The Contribution of Thyroxine-Binding Prealbumin to the Binding of Thyroxine in Human Serum, as Assessed by Immunoadsorption

KENNETH A. WOEBER and SIDNEY H. INGBAR
with the technical assistance of URDA I. TRAENKLE †

From the Thorndike Memorial Laboratory and the Second and Fourth (Harvard) Medical Services, Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02118

ABSTRACT An immunoadsorption technique employing a rabbit antiserum specific for human serum prealbumin has been devised to remove thyroxine (T_4)-binding prealbumin (TBPA) from serum completely without affecting the T_4-binding activity of thyroxine-binding globulin (TBG) or the concentration of the other major proteins in serum. As judged from the proportion of T_4 associated with the antigen-antibody precipitate, only about 15% of the endogenous T_4 is bound by TBPA, a value considerably less than that indicated by electrophoretic methods. As judged from the increase in the proportion of free T_4 that followed immunoadsorption of TBPA, TBPA does act as one determinant of the proportion of free T_4 but is far less important than TBG in this respect. A decrease in the T_4-binding capacity of TBPA cannot solely account for the increase in the proportion of free T_4 in the sera of ill patients, since a comparable increase does not occur in normal sera after complete removal of TBPA. From data obtained in normal and abnormal sera before and after immunoadsorption of TBPA, estimates of the equilibrium constants for the interactions between T_4 and its binding proteins, as they exist in serum, have been derived. The values obtained were: K_{ALB}, 6.2×10^5; K_{TBPA}, 2.3×10^8; and K_{TBG}, 1.7×10^{10}.

† Deceased 26 January 1968.

Received for publication 12 November 1968 and in revised form 7 February 1968.

INTRODUCTION

The existence of L-thyroxine (T_4)–binding prealbumin (TBPA) as a normal constituent of human serum has been satisfactorily demonstrated both by a variety of electrophoretic techniques and by its isolation from human serum (1–3). Nevertheless, the proportion of endogenous T_4 in serum that is normally bound by TBPA is uncertain because this has been assessed only by electrophoretic methods, which give highly variable results depending upon the method employed. For example, of an essentially endogenous concentration of T_4, about 30% migrates with TBPA in agar gel at pH 7.4 (4), 30–45% migrates with TBPA in filter paper at pH 8.6 (5), while in starch gel values which vary from 10–60% have been reported (6–8). Furthermore, in addition to the uncertainty concerning the proportion of endogenous T_4 that is bound by TBPA, the relative influence of TBPA on the proportion of free or unbound T_4 in human serum is not known. In some ill patients, or after the administration to patients or the addition to serum of certain drugs, there occur a decrease in the T_4-binding activity of TBPA and an increase in the proportion of free T_4 (9, 10). In the sera of ill patients, a good correlation has been shown to exist between the extent of decrease in T_4-binding by TBPA and the extent of increase in the proportion of free T_4 (9); nevertheless, a simple cause and effect relationship cannot be assumed. Similarly, in the case of drugs, the possibility that their effect on the proportion of free T_4 is partly due to an effect on other proteins has not been definitely excluded (10). Consequently, we sought a means for selectively removing TBPA from normal serum without affecting the concentration or activity of the other T_4-binding proteins. We achieved this by the addition to human serum of gamma globulin derived from a rabbit antihuman prealbumin antiserum, thereby taking advantage of both the specificity of immunological interactions and the virtual inability of gamma globulin to bind T_4. This technique has enabled us to study the specific contribution of TBPA to the binding of T_4 in normal and abnormal human sera.

METHODS

Preparation of rabbit antihuman prealbumin gamma globulin. Rabbit antihuman prealbumin antiserum was obtained from a commercial source.[1] The antiserum was fractionated on *O*-diethylaminoethyl (DEAE)-cellulose by the column chromatographic method of Fahey, McCoy, and Goulian (11). The eluate fractions containing gamma globulin were combined, dialyzed against distilled water, and lyophilized. Normal rabbit serum was fractionated in an identical manner.

Purification of ^{131}I-labeled T_4. ^{131}I-labeled T_4 was obtained from a commercial source.[2] On arrival, 900 μl of the ^{131}I-labeled T_4 was made up to 1 ml with human serum albumin (30 g/100 ml). After removal of a small aliquot for counting, the solution was dialyzed against a large volume of distilled water for 18 hr at 6°C to remove iodide and other degradation products, as suggested by Schussler and Plager (12). After dialysis, an aliquot of the solution was counted and the concentration of ^{131}I-labeled T_4 remaining was calculated. This solution was diluted with human serum albumin (1 g/100 ml) to provide the desired concentrations of ^{131}I-labeled T_4 for the experiments performed.

Types of sera studied (Table I). Blood samples were obtained from 10 subjects, two of whom (Nos. 9 and 10) were women. Of the five normal subjects, three were entirely healthy and two were awaiting surgery for peptic ulcer and cholecystitis, respectively, but were not ill. In these two, blood samples were obtained both before and for several days after surgery. Two subjects (Nos. 6 and 7) had chronic systemic disease, and one (No. 8) had acute pneumococcal pneumonia. Subject 9 had an idiopathic absence of T_4 binding by T_4-binding globulin (TBG) in addition to Turner's syndrome with an XO sex chromosome constitution (13). Subject 10 was 39 wk pregnant.

The blood samples were usually collected 3–4 hr after the subjects had eaten and were allowed to clot at room temperature for 1 hr. The serum was separated by centrifugation and either used immediately or stored frozen until used.

Immunoprecipitation and adsorption. In preliminary experiments, the amount of anti-prealbumin gamma globulin required to precipitate TBPA completely from normal serum was determined; this proved to be 4 mg/100 μl. The lyophilized anti-prealbumin gamma globulin was added directly to serum. The mixture was allowed to incubate for 18 hr at 6°C, and the resulting immunoprecipitate was removed by centrifugation. The same concentration of lyophilized normal rabbit gamma globulin was always added to a duplicate sample of the same serum and

[1] Hoechst Pharmaceutical Company, Kansas City, Mo.
[2] Abbott Laboratories, North Chicago, Ill.

TABLE I
Summary of the Thyroxine-Binding Characteristics of Sera of Several Types and the Contribution Thereto of TBPA, as Assessed by Immunoadsorption

Subject	State	Serum T_4 concentration	T_4-binding capacity		Serum protein concentration		Immunoprecipitable T_4	Free T_4			Adsorbed
			TBG	TBPA	Total	Albumin		Control	"Dummy"	Adsorbed	"Dummy"
		µg/100 ml	*µg/100 ml*		*g/100 ml*		*% total*		*% total*		*% increase*
1	Normal	7.5	19	238	7.5	5.0	15	0.028	0.030	0.037	23
2	Normal	6.5	19	211	7.0	4.5	13	0.028	0.028	0.034	21
3	Normal	6.5	15	231	7.3	5.4	15	0.027	0.029	0.039	34
4	Normal	7.0	19	229	7.9	5.4	13	0.031	0.033	0.037	12
4a	Postoperative	6.0	14	49	5.1	3.8	—	0.046	0.046	0.049	7
5	Normal	7.5	16	139	7.5	4.2	—	0.037	0.037	0.045	22
5a	Postoperative	8.0	15	67	6.9	3.8	—	0.047	0.047	0.049	4
6	Ill	3.0	11	21	5.7	2.0	—	0.078	0.082	0.082	0
7	Ill	4.0	15	28	8.6	3.9	7	0.046	0.048	0.051	6
8	Ill	6.0	25	36	6.0	4.0	4	0.029	0.029	0.030	3
9	A-TBG	1.0	0	232	6.3	4.5	60	0.092	0.106	0.246	132
10	Pregnant	10.0	46	119	6.9	4.1	5	0.013	0.014	0.015	7

this was treated concurrently in the same manner to serve as the "dummy" sample. In all experiments, the extent of removal of TBPA from the serum samples was assessed by electrophoresis, as described below.

Assessment of the per cent of immunoprecipitable T_4. In order to study the proportion of endogenous T_4 that is bound by TBPA, we enriched the serum samples with an exceedingly small concentration of purified ^{131}I-labeled T_4 (approximately 40 ng/100 ml) before the addition of gamma globulin. The immunoprecipitate and, in the case of the "dummy" sample, the small amount of sediment that formed during the incubation described above were washed twice with saline. The ^{131}I remaining with the washed immunoprecipitate and "dummy" sediment was measured and expressed as a per cent of that in the original sample of serum.

The effects of pH and temperature on the per cent of immunoprecipitable T_4 were studied in two sera. Here, the pH of the sera was reduced to 7.4 from its random value of approximately 8.1 by gassing with 5% CO_2, and the incubation with gamma globulin was carried out at 37°C for 18 hr. Duplicate samples of the same sera whose pH had not been corrected to 7.4 were subjected concurrently to immunoprecipitation at 6°C.

Experiments were conducted to assess the T_4-binding activities of the immunoprecipitate and "dummy" sediment. Each was suspended in a volume of human serum albumin (3 g/100 ml) equal to that of the original sample of serum. Aliquots of each suspension were enriched with 10 and 610 μg of ^{131}I-labeled T_4 per 100 ml. These were then subjected to conventional filter paper electrophoresis, as described below.

Assessment of the per cent of free T_4. The effect of immunoadsorption of TBPA on the per cent of free T_4 was studied in dilute serum at pH 7.4 and 37°C by means of the general equilibrium dialysis method of Oppenheimer, Squef, Surks, and Hauer (9). The supernatants obtained by removal of the immunoprecipitate or "dummy" sediment were diluted 1:25 with Krebs-Ringer phosphate buffer (KRP) at pH 7.4 and enriched with the equivalent of 1.8–2.0 μg of purified ^{131}I-labeled T_4 per 100 ml of undiluted serum. A sample of the same serum that had not been exposed to gamma globulin was treated in an identical manner to serve as the control. 1 ml of each diluted sample was placed in a sac made from dialysis tubing (Union Carbide Corporation, New York, size 20) and dialyzed against 5 ml of KRP in a 25 ml Erlenmeyer flask for 20 hr at 37°C. To ensure a constant pore size, the same batch of dialysis tubing was used in all experiments. After dialysis, aliquots were taken from inside and outside the dialysis sac. To these aliquots we added equal volumes of outdated serum containing carrier iodide and a pinch of propylthiouracil, and precipitated the ^{131}I-labeled T_4 with cold 20% trichloroacetic acid (TCA). The precipitates were washed twice with cold 5% TCA and then dissolved with 2 N NaOH to a standard volume for counting. Sufficient counts were obtained to reduce the probable counting error to a maximum of 3%. The amount of TCA-precipitable ^{131}I per milliliter of dialysate was expressed as a fraction of the amount of TCA-precipitable ^{131}I in the 1 ml of dilute serum within the dialysis sac. To obtain a value for the per cent of free T_4, this fraction was multiplied by 100 and divided by the dilution factor of the serum within the sac (1:25.25). In all experiments, the control, "dummy," and adsorbed samples were run concurrently and in duplicate.

In one experiment, the control, "dummy," and adsorbed samples of serum were diluted 1:25 with Krebs-Ringer bicarbonate buffer (KRB), rather than with KRP, and were dialyzed against the same buffer. Here, the contents of the Erlenmeyer flasks were gassed with 5% CO_2 at 37°C for 20 min before dialysis. Gassing of this duration was found adequate to bring the pH of KRB in a dialysis sac to 7.4, with phenol red as an indicator. For purposes of comparison, duplicate samples of the same serum were diluted with KRP and subjected concurrently to dialysis.

Filter paper electrophoresis. T_4 binding in the sera, supernatants, immunoprecipitates, and sediments was assessed by filter paper electrophoresis. The T_4-binding capacity of TBPA was assessed by enriching the samples with 610 μg of ^{131}I-labeled T_4 per 100 ml (approximately 4 μc/ml) and subjecting them to conventional electrophoresis as described previously (14), except that glycine (0.2 M)–acetate (0.13 M) buffer at pH 8.6, instead of Tris-maleate buffer, was employed. The T_4-binding capacity of TBG was assessed by enriching the samples with 160 μg of ^{131}I-labeled T_4 per 100 ml (approximately 4 μc/ml) and subjecting them to reverse-flow electrophoresis in 0.2 M glycine–0.13 M acetate buffer at pH 8.6, with the Beckman-Spinco electrophoresis cell (15). In two sera, the distribution of an essentially endogenous concentration of T_4 among the binding proteins was assessed by enriching the samples with the exceedingly small concentration of purified ^{131}I-labeled T_4 employed for immunoprecipitation (approximately 40 ng/100 ml) and subjecting them to conventional electrophoresis. Quantitation of the distribution of all concentrations of ^{131}I-labeled T_4 among the binding proteins was determined by cutting out the radioactive zones on the filter paper with the aid of radioautographs and counting them.[3] In calculating the T_4-binding capacities of TBG and TBPA, the endogenous T_4 concentration was taken into account.

Agar gel electrophoresis and immunoelectrophoresis. These techniques were performed according to the general method of Wunderly (16), using 0.073 M Tris-maleate buffer at pH 8.6.

Estimation of protein concentration. The concentrations of albumin and total protein in serum were estimated by the biuret method of Gornall, Bardawill, and David (17), using human serum mercaptalbumin as a standard.

Serum T_4 concentration. The endogenous concentra-

[3] In the samples enriched with the nanogram concentration of ^{131}I-labeled T_4, sufficient ^{131}I was not present for radioautographic darkening; accordingly, the binding proteins were localized by adjacent markers of serum containing a higher concentration of ^{131}I-labeled T_4.

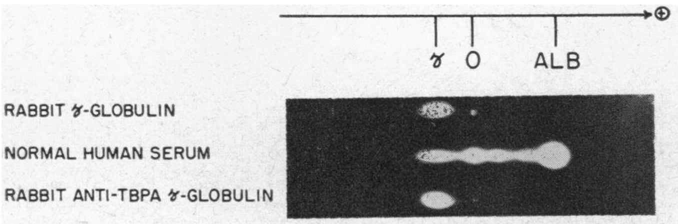

FIGURE 1 Electrophoretic patterns in agar gel of the eluate fractions containing gamma globulin obtained from normal rabbit serum and rabbit antihuman prealbumin antiserum by chromatography on *O*-diethylaminoethyl-cellulose.

tion of T_4 in serum was measured by the binding displacement method of Murphy and Pattee (18).[4]

RESULTS

As indicated by their electrophoretic patterns in agar gel, the eluate fractions containing gamma globulin were entirely free of other rabbit proteins (Fig. 1). Fig. 2 illustrates the effect of adding 4 mg of lyophilized gamma globulin per 100 μl of serum on the binding of T_4, as assessed by conventional filter paper electrophoresis. In the sample to which normal rabbit gamma globulin had been added ("dummy" sample), the binding of ^{131}I-labeled T_4 at all concentrations did not differ from that in the sample to which no gamma globulin had been added (control sample). The T_4-binding capacities of TBPA in these two samples were identical (238 μg/100 ml). By contrast, in the sample to which the anti-prealbumin gamma globulin had been added (adsorbed sample), T_4 binding by TBPA could not be demonstrated at any concentration of ^{131}I-labeled T_4, the T_4 being bound almost entirely by TBG and albumin. A very small proportion (less than 1%) of the ^{131}I-labeled T_4 appeared in the beta globulin zone, probably representing T_4 bound to soluble antigen-antibody complexes. None, however, was present in the gamma globulin zone, confirming that gamma globulin itself is devoid of significant T_4-binding activity. The T_4-binding capacity of TBG in the adsorbed sample was identical with that in the control and "dummy" samples (19 μg/100 ml), as assessed by reverse-flow electrophoresis.

Immunoelectrophoretic analysis confirmed the conclusions drawn from filter paper electrophoresis. As shown in Fig. 3, when the antibody troughs were filled with anti-prealbumin antiserum, good precipitin arcs were seen in relation to the control and "dummy" samples, but no arc was seen in

[4] Performed by the Boston Medical Laboratory, Waltham, Mass.

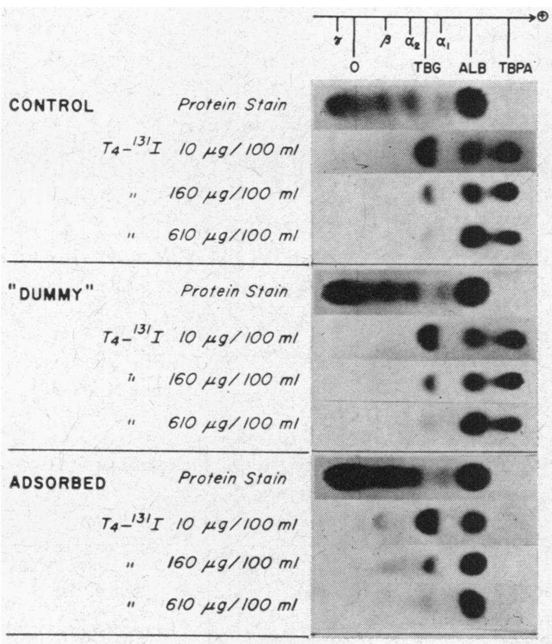

FIGURE 2 The effect of rabbit gamma globulin on the interaction between thyroxine (T_4) and the proteins of normal human serum, as assessed by conventional filter paper electrophoresis. Protein stains and radioautographs of electrophoretic strips are shown. Control, original serum; "dummy," serum enriched with normal rabbit gamma globulin; adsorbed, soluble supernatant of serum enriched with rabbit antihuman prealbumin gamma globulin. TBG, T_4-binding globulin; TBPA, T_4-binding prealbumin.

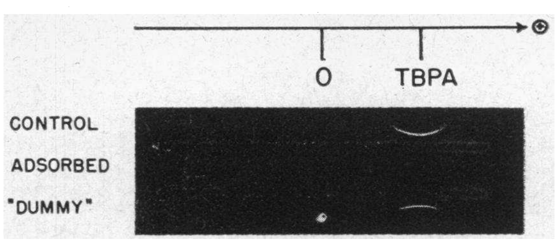

FIGURE 3 Complete removal of TBPA from normal human serum, as assessed by immunoelectrophoresis. Control, original serum; "dummy," serum enriched with normal rabbit gamma globulin; adsorbed, soluble supernatant of serum enriched with rabbit antihuman prealbumin gamma globulin.

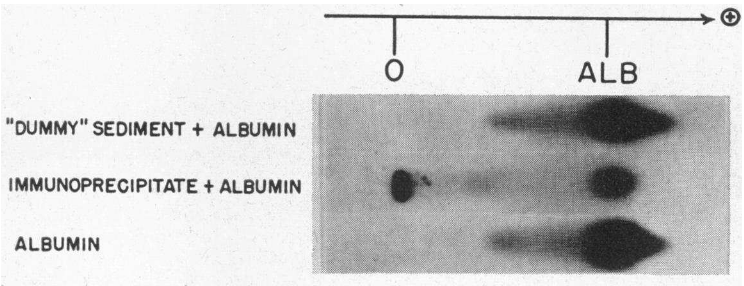

FIGURE 4 Radioautograph depicting retention of T_4 binding by the immunoprecipitate resulting from the interaction of normal human serum with rabbit antihuman prealbumin gamma globulin.

relation to the adsorbed sample. Immunoelectrophoretic analysis using rabbit total antihuman antiserum provided no evidence that immunoadsorption of TBPA was accompanied by changes in the other serum proteins.

Figure 4 compares the electrophoretic migration of ^{131}I-labeled T_4 in specimens containing either the "dummy" sediment or the specific immunoprecipitate suspended in human serum albumin with that in albumin alone. In the suspension of the "dummy" sediment, all of the ^{131}I-labeled T_4 remained associated with the albumin. By contrast, in the suspension of the immunoprecipitate, much of the ^{131}I-labeled T_4 was removed from the albumin and, during electrophoresis, remained at the origin with the antibody-bound TBPA. The T_4-binding capacity of the antibody-bound TBPA derived from three different sera averaged approximately 75% of that of the TBPA in the original samples.

Table II compares the value for the proportion of an exceedingly small concentration of ^{131}I-labeled T_4 (approximately 40 ng/100 ml) that was precipitated from normal human serum by rabbit anti-prealbumin gamma globulin at pH 7.4 and 37°C with that precipitated at pH 8.1 and 6°C. In the two sera studied, the per cent of immunoprecipitable T_4 was essentially the same under both conditions of pH and temperature. Consequently, all subsequent immunoprecipitation experiments were performed at 6°C in sera whose pH had not been corrected to 7.4. As shown in Table II, the values for the per cent of immunoprecipitable T_4 were considerably less than those for the per cent of T_4 bound by TBPA, as assessed by conventional filter paper electrophoresis of the same sera at pH 8.6 employing the same small concentration of ^{131}I-labeled T_4 (approximately 40 ng/100 ml).

Table I summarizes the principal data obtained in the various sera studied. In sera from four normal subjects (Nos. 1–4), the values for the per cent of immunoprecipitable T_4 were very similar, ranging between 13 and 15%. In sera from two ill subjects (Nos. 7 and 8) in which the T_4-binding capacity of TBPA was greatly decreased, only 7 and 4%, respectively, of the T_4 was immunoprecipitable. In the serum in which T_4 binding by TBG was absent but in which the binding capacity of TBPA was normal, 60% of the T_4 was immunoprecipitable, whereas in the serum from the pregnant subject, in which the binding capacity of TBG was greatly increased, only 5% of the T_4 was immunoprecipitable. In contrast to the values obtained for the per cent of T_4 in the specific immunoprecipitates, the per cent of T_4 in the washed sediment of the "dummy" samples was always only a fraction of 1%.

Values for the per cent of free T_4 before and after immunoadsorption of TBPA are also presented in Table I. In the five normal sera, the mean control value for the per cent of free T_4 was 0.030, and this value was essentially unaltered by ex-

TABLE II

*The Effects of pH, Temperature, and the Method of Measurement on the Proportion of Endogenous Thyroxine Bound by TBPA**

Subject	% Immunoprecipitable T_4		% T_4-TBPA as assessed by electrophoresis at pH 8.6
	At pH 7.4 and 37°C	At random pH‡ and 6°C	
1	15	15	31
4	17	13	32

* Experiments conducted in sera enriched with 40 ng of ^{131}I-labeled T_4 per 100 ml.

‡ Random pH indicates the pH spontaneously achieved in sera allowed to stand in room air. Direct measurement revealed this to be approximately pH 8.1.

posure to normal rabbit gamma globulin in the "dummy" samples. Nevertheless, since the values in some "dummy" samples were slightly higher than those in the corresponding control samples, values for the per cent of free T_4 in the adsorbed samples were always compared to those in the "dummy," rather than in the control, samples. After immunoadsorption of TBPA, values for the per cent of free T_4 were 12–34% (mean, 22%) greater than those in the "dummy" samples. Although the values for the percent of free T_4 were approximately 20% lower in KRB than in KRP, the increase in the per cent of free T_4 after immunoadsorption of TBPA was the same in the two buffers.

In the two sera obtained from subjects in the postoperative state and in two of the three sera obtained from ill subjects, in all of which T_4 binding by TBPA was demonstrable but greatly decreased, values for the per cent of free T_4 before immunoadsorption of TBPA exceeded those in normal sera from which TBPA had been completely removed. In the serum from the third ill subject (No. 8), the value for the per cent of free T_4 was normal, although the T_4-binding capacity of TBPA was greatly decreased. This serum differed from the other four sera from ill subjects in that the binding capacity of TBG was by far the highest. In all five of these sera, the increase in the per cent of free T_4 after immunoadsorption of TBPA was small compared to that obtained in normal sera.

The effect of immunoadsorption of TBPA on the binding of T_4 in the two sera characterized by primary alterations in T_4 binding by TBG is also presented in Table I. In the serum in which T_4 binding by TBG was absent but in which the binding capacity of TBPA was normal, the value for the per cent of free T_4 was three times that in normal serum and increased by 132% after immunoadsorption of TBPA. By contrast, in the serum from the pregnant subject, in which the binding capacity of TBG was greatly increased, the value for the per cent of free T_4 was about one-half normal and increased by only 7% after immunoadsorption of TBPA.

DISCUSSION

An evaluation of the precise contribution of TBPA to the over-all binding of T_4 in human serum has been difficult. Electrophoretic methods which have commonly been employed to assess the apportionment of endogenous T_4 among the binding proteins inevitably raise the possibility that artifacts are produced by the pH, the supporting media or buffers used, the separation of proteins from one another, or the electrical field itself. It is not surprising, therefore, that the proporton of endogenous T_4 associated with TBPA, as judged from electrophoretic analyses, has varied greatly (4–8). In addition, unlike the case with TBG, instances of uncomplicated, idiopathic alterations in the concentration of TBPA have not been reported. Hence, it has not been possible to analyze the influence of alterations in TBPA, in the absence of known or possible alterations in other factors, upon the proportion of free T_4 in serum. Although the concentration and T_4-binding capacity of TBPA are decreased in the sera of many ill patients (9, 19), and this is usually accompanied by an increase in the proportion of free T_4 (9, 20, 21), concomitant decreases in T_4 binding by TBG or albumin may also occur and could contribute to or even account completely for the increase in the proportion of free T_4 observed. By the same token, there is no conclusive evidence that drugs which both inhibit binding of T_4 by TBPA and increase the proportion of free T_4 do not also decrease the binding of T_4 by other proteins (10). That factors other than TBPA may, in certain circumstances, be more important determinants of the proportion of free T_4 in serum is indicated by the studies of Inada and Sterling, in which changes in the proportion of free T_4 in the sera of patients with hyperthyroidism or hypothyroidism were shown to correlate more closely with variations in TBG than in TBPA (22).

The technique herein described has afforded a means of rendering normal serum free of TBPA without altering the content of TBG, as judged by T_4 binding, or the content of albumin, as judged by immunoelectrophoresis. Both immunoelectrophoresis and assessment of T_4 binding have indicated that the immunoadsorption technique removes TBPA from serum completely. This technique has, therefore, made possible an evaluation of two aspects of T_4 binding by TBPA: the proportion of endogenous T_4 bound by TBPA and the contribution of TBPA as a determinant of the proportion of free T_4 in serum. The first aspect was evaluated by determining the proportion of an exceedingly small concentration of tracer T_4 removed from serum by the immunoprecipitation of TBPA. The validity of this approach depends upon the ability of the antibody-bound TBPA to bind T_4 as strongly as does the native TBPA in the original serum. Electrophoretic analyses revealed that suspensions of the TBPA-antibody precipitate retained an average of 75% of the original binding capacity of TBPA. In all likelihood, this value represents an underestimate owing to limited access of T_4 to unoccupied binding sites within the insoluble aggregated TBPA-antibody complexes. It would have been indeed surprising if the enriching T_4 were to have had as ready access to binding sites within the macroscopic immunoprecipitate as it does to the protein in free solution. Nevertheless, the possibility cannot be excluded that slight loss of binding activity may have resulted from immunoprecipitation, leading to a slight underestimation of the proportion of T_4 actually bound by TBPA. Even if this were true, however, the inaccuracy introduced in the calculation of the equilibrium constants for the several proteins (see below) would be negligible.

The validity of the immunoprecipitation technique for assessing T_4 binding by TBPA is further supported by the results obtained in abnormal sera. In the sera from two ill subjects, in which the T_4-binding capacities of TBPA were greatly decreased, values for the per cent of immunoprecipitable T_4 were very low. Similarly, by virtue of its increased binding capacity, TBG in the serum from the pregnant subject should assume a much greater role than TBPA in T_4 binding relative to its role in normal serum. Here, the value for the per cent of immunoprecipitable T_4 was also low. Conversely, in the serum devoid of TBG, in which TBPA should assume a much greater role in T_4-binding than in normal serum, the value for the per cent of immunoprecipitable T_4 was very high. We would suggest, therefore, that the immunoprecipitation technique affords the most nearly reliable estimate of the proportion of endogenous T_4 actually bound by TBPA in human serum. In normal serum, the value of approximately 15% is considerably less than that which has generally been accepted, and is only about one-half of that found by conventional electrophoresis of the same samples in filter paper at pH 8.6. Possible difficulties in electrophoretic assessment, which may lead to overestimation of T_4-binding by TBPA, have been cited above.

The second aspect of T_4 binding that was studied was the contribution of TBPA towards determining the proportion of free T_4 in serum. For this purpose, an equilibrium dialysis method employing dilute serum was chosen. The decision to employ this method stemmed from the limited availability and high cost of the rabbit antihuman prealbumin antiserum. This dictated that TBPA be removed from only small volumes of human serum and, in turn, that dilutions thereof be employed for equilibrium dialysis. Had undiluted serum been employed, at least 25 times the volume of antiserum would have been required for each experiment. In the sera from five normal subjects, immunoadsorption of TBPA increased the per cent of free T_4 by 12–34% (mean, 22%). In the five sera in which the T_4-binding capacity of TBPA was greatly decreased, the increase in the per cent of free T_4 after immunoadsorption of TBPA ranged between 0 and 7% (mean, 4%). Similarly, as would be expected, only a small increase (7%) in the per cent of free T_4 was obtained in the serum from the pregnant subject, in which the binding capacity of TBG was greatly increased. On the other hand, in the serum devoid of TBG, the greatly increased initial value for the per cent of free T_4 was further increased by 132% by immunoadsorption of TBPA. These findings in abnormal sera support the conclusion that the changes in the proportion of free T_4 induced by immunoadsorption are indeed the result of removal of TBPA. This conclusion is further strengthened by the relationship, shown in Fig. 5, between the increase in the per cent of free T_4 after immunoadsorption of TBPA and the per cent of immunoprecipitable T_4. For the eight sera in which both values were measured, this relationship conformed to a simple linear function with a correlation coefficient that approached 1.0.

From a comparison of the per cent of free T_4 in the serum spontaneously devoid of TBG with the values found in normal sera rendered free of TBPA, it is clear that TBG is the much more important determinant of the proportion of free T_4 in normal human serum. Hence, as would be expected, the per cent of free T_4 in individual samples of serum rendered free of TBPA displayed

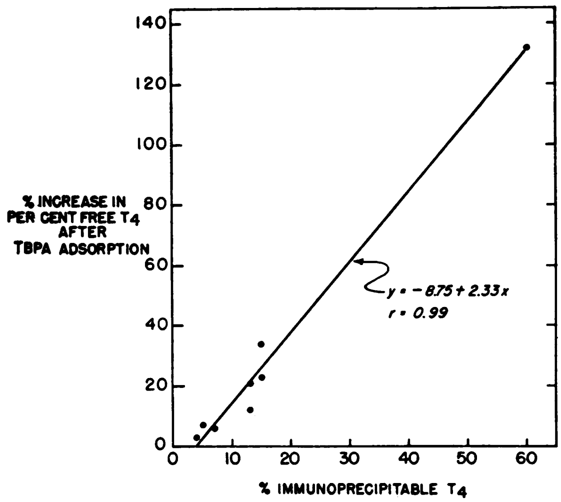

FIGURE 5 The relationship between the increase in the per cent of free T_4 in serum after immunoadsorption of TBPA and the per cent of T_4 that had been immunoprecipitated by antihuman prealbumin gamma globulin.

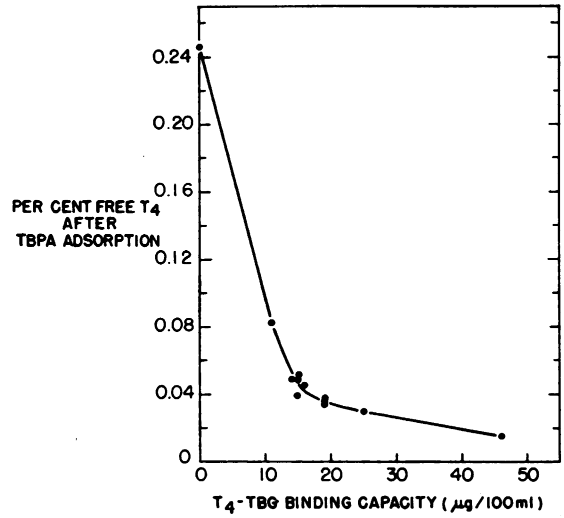

FIGURE 6 The relationship between the per cent of free T_4 in serum after immunoadsorption of TBPA and the T_4-binding capacity of TBG.

a close inverse relationship to the T_4-binding capacity of TBG (Fig. 6). The curvilinear nature of this relationship would be predicted from the mass law expression governing binding interactions between T_4 and one or more binding components (23).

From observations in the literature, the inference has generally been drawn that the decrease in TBPA in the sera of ill patients is largely responsible for the increase in the proportion of free T_4 that such sera display. The present findings indicate that the cause of the increased proportion of free T_4 in these sera is considerably more complex. Values for the per cent of free T_4 in the sera of ill patients were generally greater than normal, as has previously been reported (9, 20, 21). However, comparably increased values were not produced in normal sera by complete removal of TBPA, indicating that a decrease in TBPA alone cannot account for the increased proportion of free T_4 in the sera of ill patients. Evidence from the present studies cited above, as well as that obtained by others (22), indicates that TBG is the more important determinant of the proportion of free T_4 in serum, other factors being equal. Consonant with this conclusion is the observation that, in ill patients, the proportion of free T_4 was highest when the binding capacity of TBG was low, and vice versa. Nevertheless, the participation of still other factors in decreasing the over-all binding of T_4 in the sera of ill patients cannot be excluded.

From the several measurements made in the various sera studied before and after immunoadsorption of TBPA, it has been possible to derive estimates of the equilibrium constants for the interactions of T_4 with albumin, TBPA, and TBG, respectively. These values were calculated from the general formulation described by Robbins and Rall (23). For whole serum,

$$(T_4) = \frac{(T_4\cdot TBG) + (T_4\cdot TBPA) + (T_4\cdot ALB)}{[K_{TBG}(TBG)] + [K_{TBPA}(TBPA)] + [K_{ALB}(ALB)]}$$

where T_4 = concentration of free or unbound T_4; T_4·protein = concentration of binding sites occupied by T_4 on the protein indicated; protein = concentration of unoccupied binding sites on the protein indicated; $K_{protein}$ = equilibrium constant for the binding interaction between T_4 and the protein indicated. Sample calculations, including relevant assumptions, are presented in the appendix. In general, however, the calculations were carried out in the following sequence. An estimate of K_{ALB} was derived from the measured concentrations of free and bound T_4 in the serum devoid of

TBG after immunoadsorption of TBPA, assuming that albumin was the only remaining binding protein. From this value of K_{ALB} and the measured concentrations of free and bound T_4 in the same serum before immunoadsorption of TBPA, an estimate of K_{TBPA} was derived. From the estimates of K_{ALB} and K_{TBPA}, it was possible to derive in a similar manner estimates of K_{TBG} in each of the five normal sera and also in each of the six abnormal sera (exclusive of the serum devoid of TBG). Finally, from the equilibrium constants obtained, predicted values for the per cent of free T_4 after immunoadsorption of TBPA from these 11 sera were derived, and these were compared to the values obtained by direct measurement.

The foregoing calculations yielded an estimated K_{ALB} of 6.2×10^5. Although previously reported estimates of K_{ALB}, based on studies with the purified protein, have varied somewhat, values of 1.6×10^6 (24) and 1.4×10^6 (25) have been described. Since chloride ions apparently decrease the affinity of albumin for T_4, it is of interest that the value we have derived, obtained in the presence of chloride ions, is very similar to that found by Tabachnick (25) for purified albumin in the presence of chloride ions.

The estimated value for K_{TBPA} of 2.3×10^8 is of the same order of magnitude as that derived by Oppenheimer and Surks (3.56×10^8) (26) and indicates a net affinity for T_4 several hundred times that of albumin.

The derived values for K_{TBG} in the five normal sera averaged 1.7×10^{10} with a range of 1.3–2.3 $\times 10^{10}$. In a recently published abstract describing studies with a purified TBG, Sterling, Hamada, Newman, Brenner, and Inada state that K_{TBG} is of an order of magnitude of 10^{10} (27). Derived values in the six abnormal sera (mean, 1.5×10^{10}; range, $1.2–1.9 \times 10^{10}$), in which the T_4-binding capacities of TBG ranged between 11 and 46 μg/100 ml, agreed closely with those obtained in the normal sera. This indicates that the alterations in the concentration of T_4-binding sites on TBG, which occur in ill patients or in pregnancy, are not accompanied by changes in the specific affinity of such sites for T_4.

Since the value for K_{TBPA} was only derived from one serum (that devoid of TBG), an estimate of its reliability was sought in a comparison of the predicted and observed effects of immunoadsorption of TBPA on the per cent of free T_4 in each of the 11 remaining sera. For the five normal sera, the differences between the predicted and measured values, expressed as a per cent of the latter, ranged between 2 and 13% and averaged 6%. For the six abnormal sera, the differences ranged between 0 and 8% and averaged 4%. This close concordance indicates that the value for K_{TBPA} derived from the single serum devoid of TBG can be reliably applied to the TBPA in normal sera. Moreover, it suggests that the affinity for T_4 of the binding sites on TBPA in the sera of ill patients is not greatly different from normal.

From the estimates of K_{TBG}, K_{TBPA}, and K_{ALB}, one may predict that in normal serum approximately 75% of the endogenous T_4 is bound by TBG, 15% by TBPA, and 10% by albumin. The latter value is considerably less than that which would be suggested by the marked elevation of the per cent of free T_4 that has been found in analbuminemic serum (28). Therefore, a direct evaluation of the per cent of free T_4 in normal serum rendered free of albumin would be of interest, and we are currently engaged in efforts to apply the immunoadsorption technique to this problem.

APPENDIX

For calculating the equilibrium constants of albumin, TBPA, and TBG, several general assumptions were made. First, it was assumed that TBG and TBPA possess only one T_4-binding site per molecule; direct analysis has indicated that this is true in the case of TBPA (2). Hence, the molar concentrations of these proteins in serum could be directly equated with their measured molar T_4-binding capacities. Second, albumin was assumed to have a mol wt of 69,000 and a single primary T_4-binding site. Other assumptions will be indicated in the ensuing presentation of sample calculations.

Calculation of K_{ALB}. K_{ALB} was calculated from the data obtained in the serum devoid of TBG after immunoadsorption of TBPA, according to the formula:

$$(T_4) = \frac{(T_4 \cdot ALB)}{K_{ALB}(ALB)}$$

Here, 10 μg/liter = original endogenous T_4 concentration; 0.4 = fraction of nonimmunoprecipitable T_4; 19 μg/liter = contribution of ^{131}I-labeled

T_4 used for measurement of the per cent of free T_4 (common to all experiments). Hence, the final total T_4 concentration equals:

$$(10 \times 0.4) + 19\ \mu g/\text{liter}$$
$$= 23\ \mu g/\text{liter or } 29.60 \times 10^{-9}\ \text{mole/liter}$$
$$(T_4) = 29.60 \times 10^{-9} \times \text{measured free } T_4 \text{ fraction}$$
$$= 29.60 \times 10^{-9} \times 0.00246$$
$$= 72.82 \times 10^{-12}\ \text{mole/liter}$$
$$(T_4\cdot ALB) = (29.60 \times 10^{-9}) - (72.82 \times 10^{-12})$$
$$= 29.53 \times 10^{-9}\ \text{mole/liter.}$$

Since $(T_4\cdot ALB)$ represents a vanishingly small proportion of the total albumin concentration, in this and all subsequent calculations the total albumin concentration and (ALB) will be considered equivalent. Hence,

$$(ALB) = 45 \div 69{,}000$$
$$= 6.52 \times 10^{-4}\ \text{mole/liter.}$$

From which,

$$K_{ALB} = \frac{(29.53 \times 10^{-9})}{(72.82 \times 10^{-12}) \times (6.52 \times 10^{-4})}$$
$$= 6.22 \times 10^5.$$

Calculation of K_{TBPA}. K_{TBPA} was calculated from the data obtained in the same serum before immunoadsorption of TBPA, according to the formula:

$$(T_4) = \frac{(T_4\cdot ALB) + (T_4\cdot TBPA)}{[K_{ALB}(ALB)] + [K_{TBPA}(TBPA)]}$$

Here, the final total T_4 concentration equals:

$$10 + 19\ \mu g/\text{liter}$$
$$= 29\ \mu g/\text{liter or } 37.32 \times 10^{-9}\ \text{mole/liter}$$
$$(T_4) = 37.32 \times 10^{-9} \times 0.00092$$
$$= 34.33 \times 10^{-12}\ \text{mole/liter.}$$
$$(T_4\cdot ALB) + (T_4\cdot TBPA)$$
$$= (37.32 \times 10^{-9}) - (34.33 \times 10^{-12})$$
$$= 37.29 \times 10^{-9}\ \text{mole/liter.}$$

In calculating (TBPA), the proportion of immunoprecipitable T_4 was considered to indicate the proportionate distribution of T_4 between TBPA and albumin. Hence,

$$(TBPA) = (2320 \times 10^{-6} \div 777) - (37.29 \times 10^{-9} \times 0.6)$$
$$= 2.97 \times 10^{-6}\ \text{mole/liter.}$$

From which,

$$K_{TBPA} = 2.29 \times 10^8.$$

Calculation of K_{TBG}. The calculation for K_{TBG} in serum No. 1 will be presented.

$$(T_4) = \frac{(T_4\cdot ALB) + (T_4\cdot TBPA) + (T_4\cdot TBG)}{[K_{ALB}(ALB)] + [K_{TBPA}(TBPA)] + [K_{TBG}(TBG)]}$$

Here, the final total T_4 concentration equals:

$$75 + 19\ \mu g/\text{liter}$$
$$= 94\ \mu g/\text{liter or } 120.98 \times 10^{-9}\ \text{mole/liter}$$
$$(T_4) = 120.98 \times 10^{-9} \times 0.00028$$
$$= 33.87 \times 10^{-12}\ \text{mole/liter.}$$
$$(T_4\cdot ALB) + (T_4\cdot TBPA) + (T_4\cdot TBG)$$
$$= (120.98 \times 10^{-9}) - (33.87 \times 10^{-12})$$
$$= 120.95 \times 10^{-9}\ \text{mole/liter.}$$

From the fraction of immunoprecipitable T_4 (0.15),

$$(T_4\cdot TBPA) = 120.95 \times 10^{-9} \times 0.15$$
$$= 18.14 \times 10^{-9}\ \text{mole/liter}$$

Therefore,

$$(TBPA) = (2380 \times 10^{-6} \div 777) - (18.14 \times 10^{-9})$$
$$= 3.04 \times 10^{-6}\ \text{mole/liter}$$
$$(ALB) = 50 \div 69{,}000$$
$$= 7.25 \times 10^{-4}\ \text{mole/liter.}$$

To calculate (TBG), the approximate apportionment of T_4 between TBPA and albumin was first calculated as follows:

$$\frac{(T_4\cdot TBPA)}{(T_4\cdot ALB)} = \frac{K_{TBPA}(TBPA)}{K_{ALB}(ALB)}$$
$$= \frac{(2.29 \times 10^8) \times (3.04 \times 10^{-6})}{(6.22 \times 10^5) \times (7.25 \times 10^{-4})}$$
$$= \frac{1.54}{1}.$$

Since 15% of the bound T_4 is associated with TBPA, $15 \div 1.54$ or approximately 10% represents the proportion of bound T_4 associated with albumin. Hence, 75% represents the proportion associated with TBG. Therefore,

$$(T_4\cdot TBG) = 120.95 \times 10^{-9} \times 0.75$$
$$= 90.71 \times 10^{-9}\ \text{mole/liter}$$
$$(TBG) = (190 \times 10^{-6} \div 777) - (90.71 \times 10^{-9})$$
$$= 1.54 \times 10^{-7}\ \text{mole/liter.}$$

From which,

$$K_{TBG} = 1.57 \times 10^{10}.$$

Prediction of the per cent of free T_4 after immunoadsorption of TBPA. The calculation presented is also based on the data obtained in serum No. 1.

$$(T_4) = \frac{(T_4\cdot ALB) + (T_4\cdot TBG)}{[K_{ALB}(ALB)] + [(K_{TBG}(TBG)]}$$

Here, the final total T_4 concentration is reduced by immunoprecipitation of 15% of the endogenous T_4 concentration. Therefore, it equals $(75 \times 0.85) + 19 = 83\ \mu g/\text{liter}$ or 106.82×10^{-9} mole/liter. It is assumed, furthermore, that the total and bound T_4 concentrations are equal. Since the per cent of free T_4 is less than 0.1, the error introduced by this assumption is negligible.

In the same serum before immunoadsorption of TBPA, the ratio of T_4 bound by TBG to that bound by albumin was 7.5:1. It is assumed that the residual T_4 after immunoadsorption of TBPA is distributed between TBG and albumin in the same ratio. Hence, approximately 88% would be associated with TBG and 12% with albumin. Therefore,

$$(\mathrm{TBG}) = (190 \times 10^{-6} \div 777) - (106.82 \times 10^{-9} \times 0.88)$$
$$= 1.51 \times 10^{-7} \text{ mole/liter}$$

and

$$(\mathrm{T_4}) = \frac{(106.82 \times 10^{-9})}{(6.22 \times 10^{-5}) \times (7.25 \times 10^{-4}) + (1.57 \times 10^{10}) \times (1.51 \times 10^{-7})}$$
$$= 37.86 \times 10^{-12} \text{ mole/liter.}$$

Therefore,

$$\text{per cent free } \mathrm{T_4} = \frac{37.86 \times 10^{-12} \times 100}{106.82 \times 10^{-9}}$$
$$= 0.035.$$

ACKNOWLEDGMENTS

The authors are indebted to Dr. Bernard J. Ransil for his valuable assistance with the mathematical aspects of the study.

This work was supported in part by research grant AM-09753 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

REFERENCES

1. Ingbar, S. H. 1963. Observations concerning the binding of thyroid hormones by human serum prealbumin. *J. Clin. Invest.* **42**: 143.
2. Oppenheimer, J. H., M. I. Surks, J. C. Smith, and R. Squef. 1965. Isolation and characterization of human thyroxine-binding prealbumin. *J. Biol. Chem.* **240**: 173.
3. Purdy, R. H., K. A. Woeber, M. T. Holloway, and S. H. Ingbar. 1965. Preparation of crystalline thyroxine-binding prealbumin from human plasma. *Biochemistry.* **4**: 1888.
4. Hollander, C. S., V. V. Odak, T. E. Prout, and S. P. Asper. 1962. An evaluation of the role of prealbumin in the binding of thyroxine. *J. Clin. Endocrinol. Metab.* **22**: 617.
5. Ingbar, S. H., and N. Freinkel. 1960. Regulation of the peripheral metabolism of the thyroid hormones. *Recent Progr. Hormone Res.* **16**: 353.
6. Blumberg, B. S., and J. Robbins. 1960. Thyroxine-serum protein complexes: single dimension gel and paper electrophoresis studies. *Endocrinology.* **67**: 368.
7. Squef, R., M. Martinez, and J. H. Oppenheimer. 1963. Use of thyroxine-displacing drugs in identifying serum thyroxine-binding proteins separated by starch gel electrophoresis. *Proc. Soc. Exptl. Biol. Med.* **113**: 837.
8. Rich, C., and A. G. Bearn. 1958. Localization of the thyroxine-binding protein of serum by starch gel electrophoresis. *Endocrinology.* **62**: 687.
9. Oppenheimer, J. H., R. Squef, M. I. Surks, and H. Hauer. 1963. Binding of thyroxine by serum proteins evaluated by equilibrium dialysis and electrophoretic techniques. Alterations in non-thyroidal illness. *J. Clin. Invest.* **42**: 1769.
10. Woeber, K. A., and S. H. Ingbar. 1964. The effects of noncalorigenic congeners of salicylate on the peripheral metabolism of thyroxine. *J. Clin. Invest.* **43**: 931.
11. Fahey, J. L., P. F. McCoy, and M. Goulian. 1958. Chromatography of serum proteins in normal and pathologic sera: the distribution of protein-bound carbohydrate and cholesterol, siderophilin, thyroxin-binding protein, B_{12}-binding protein, alkaline and acid phosphatases, radio-iodinated albumin, and myeloma proteins. *J. Clin. Invest.* **37**: 272.
12. Schussler, G. C., and J. E. Plager. 1967. Effect of preliminary purification of ^{131}I-thyroxine on the determination of free thyroxine in serum. *J. Clin. Endocrinol. Metab.* **27**: 242.
13. Refetoff, S., and H. A. Selenkow. 1967. Familial thyroxine binding globulin (TBG) deficiency in a patient with Turner's syndrome (XO): genetic study of a kindred. Program, Annual Meeting of the American Thyroid Association. 89. (Abstr.)
14. Ingbar, S. H. 1961. Clinical and physiological observations in a patient with an idiopathic decrease in the thyroxine-binding globulin of plasma. *J. Clin. Invest.* **40**: 2053.
15. Elzinga, K. E., E. A. Carr, Jr., and W. H. Beierwaltes. 1961. Adaptation of the standard Durrum-type cell for reverse-flow paper electrophoresis. *Am. J. Clin. Pathol.* **36**: 125.
16. Wunderly, C. 1960. The technique of immun-electrophoresis in agar gel. *In* A Laboratory Manual of Analytical Methods of Protein Chemistry. P. Alexander and R. J. Block, editors. Pergamon Press, Inc., New York. **2**: 231.

17. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751.
18. Murphy, B. E. P., and C. J. Pattee. 1964. Determination of thyroxine utilizing the property of protein-binding. *J. Clin. Endocrinol. Metab.* **24:** 187.
19. Richards, J. B., J. T. Dowling, and S. H. Ingbar. 1959. Alterations in the plasma transport of thyroxine in sick patients and their relation to the abnormality in Graves' disease. *J. Clin. Invest.* **38:** 1035. (Abstr.)
20. Ingbar, S. H., L. E. Braverman, N. A. Dawber, and G. Y. Lee. 1965. A new method for measuring the free thyroid hormone in human serum and an analysis of the factors that influence its concentration. *J. Clin. Invest.* **44:** 1679.
21. Sterling, K., and M. A. Brenner. 1966. Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. *J. Clin. Invest.* **45:** 153.
22. Inada, M., and K. Sterling. 1967. Thyroxine transport in thyrotoxicosis and hypothyroidism. *J. Clin. Invest.* **46:** 1442.
23. Robbins, J., and J. E. Rall. 1960. Proteins associated with the thyroid hormones. *Physiol. Rev.* **40:** 415.
24. Steiner, R. F., J. Roth, and J. Robbins. 1966. The binding of thyroxine by serum albumin as measured by fluorescence quenching. *J. Biol. Chem.* **241:** 560.
25. Tabachnick, M. 1967. Thyroxine-protein interactions. IV. Thermodynamic values for the association of thyroxine with human serum albumin. *J. Biol. Chem.* **242:** 1646.
26. Oppenheimer, J. H., and M. I. Surks. 1964. Determination of free thyroxine in human serum: a theoretical and experimental analysis. *J. Clin. Endocrinol. Metab.* **24:** 785.
27. Sterling, K., S. Hamada, E. S. Newman, M. A. Brenner, and M. Inada. 1967. Properties of the thyroxine binding alpha-globulin (TBG). *Clin. Res.* **15:** 457 (Abstr.)
28. Hollander, C. S., G. Bernstein, and J. Oppenheimer. 1966. Thyroxine binding in analbuminemia. Program, 48th Meeting of the Endocrine Society, Abstract No. 55.