluded (cf 6, below).

3. The effect of temperature. The results at 30° and 38°C did not differ appreciably; as illustrated in Figure 4, however, low temperature (5°C) resulted in appreciably tighter binding. The free energy of binding (−$\Delta F^\circ$), computed for 38°C, approximated 7,200 calories per mole, a value somewhat greater than that for the association of bovine serum albumin with methyl orange (5).

4. Second binding site on albumin. Lein (23, 24) has described two classes of binding sites for the interaction of L-thyroxine with bovine serum albumin at pH 9.75 in glycinate buffer. Since the solubility of thyroxine is increased at an alkaline pH, it was possible to extend the experimental points by using more concentrated thyroxine solutions. A study at pH 9.8 in 0.2 M glycinate buffer is illustrated in Figure 5, in which the flatter segment of the curve with greater amounts of thyroxine is clearly evident. The data at pH 9.8 are

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Effect of temperature upon the binding of thyroxine by human serum albumin. pH 8.6, 0.05 M Tris-0.1 M NaCl.
best described by assuming the existence of two binding sites: \( n_1 = 3, k_1 = 143,000; n_2 = \text{approximately } 20, k_2 = \text{approximately } 1,000. \) The values for \( n_2 \) and \( k_2 \) represent estimates based upon extrapolations of the flat segment. Theoretical curves constructed with these constants resulted in the closest approximation to the experimental points. Owing to the extremely low concentration of free thyroxine in serum, only the first binding site has physiological importance (cf Discussion).

5. The effect of pH. A series of amorphous albumin experiments with varying pH is presented in Table I. It should be pointed out that at pH 7.0 the limitation of thyroxine solubility permitted only a few satisfactorily equilibrated points at the lowest values for \( \bar{v} \); therefore, lines could be drawn with reasonable certainty in only two of four experiments attempted. The results in Table I represent the single most satisfactory experiment of the four, and these values should be regarded as the best approximations obtainable at pH 7.0. The appreciable diminution in binding at pH 10.9 is evident. At pH 11.2 with phosphate buffer, the data yielded a low, essentially horizontal curve indicative of pronounced reduction in binding. As discussed below, the reduction in binding with strongly alkaline pH is compatible with removal of protons from free \(-\text{NH}_3^+\) groups on the protein.

6. Isooctane-extracted albumin. Goodman (12) has reported that serum albumin has a primary class of two binding sites for fatty acids, with an association constant of \( 6 \times 10^6 \) for palmitate. To investigate the possibility that fatty acids might interfere with the binding of thyroxine, studies were carried out with deionized amorphous albumin preparations which had been subjected to isooctane extraction to free them of bound fatty acids.

The equilibrium dialysis study in Figure 6 revealed no diminution in the number of primary binding sites (\( n = 4.2 \)) and an increased association constant (\( k = 148,000 \)). Similar findings were obtained at pH 7.4. The mean values of four studies with isooctane-extracted albumin were \( n = 4.2 \) and \( k = 163,000 \). The reason for the firmer binding is not evident; possibly, removal of the long-chain fatty acid may free a nonpolar part of the protein molecule important in the interaction. Addition of 2 moles of palmitate per mole of protein to an isooctane-extracted albumin preparation resulted in a curve indistinguishable from the original, as shown (Figure 6).

7. Nature of the principal binding sites of albumin. Since Klotz and Walker (25) presented evidence in support of the interaction of the anionic dye, methyl orange, and the \( \epsilon \)-amino groups of lysine residues of serum albumin, it seemed reasonable to suppose that these groups might also be involved in the binding of the anionic molecule,
thyroxine. A means of testing this hypothesis was provided by studies in which the free amino groups of the protein were blocked by acetylation (14). As illustrated in Figure 7, acetylation of 11 per cent of the lysyl ε-amino groups resulted in reduction of $n$ from 4 to 3, and reduction of the association constant by one-third (from 100,000 to 70,000). Heavier acetylation, which produced blocking of 45 to 75 per cent of the protein amino groups, resulted in more pronounced reduction of binding. The behavior as a function of variation of pH is consistent with an interaction of free $-NH_3^+$ groups and the small anionic molecule, thyroxine. Thus, at very strongly alkaline reaction (pH 10.9 and 11.2) in the presence of isotonic phosphate buffer, the binding of L-thyroxine is markedly reduced, presumably because most of the $-NH_3^+$ groups have lost a proton to become $-NH_2$ groups.

As previously reported (1), the free cationic groups on the protein molecule are believed to interact with the anionic phenolate groups of thyroxine and triiodothyronine. At pH 7.4 triiodothyronine was bound only one-tenth as tightly as L-thyroxine and D-thyroxine. The difference in behavior was attributed to the lesser degree of ionization of the phenolic hydroxyl group of triiodothyronine, which at pH 7.4 is only one-tenth as highly ionized as that of thyroxine. At pH 8.6, with greater ionization to the phenolate form, triiodothyronine was found to be more firmly bound by albumin. While other factors besides electrostatic attraction may be involved in binding, the data indicate an important role for the interaction of free cationic groups on the protein molecule and the anionic phenolate groups of thyroxine and triiodothyronine. The interaction suggested in Figure 8 may be regarded as a tentative and simplified hypothesis, consistent with the data at hand but not definitely proven. An alternative explanation may be advanced for the observations of reduced binding at strongly alkaline reaction (pH 10.9 and 11.2), as well as for the behavior of the acetylated proteins. In both circumstances the protein molecule should have a greater net negative charge because of the loss of free cationic

Fig. 6. Effect of palmitate on the binding of L-thyroxine by isooctane-extracted human serum albumin. 30°C, pH 8.6, 0.05 M Tris-0.1 M NaCl.

Fig. 7. The binding of thyroxine by acetylated human serum albumin preparations. 30°C, pH 8.6, 0.05 M Tris-0.1 M NaCl.

Fig. 8. Schematic representation of hypothesis of thyroxine-albumin interaction.
(--NH₃⁺) groups, whether by acetylation or by loss of a proton. Thus, the reduction of affinity between a more negatively charged protein molecule and the thyroxine anion does not require the specific hypothesis suggested in Figure 8. Moreover, configurational changes or other possible alterations have not been assessed. Further studies with modified proteins and with thyroxine analogs may provide more evidence concerning the nature of the interaction.

DISCUSSION

The theoretical treatment of the interaction of small molecules with proteins has been considered extensively by Scatchard (2–4), Klotz (5), and many other workers. Scatchard has simplified the theoretical consideration of the problem by discussing it under the four questions listed below; the answers for the thyroxine–albumin interaction are provided by the present data.

1. "How many?"  (How many small molecules are bound by each protein molecule?) At physiological pH, in isotonic phosphate or Tris-NaCl buffer, n = 4, signifying that the human serum albumin molecule may bind four thyroxine molecules at its primary binding sites.

2. "How tightly?"  (How tightly are the small molecules bound to the protein?) The apparent association constant of approximately 100,000 is of an order of magnitude comparable with the binding of many drugs and dyes. Albumin binds thyroxine somewhat more tightly than it binds the anionic dye, methyl orange (5), or tryptophan (6), but much less tightly than it binds the free fatty acids of serum (12). In contrast, triiodothyronine is bound only one-tenth as tightly as thyroxine at pH 7.4, a difference attributed to the greater degree of dissociation of the phenolic hydroxyl group of thyroxine.

3. "Where?"  (At what sites on the protein molecule does binding occur?) Since the negatively charged phenolic hydroxyl group appears to be one site of attachment of the iodothyronine molecule, it seems reasonable to suppose that free cationic groups on the protein are binding sites. The reduction of binding at strongly alkaline reaction (pH 10.9 and 11.2) is compatible with such a hypothesis. Experimental support is afforded by the studies in which diminished binding was observed after lysyl ε-amino groups of the protein had been blocked by acetylation. The evidence is not, however, a conclusive demonstration that --NH₃⁺ groups are the specific binding sites. In strongly alkaline solutions where --NH₃⁺ groups lose protons, or with these groups blocked, the protein molecule would have a greater net negative charge that could reduce its affinity for the thyroxine anion.

4. "Why?"  (What is the biological significance of binding, if any?) In normal serum, the albumin molecules are present in vast excess. The ratio approximates 10,000 albumin molecules to one of thyroxine. Under these circumstances an even smaller minority than 1/10,000 of the albumin molecules would carry a bound thyroxine molecule, since most of the serum thyroxine is bound by the thyroxine-binding α-globulin and prealbumin fractions. Therefore, the tightness of albumin binding is not a limiting factor. Nevertheless, it has biological relevance as a secondary carrier.

From the binding constants established for human serum albumin it is possible to estimate the free thyroxine concentration of serum by employing the observation that approximately 15 per cent of serum thyroxine is albumin-bound upon electrophoresis. With the addition of tracer amounts of thyroxine, the mean value of 15 per cent has been obtained both by Ingbart and Freinkel (7) and Robbins and Rall (26) with respective buffer systems of pH 8.6 and 8.4; the precise value at pH 7.4 has not thus far been determined in a system that separates the prealbumin carrier. However, the use of 15 per cent seems reasonable as a first-order approximation, in view of the similarity of albumin binding of thyroxine at pH 7.4 and pH 8.6. The 85 per cent bound by the other carriers was ignored for the present calculation.

The equation most conveniently applied is that of Scatchard — v = nkA/(1 + kA) — which represents the form from which the equation v/A = kn/kv is derived algebraically. If an average normal butanol-extractable iodine of 5.1 μg per 100 ml is assumed, this would correspond to a serum thyroxine concentration of 7.78 μg per 100 ml, or 1 × 10⁻² M. Since approximately 15 per cent of the total serum thyroxine is albumin-
bound, the value for the thyroxine-albumin complex would be $1.5 \times 10^{-8}$ M. The mean normal concentration of albumin of 4.5 g per 100 ml corresponds to a concentration of $6.5 \times 10^{-4}$ M. Therefore $k = (1.5 \times 10^{-8} \text{ M}) / (6.5 \times 10^{-4} \text{ M}) = 2.3 \times 10^{-5}$. Since the equilibrium dialysis studies reported have given values of 4 for $n$ and 100,000 for $k$, we may substitute in the equation and solve for $A$, the free thyroxine concentration of serum. The value of $0.6 \times 10^{-10}$ M obtained corresponds to the predictions of Robbins and Rall (8, 9). A value of $1.3 \times 10^{-10}$ M has been obtained in this laboratory by dialysis of whole serum, as reported separately (21). The discrepancy may be attributed to the extrapolation of binding data to undiluted serum with its many small molecules (27), the partial removal of fatty acids bound to albumin, and the use of data from electrophoresis at pH 8.6.

**SUMMARY**

1. Equilibrium dialysis has been employed to investigate the binding of thyroxine by human serum albumin. Various temperatures and buffers were used, including 38°C, pH 7.4, and 0.15 ionic strength (isotonic).

2. Detailed studies of various albumin preparations yielded results consistent with four primary binding sites per molecule, with an apparent association constant of approximately 100,000.

3. Studies exploring variation in pH and behavior of acetylated albumin preparations suggested that four cationic groups on the protein molecule interact with the anionic phenolate groups of thyroxine and its analogs. The evidence is compatible with the hypothesis that the four primary binding sites are ε-amino groups of lysine residues of albumin, but the nature of the binding sites is not considered proven.

4. With the observation that 15 per cent of the total serum thyroxine is bound to albumin, it was possible to employ the binding constants obtained to compute the theoretical free thyroxine concentration of serum; the value calculated was $0.6 \times 10^{-10}$ M.

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