Preparation of ^{125}I-Labeled Human Thyroxine-Binding Alpha Globulin and Its Turnover in Normal and Hypothyroid Subjects

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ABSTRACT A protein with the electrophoretic, immunologic, and hormone-binding properties of thyroxine-binding globulin (TBG) has been prepared from human plasma and labeled with radioiodine (^{125}I) by an enzymatic method of iodination. The [^{125}I]TBG retained the electrophoretic and immunologic characteristics of unlabeled TBG but exhibited a partial loss of thyroxine-binding activity, as assessed by affinity chromatography. The in vivo behavior of [^{125}I]TBG was studied in six euthyroid subjects (controls) with normal serum levels of TBG as measured both by radioimmunoassay and by determination of maximal T_4-binding capacity and in four male patients with untreated primary hyperthyroidism, three of whom had elevated serum TBG. The half-time of the final slope of the plasma disappearance curve averaged 5.0 days ±1.2 (SD) in the controls and ranged from 3.9 to 10.9 days in the hypothyroid patients. The distribution volume was similar in the two groups, 6.7±1.3 vs. 7.1±2.1 liters. The catabolic clearance rate averaged 0.99±0.33 liters plasma/24 h in the controls and 0.92±0.46 in the hypothyroids. The absolute turnover rate of TBG, calculated from the catabolic clearance rate multiplied by the serum concentration of radioimmunoassayable TBG, averaged 17.8±2.1 mg/day in the controls and ranged from 14.8 to 33.2 mg/day in the hypothyroids. Among the entire group of subjects there was no correlation between the serum TBG concentration and the absolute turnover rate of TBG.

INTRODUCTION

Thyroxine-binding globulin (TBG),[1] the major extracellular thyroid hormone-binding protein in man, has been isolated and studied by several groups (2–7). It is a glycoprotein with a single binding site for thyroxine. Most workers place its molecular weight at about 60,000. The plasma concentration of TBG, measured by maximal T_4-binding capacity (8) or by radioimmunoassay (9), varies over a wide range depending upon genetic and environmental factors. As is true for all plasma proteins, the level of TBG in the plasma is determined by the relative rates of synthesis and removal and by the distribution of the protein between intravascular and extravascular compartments.

The present study was undertaken in order to define the in vivo distribution and metabolism of TBG in humans. This report describes a method of preparing TBG from human plasma and of labeling the protein with radioiodine. Some of the chemical and immunologic properties of the labeled TBG will be presented as well as the in vivo behavior of this tracer in human subjects. While this investigation was in progress, Refetoff, Fang, Robin, and Marshall published findings of a similar study in abstract form (10).

METHODS

Materials

The starting material for the purification of TBG was blood bank plasma, collected from volunteer donors and

[1] *Abbreviations used in this paper:* HSA, human serum albumin; T_3, 3,5,3'-triiodo-L-thyronine; T_4, thyroxine; TBPA, thyroxine-binding prealbumin; PAGE, polyacrylamide gel electrophoresis; PB ^{125}I, trichloroacetic acid-precipitable ^{125}I.

stored in acid-citrate-dextrose at 4°C. Sepharose 2B and Sephadex G-75 and G-25, were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Cyanogen bromide was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y., and used only from newly opened bottles. 3,5,3'-Triiodo-L-thyronine (T_3) and L-thyroxine (T_4), both in the form of the sodium salt, were supplied by Sigma Chemical Co., St. Louis, Mo. Acrylamide and bisacrylamide (N,N'-methylene bisacrylamide) were obtained from Bio-Rad Laboratories, Richmond, Calif., tetramethylethylenediamine was from Eastman Organic Chemicals Div., and ammonium persulfate was from Mallinckrodt Chemical Works, St. Louis, Mo. Lactoperoxidase was obtained from Calbiochem, La Jolla, Calif. Other chemicals used were reagent-grade and purchased from commercial suppliers. [^{125}I]iodide (for iodination) was obtained in carrier-free form from New England Nuclear, Boston, Mass. ^{131}I-labeled T_4 (sp act 20–50 μCi/μg) and ^{131}I-labeled T_3 (sp act 10–30 μCi/μg) were obtained from Amersham/Searle Corp., Arlington Heights, Ill.

Purification of TBG

Preparation of T_3-substituted Sepharose. The method described by Pensky and Marshall (11) for the preparation of T_4-substituted Sepharose was modified as follows: T_3, 100 mg, labeled with approximately 10 μCi [^{131}I]T_3 and dissolved in 5 ml 0.1 M KOH, was added to 100 ml cyanogen bromide-activated Sepharose 2B. The mixture was stirred gently for 16 h at 4°C. Unreacted T_3 was removed by washing the settled Sepharose with 100 vol of 0.1 M NaHCO$_3$, pH 8.6. The final yield of T_3 coupled to Sepharose, determined from the [^{131}I]T_3 content in the washed Sepharose, ranged from 25 to 55% (0.30–0.66 mg T_3/ml Sepharose).

Affinity chromatography. Approximately 180 ml of human plasma diluted with an equal volume of 0.05 M sodium phosphate buffer, pH 7.0, was applied to a 1 × 10-cm column containing T_3-Sepharose (bed volume 2.5 ml) at a rate of approximately 1 ml/min, at room temperature. The column was washed with about 100 ml of phosphate buffer in order to remove weakly adsorbed protein. Completeness of wash was determined by measuring the optical density at 280 nm of the column eluate and finding a constant minimal value. TBG together with small quantities of other proteins, mainly albumin, were eluted with 15 ml sodium glycinate buffer, pH 9.2, containing 0.5 μg T_4/ml. This eluate was dialyzed at 4°C for 4 h against 2,000 ml 0.005 M glycinate and then lyophilized and stored until the following step of purification.

Preparative polyacrylamide gel electrophoresis (PAGE). The lyophilized affinity-column eluate from 3–4 T_3-Sepharose columns, representing about 600 ml plasma, was reconstituted with deionized water. ^{131}I-labeled T_4 was added as a marker. This mixture (about 2 ml) was applied to a 15 × 25 × 0.6-cm polyacrylamide gel slab and subjected to electrophoresis at 200 V for 30 min, then at 400 V for 90 min in a water-cooled vertical gel electrophoresis chamber (12) obtained from Buchler Instruments, Fort Lee, N. J. Immediately after preparative gel electrophoresis, the gel was sliced into 3-mm-wide segments. Those segments containing the highest concentration of [^{131}I]T_4, as determined in a well-counter were minced, suspended in 4 ml 0.1 M sodium phosphate buffer, pH 7.4, and subjected to elution by electrophoresis in a specially constructed apparatus.[2] The protein eluted by this method was concentrated into a volume of 0.5–1.0 ml with a Minicon® concentrator (Ami-

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[2] McMahon, F. A. Unpublished.

con Corp., Scientific Sys. Div., Lexington, Mass.). Aliquots were removed for determination of total protein by the method of Lowry, Rosebrough, Farr, and Randall (13) and for analysis by disk electrophoresis by the method of Davis (14).

Iodination of TBG

To approximately 300 μg purified TBG, in 0.05 M sodium phosphate buffer, pH 7.4, were added 6 μg lactoperoxidase and 5 mCi [^{125}I]iodide, all in a total volume of 0.5 ml. The iodination reaction, which was carried out at room temperature, was initiated and maintained by two additions, 20 ng each, of H$_2$O$_2$ at intervals of 60 s. 60 s after the second addition of H$_2$O$_2$ the reaction was stopped with sodium metabisulfite. Unreacted iodide was separated from protein on a 1 × 12-cm column of Sephadex G-75 medium grade that had previously been washed with 1% human serum albumin (HSA) in 0.05 M sodium phosphate buffer, pH 7.4. The iodination yield ranged from 20 to 40%.

Final purification of [^{125}I]TBG was performed by polyacrylamide gel (slab) electrophoresis in the same Buchler apparatus. [^{131}I]T_4 was added to the [^{125}I]TBG before electrophoresis. Gel sections containing ^{125}I were subjected to elution. The [^{125}I]TBG was stored at 4°C in 0.05 M sodium phosphate, pH 7.4, containing HSA, 10 mg/ml.

Antibody preparation

Two 2-kg male New Zealand albino rabbits were immunized with 200–400 μg purified TBG, emulsified in 1 ml complete Freund's adjuvant. Inoculation was done in 15–20 sites over the back of each animal at intervals of 2–3 wk for a total of four inoculations. One rabbit was given a final "booster" immunization of 700 μg partially purified TBG (affinity-column eluate). Blood was collected at 10–14 days after each inoculation. The titer of the rabbit antisera was determined from the dilution of antiserum that caused precipitation of 50% of added [^{125}I]TBG (in a minimal tracer amount) with either polyethylene glycol or antirabbit goat serum used to precipitate the immune complex. Immunoelectrophoresis was performed according to the method of Scheidegger (15).

Radioimmunoassay of serum TBG

The assay was carried out in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1% normal rabbit serum, rabbit antiserum against human TBG, produced as described above, in a final dilution of 1:72,000, [^{125}I]TBG (1–2 ng TBG protein), and a diluted sample of test serum, equivalent to 0.5–1 μl of undiluted serum. After incubation of the above mixture for 48 h at 4°C, a second antibody (goat antirabbit serum) was added. After an additional 24 h, the immune precipitate was separated by centrifugation and assayed for ^{125}I. A standard curve was constructed with each assay by using a pool of normal sera as a reference standard. The concentration of TBG in this serum pool was determined to be 1.82 mg/100 ml by using highly purified TBG as a primary standard. The results in patients' sera were expressed in mg TBG/100 ml by referring to this serum pool. All samples were run in triplicate. The minimum concentration of TBG detectable by this assay is 1.0 ng TBG (0.067 μl serum). The specificity of the assay for TBG was established by finding no significant displacement of [^{125}I]TBG from the antibody when sera from two individuals

with congenital, isolated absence of TBG[3] were added in amounts up to 25 times the concentration of normal serum required to displace approximately 50% of added tracer.

Determination of T_4-binding capacity of purified TBG

Small columns, fashioned from 1 ml tuberculin syringes, were filled with Sephadex, G-25 fine grade, to a bed-volume of 0.7 ml. These were equilibrated with 0.1 M Tris-glycinate buffer, pH 8.3. To solutions of a known concentration of purified TBG and a tracer quantity of $[^{131}I]T_4$ in the same buffer were added various known amounts of unlabeled L-T_4. After equilibration of these mixtures for 30 min at room temperature, 50 μl of each was applied to a Sephadex column and allowed to enter the gel. The protein (TBG) was eluted from the column with 1.5 ml Tris-glycinate buffer. The proportion of $[^{131}I]T_4$ eluted with protein was determined and used to calculate the amount of total T_4 bound to TBG. The maximal T_4-binding capacity of TBG was computed from the maximal amount of T_4 bound per unit quantity of TBG protein.

Patients

There were six subjects in the control group. All were eumetabolic by clinical assessment, had normal levels of serum T_4 and free T_4 index, and normal serum TBG T_4-binding capacity. One patient in this group (E. L.) had undergone a total thyroidectomy 20 yr previously and had been taking L-T_4, 0.2 mg/day, continuously since his operation. The other five subjects had no thyroid disease.

Four patients with primary thyroid failure were studied. All exhibited clinical features of hypothyroidism in varying degrees, had low levels of serum T_4 and free T_4 index and elevated serum thyroid-stimulating hormone (radioimmunoassay). Serum TBG T_4-binding capacity was above the normal range in three members of this group.

In vivo kinetics studies

$[^{125}I]$TBG, from 5 to 15 μCi (1–3 μg TBG protein), in 0.1% HSA, was sterilized by Millipore filtration immediately before intravenous administration to each subject. Samples of blood were collected at 30 min and 4, 6, and 24 h postdose and at 24-h intervals thereafter for a period of 10–14 days. Complete 24-h collections of urine were made for the same period. Each subject received saturated solutions of potassium iodide, five drops twice daily for the duration of the study. Plasma samples were assayed for total ^{125}I concentration, trichloroacetic acid-precipitable ^{125}I (PB ^{125}I), and ethanol-insoluble ^{125}I. Aliquots of the injected dose, diluted in normal human plasma, were subjected to the above procedure.

Analysis of kinetics data

After an initial phase of 2 or 3 days, the plasma disappearance curve of PB ^{125}I approximated a single exponential slope from which a fractional turnover rate, k, was calculated ($k = \ln 2/t_{\frac{1}{2}}$). The daily urinary excretion of

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³ A serum sample from one of these individuals was generously supplied by Dr. Samuel Refetoff, who found no immunoreactive TBG as well as no T_4-binding activity by the alpha-globulins of this patient's serum (16).
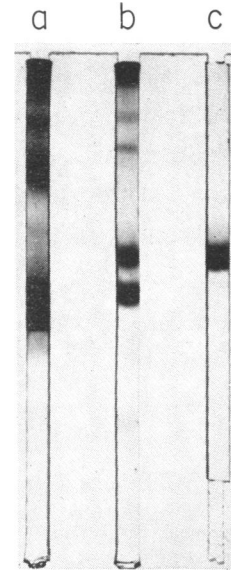

FIGURE 1 Disk electrophoretic analysis of proteins during the purification of TBG. The origin is at the top and the anode at the bottom. Proteins were stained with amido black. (a) Whole serum for reference. (b) Eluate of the affinity chromatographic step showing a prominent albumin band, an equally dense TBG band, and several other components. (c) Final preparation, following preparative PAGE, showing a single stainable component corresponding to TBG.

^{125}I, from day 3 to the end of each study, declined in parallel with the plasma PB ^{125}I concentration. A catabolic clearance rate, in liters plasma per day, was calculated, for each 24-h interval after day 2, from the urinary ^{125}I divided by the mean plasma PB ^{125}I concentration during that interval. The clearance rate, so calculated, was reasonably constant during the study in each case. The total distribution volume of $[^{125}I]$TBG was computed from the mean clearance rate (liters/day) divided by the fractional turnover rate, k (days^{-1}). This method of analysis assumes that the final plasma PB ^{125}I slope accurately reflects the turnover rate of the TBG pool and that isotopic equilibrium is achieved during the initial 2–3 days postinjection. Furthermore, fecal (nonurinary) losses of tracer and tracee are neglected. The absolute turnover rate was calculated as the product of the serum TBG concentration (determined by radioimmunoassay) and the catabolic clearance rate.

Other procedures

Total serum T_4 was measured by a competitive-binding technique (17). Serum free T_4 index was determined as the product of total T_4 times the uptake of labeled T_4 by Sephadex, both values expressed as a percentage of the value obtained in a pool of normal sera (18). The maximal T_4-binding capacity of serum TBG was determined by using reverse-flow paper electrophoresis in ammonium carbonate buffer, pH 8.4, according to Robbins (8).

RESULTS

Purification of TBG. The protein eluted from the affinity columns included albumin, globulins, and a component with the electrophoretic mobility of TBG (Fig.
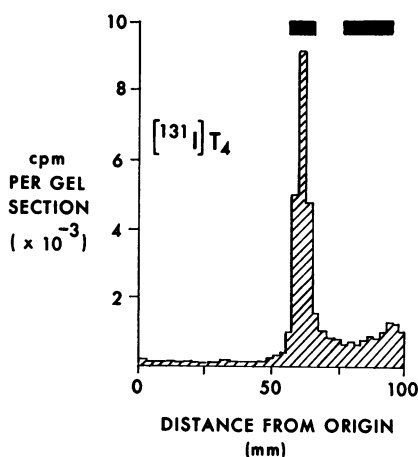
FIGURE 2 Analytic PAGE of the T_4-binding protein in the eluate of the affinity column. The positions of the major stained protein components are shown in solid black. This single large peak containing most of the [^{131}I]T_4 (hatched area) corresponds to the position of the stainable protein cathodal to serum albumin.

1). Labeled T_4, added to this affinity-column eluate, migrated on analytical PAGE to a position corresponding exactly to that of TBG (Fig. 2). Final purification of preparative PAGE yielded a product with a single stainable protein on analytical disk gel electrophoresis (Fig. 1c). The yield of TBG, based upon protein recovered in the final purification step and assuming an initial concentration of TBG of 1.5 mg/100 ml plasma, ranged from 11 to 17%.

Characterization of purified TBG. The maximal T_4-binding capacity of purified TBG was determined to be 11.0 μg T_4/mg TBG protein. If we assume a molecular weight of 60,000 for TBG (6), this result indicates that
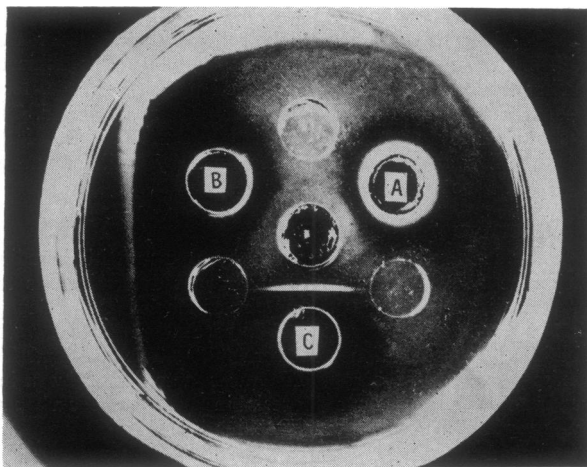

FIGURE 3 Ouchterlony double-diffusion analysis of purified TBG. Center well, purified human TBG. (A) Rabbit antiserum against whole human serum. (B) Rabbit antiserum against HSA. (C) Rabbit antiserum against purified TBG.
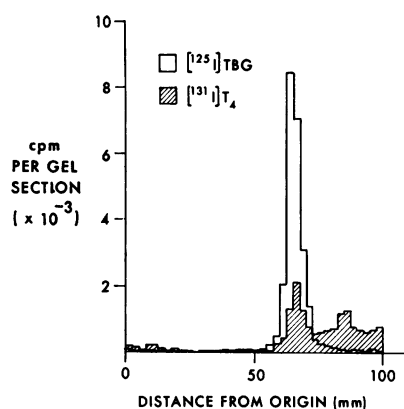

FIGURE 4 Analytical gel electrophoresis of ^{125}I-labeled TBG. A small quantity of serum albumin was added after the iodination reaction. [^{131}I]T_4 was added before analysis in order to identify hormone-binding proteins. After electrophoresis, the gel was sliced into 3-m sections. The [^{131}I]T_4 migrating anodal (to the right) of the [^{125}I]TBG peak represents both albumin-bound and free T_4.

0.85 mol of T_4 are bound per mole of TBG. Upon gel filtration of purified TBG labeled with [^{131}I]T_4 on a Bio-Gel P-60 column the protein appeared at an elution volume identical to that of HSA, indicating a similar molecular size of the two proteins. As further evidence of purity of the final TBG product, Ouchterlony double-diffusion analysis of purified TBG exhibited a single precipitin line against rabbit antiserum to TBG and no visible precipitation against either a highly potent antiserum to HSA or an antiserum to whole serum (Fig. 3). (Presumably, the latter antiserum contained too low a titer of antibodies against TBG to give a visible precipitin line.)

Characterization of [^{125}I]TBG. The yield of iodinated TBG, determined by gel filtration of the iodination mixture on Sephadex G-75, ranged from 24 to 38% of total [^{125}I]iodide in the mixture. Analytical gel (slab) electrophoresis of the [^{125}I]TBG, to which [^{131}I]T_4 was added as marker, showed a single peak of ^{125}I corresponding to the main T_4-binding component (Fig. 4). To determine the iodoamino acid composition of [^{125}I]TBG, samples of the iodinated protein were hydrolyzed with Pronase (Calbiochem, La Jolla, Calif.) for 18 h, and the digests were analyzed by single -dimension ascending paper chromatography in butanol-acetic acid-water (4:1:1). 90% of the ^{125}I on the chromatograms migrated as monoiodotyrosine, 4% migrated as diiodotyrosine, and the remainder stayed at the origin. No [^{125}I]T_4 or T_3 was detected. The virtual absence of [^{125}I]iodothyronine was confirmed by finding that less than 4% of the ^{125}I in these preparations of labeled TBG was soluble in ethanol.

[^{125}I]TBG mixed with normal human serum was subjected to immunoelectrophoresis with each of the fol-

lowing three different antisera in the troughs: anti-HSA, anti-TBG (raised in rabbits against our purified TBG preparations), and commercial anti-whole serum. The results are shown in Fig. 5. The protein stain shows the albumin arc and minor globulin arcs contributed by the carrier serum. The autoradiograph shows most of the radioactivity to be concentrated in the postalbumin region where TBG migrates and a small amount (less than 5% of the label) associated with albumin. The latter probably represents radioiodine which had been transferred to albumin during contact with serum before electrophoresis.

Similar findings were obtained from immunoprecipitation experiments. Small aliquots of [^{125}I]TBG, containing no carrier albumin or serum, were incubated for 72 h with either anti-TBG or anti-HSA rabbit sera (dilutions 1:5–1:100). Goat anti-rabbit serum was added, and incubation was continued for an additional 48 h. An average of 90.2% of the ^{125}I was precipitated at the lowest dilution of anti-TBG antiserum and only 9.0% in the anti-HSA.

At the specific activity of [^{125}I]TBG obtained, less than one molecule in 40 contains an atom of iodine-125. To assess the hormone-binding activity of the iodine-labeled TBG as distinct from unlabeled TBG, we employed affinity chromatography. A tracer quantity of [^{125}I]TBG mixed with 2 ml normal human serum was applied to a column of T₄-Sepharose (2 ml bed volume) equilibrated with 0.05 M phosphate buffer, pH 7.4.

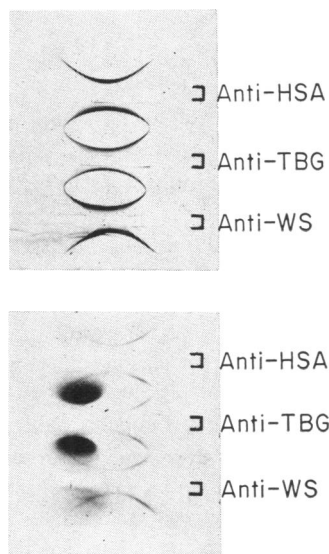

FIGURE 5 Immunoelectrophoresis of [^{125}I]TBG. Each origin well contained [^{125}I]TBG in normal human serum. Troughs contained, from top to bottom in order, rabbit antiserum vs. HSA, rabbit anti-human TBG, and rabbit anti-human whole serum (WS). The top half of the figure shows protein pattern, and the bottom half the corresponding autoradiograph.

After a brief wash with the buffer to remove loosely bound serum proteins, the proportion of total ^{125}I that was retained on the column was determined. (This was taken to be a measure of the T₄-binding activity of the [^{125}I]TBG.) As shown in Table I, depending on the lot, from 20.2 to 34.1% of the added [^{125}I]TBG was retained on the T₄ column. When an excess of T₄ was added to the serum-[^{125}I]TBG mixture before application to the affinity column, only 0.1% was retained, indicating that retention on the column depends on the specific property of T₄-binding activity. In other experiments, heating of [^{125}I]TBG-serum at 60°C virtually eliminated T₄ binding, consistent with known susceptibility of TBG to heat-inactivation (19). Sera collected from patients 3 or 5 days after they had received [^{125}I]-TBG for turnover studies exhibited at least as much T₄-binding activity as the original preparation of [^{125}I]-TBG. Radioiodinated HSA applied to the T₄-Sepharose column under the same conditions, showed less than 1% retention, a finding consistent with the relatively low affinity of albumin for T₄.

In separate experiments, the efficiency of the T₄-Sepharose columns in retaining native unlabeled TBG was tested as follows: 2 ml of normal serum was applied to a column, and the unadsorbed proteins were eluted in an identical fashion to that used in analysis of [^{125}I]TBG preparations. The maximal T₄-binding capacity of TBG in aliquots of the normal serum was measured by reverse-flow electrophoresis before and after exposure to the affinity column. (Suitable corrections were made for dilution on the column.) An average of 72% of the TBG binding capacity was removed by the column. These findings indicate that the affinity column retained about twice as much unlabeled TBG as [^{125}I]-TBG. The apparent loss in T₄-binding activity by radioiodinated TBG could be a consequence of a decrease in

TABLE II

Serum T_4, Free T_4, TBG Capacity, and in Vivo Kinetics of $[^{125}I]TBG$ Metabolism

Subject	Body wt	Serum T_4	Free T_4 index	Serum TBG capacity	Serum TBG concn	$[^{125}I]$TBG lot no.	Early urinary fraction*	Final $t_{\frac{1}{2}}$	Catabolic clearance‡	Distribution volume‡	TBG turn-over rate‡
	kg	*µg/100 ml*	*% of normal*	*µg T_4/ 100 ml*	*mg/100 ml*		*% of dose*	*days*	*liters/day*	*liters*	*mg/ day*
Controls											
R. C.	77	5.7	75	19.9	2.11	66	51.5	4.90	0.902	6.36	19.0
J. L.	67	6.3	108	18.3	—	67	29.9	3.68	1.61	8.59	—
C. M.	75	6.6	86	17.2	1.41	67	33.1	3.61	1.21	6.32	17.0
R. P.	65	9.2	92	20.0	2.80	69	7.1	5.32	0.634	4.86	17.8
E. L.	88	9.1	104	15.1	2.39	77	43.4	6.64	0.851	7.74	20.3
F. O.	52	5.8	98	17.0	2.04	88	9.3	5.90	0.729	6.21	14.8
Mean		7.12	93.8	17.92	2.15			5.01	0.989	6.68	17.8
±SD		±1.61	±12.2	±1.88	±0.51			±1.21	±0.326	±1.31	±2.1
Hypothyroids											
J. R.	70	3.5	42	25.0	2.64	63	9.1	5.10	0.560	4.12	14.8
W. S.	68	3.6	40	24.8	2.58	65	5.0	4.73	1.05	7.13	27.1
W. M.	73	2.6	26	18.5	2.18	67	21.1	3.90	1.52	8.58	33.2
L. H.	86	0.76	21	29.9	3.20	88	13.7	10.9	0.554	8.71	17.7
Mean		2.62	32.2	24.6	2.65			6.16	0.921	7.14	23.2
±SD		±1.32	±10.3	±4.67	±0.42			±3.20	±0.462	±2.13	±8.5

* Early urinary fraction = percent of dose excreted in urine during initial 48 h in excess of that expected from the extrapolated final disappearance rate.
‡ Values have been corrected to 70 kg body wt.

affinity for T_4 on the part of *all* molecules of $[^{125}I]$TBG, a complete loss of T_4-binding activity by some molecules, or a combination of these effects.

Serum TBG levels. There was an approximate correlation between the individual values for maximal T_4-binding capacity of TBG and the serum TBG concentration, as measured by radioimmunoassay. Patient

FIGURE 6 Plasma PB ^{125}I concentration (in percent of dose per liter) and urinary ^{125}I excretion (in percent of dose per 24 h) from day 1 through 11 after injection of $[^{125}I]$TBG in a control subject. The ordinate is a logarithmic scale.

L. H., who had the highest T_4 binding capacity, showed the highest TBG by immunoassay. Among the controls, however, the correlation was less perfect, for which we have no explanation.

In vivo metabolism. Fig. 6 shows the time-course of plasma PB ^{125}I concentration and the daily urinary excretion of ^{125}I after injection of $[^{125}I]$TBG in a control subject. Nearly 30% of the injected dose appeared in the urine during the initial 48 h. Thereafter, the rate of excretion paralleled the plasma disappearance of PB ^{125}I. Presumably, the "excessive" urinary excretion of ^{125}I in the initial 2 days was a result of the relatively rapid clearance from the plasma of damaged or denatured $[^{125}I]$TBG. This phenomenon was observed to a variable degree in every subject (Table II).

The plasma disappearance curves for all studies are illustrated in Fig. 7. In every case a single exponential slope was obtained from day 3 onward. The time-zero intercept of the final exponential varied from one study to another, depending largely upon the $[^{125}I]$TBG preparation administered.

The values obtained for various parameters of $[^{125}I]$-TBG kinetics are given in Table II. Among the control subjects, the half-time of the final slope of the plasma disappearance curve ranged from 3.61 to 6.64 days
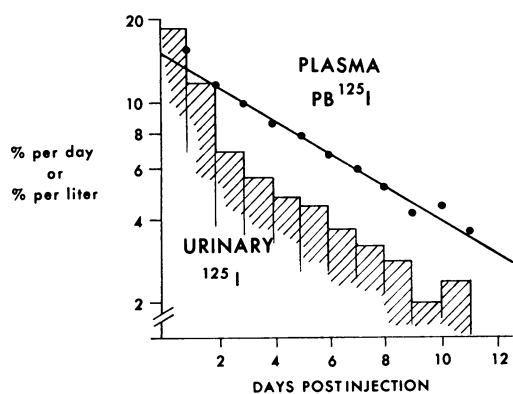
(mean = 5.01), the catabolic clearance rate ranged from 0.634 to 1.61 liters/day (mean = 0.989), and the volume of distribution, from 4.86 to 8.59 liters (mean = 6.68). The absolute rate of turnover of TBG ranged from 14.8 to 20.3 mg/day (mean = 17.8±2.1 mg/day).

Among the hypothyroid subjects as a group, there was no significant difference from the controls in any of the parameters of TBG turnover. However, two of these patients (J. R. and L. H.) exhibited catabolic clearance rates below the normal range. In patient L. H. this was due to a prolonged $t_{\frac{1}{2}}$, more than twice the normal mean, while in the other case (J. R.) a small distribution volume was responsible. The values for the absolute rate of TBG turnover in both of these subjects were within control range. The findings in patient L. H. are especially significant in view of the fact that this patient received the same lot of $[^{125}I]$TBG as one of the control subjects (F. O.).

DISCUSSION

The method employed in the present study for preparing human TBG in relatively pure form is similar to that used by Pensky and Marshall (11), but there are several points of difference. T_3-agarose rather than T_4-agarose was employed for affinity chromatography. The presence of T_4 in the eluting buffer increases the recovery of TBG in this step and may tend to protect eluted TBG against denaturation. Sterling et al. have reported that removal of T_4 from preparations of purified TBG results in a loss of binding capacity (7). The final purification step involving preparative electrophoresis on a slab of polyacrylamide gel was adopted to obtain a highly purified product in yield sufficiently high for the purpose of trace-labeling of TBG. This method of purification is not well-suited, however, for the preparation of large quantities (more than 500 µg) of pure TBG. For this purpose it is preferable to employ column chromatography and/or gel filtration (5–6).

The product obtained by the present method showed a high T_4-binding capacity, 0.85 mol T_4/mol TBG, which is evidence that the protein did not undergo extensive denaturation during purification. This is similar to the approximate ratio of 1 mol T_4/mol TBG obtained by Marshall and Pensky in their preparations of TBG purified by affinity chromatography (20).

The iodinated preparations of TBG showed no evidence of alteration from native TBG in terms of the electrophoretic mobility in polyacrylamide gel or in immunologic reactivity. In another respect, however, there was evidence of "damage" or at least a deviation from the properties of unlabeled TBG. The hormone binding of $[^{125}I]$TBG was only a fraction (about one-half) of that of native TBG (Table I). The method used to assess binding of the labeled protein does not distin-
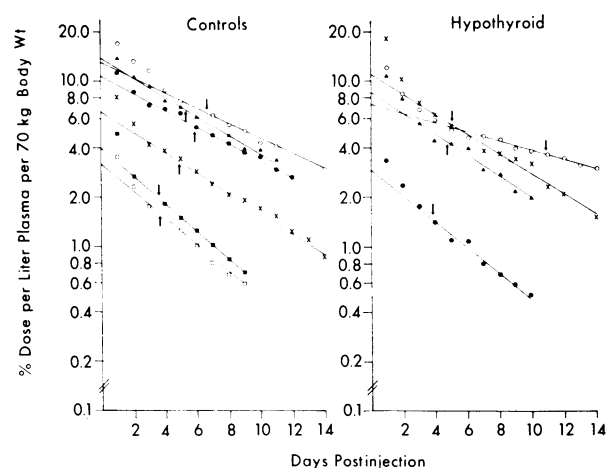

FIGURE 7 Semilogarithmic plot of the plasma PB ^{125}I disappearance curves in six control subjects, on the left, and four hypothyroid patients on the right. The final exponential slope, indicated by a solid line, was calculated by the method of least squares. The half-time of each slope is shown by a vertical arrow.

guish between binding affinity and capacity. It is not known, therefore, whether some molecules of $[^{125}I]$TBG suffered a complete loss of T_4-binding activity with the remainder having normal activity or all labeled TBG retained some ability to bind hormone but with diminished avidity.

The in vivo behavior of a labeled protein is probably the severest test of its biological integrity (21). The protein may be altered during isolation or iodination by the procedures employed, by the presence of a foreign constituent (iodine), or by self-irradiation. There was evidence of "damaged" $[^{125}I]$TBG in all of the lots used for turnover studies, judging from the appearance of disproportionate amounts of radioactivity in the urine during the initial 24–48 h after administration, presumably reflecting rapidly cleared and degraded tracer. In some of our lots, however, this rapidly cleared component represented less than 10% of the total (Table II). This suggests that the presence of an iodine atom does not necessarily cause gross biological denaturation. It is noteworthy that Refetoff, Fang, and Marshall (22) have reported that the introduction of up to six atoms of iodine per molecule of human TBG, using the chloramine-T method, did not shorten the survival of the labeled protein in the circulation of rabbits, compared to TBG labeled with one atom per molecule.

Whatever the reason for the variable degree of "damage" in the preparations of $[^{125}I]$TBG, it would appear that the relative hormone-binding activity, as assessed by affinity chromatographic analysis, correlated with the proportion of slowly cleared (relatively undamaged) $[^{125}I]$TBG in the different lots. The data pre-

sented in Table I in regard to preparations 67 and 88 can be compared with the early urinary excretion values given in Table II. Thus, in lot no. 67, nearly one-third of the dose was rapidly cleared ("damaged"), and the hormone-binding potency of this lot was relatively low. Residual label in the serum of C. M. 5 days after he received [^{125}I]TBG from this lot showed binding activity more similar to that of other lots. The T_4-binding potency of preparation no. 88 was relatively high and the rapidly cleared fractions in two patients (F. O. and L. H.) who received doses from this lot were low. These results are consistent with the view that the rapidly catabolized component of [^{125}I]TBG is relatively less active in terms of hormone binding.

By the method used to analyze the kinetics data, the catabolic clearance rate and the volume of distribution are not influenced by variations from one lot to another in the proportion of rapidly cleared tracer, i.e., that fraction of injected label which appeared in the urine within the initial 48 h. In fact, the catabolic clearance rate did not correlate with the percentage of "early" cleared tracer (Table II). Therefore, it would seem reasonably justified to accept the results at least as a first approximation of the kinetic behavior of TBG in man.

Thyroxine-binding prealbumin (TBPA) is generally accepted to be of lesser importance than TBG in the transport of T_4; this protein binds a smaller proportion of circulating T_4 than does TBG (23). Studies of TBPA metabolism have been reported by two groups, each working with a radioiodinated preparation of pure human TBPA. Socolow, Woeber, Purdy, Holloway, and Ingbar (24) found in normal individuals a fractional turnover of TBPA of 27%/day ($t_{\frac{1}{2}} = 2.6$ days) and a distribution volume of 9.4 ± 1.6 liters. In an independent study, Oppenheimer, Surks, Bernstein, and Smith (25) obtained similar results.

Transcortin, the cortisol-binding protein in human plasma, has also been studied in regard to in vivo kinetics. The physical and chemical properties of this protein are similar to those of TBG. Transcortin has a molecular weight of approximately 50,000, is a glycoprotein containing sialic acid (4), and binds 1 mol of cortisol/mol of protein with relatively high affinity (26). Sandberg, Woodruff, Rosenthal, Neinhouse, and Slaunwhite (27) prepared ^{125}I-labeled human transcortin and studied its metabolism in man. The final plasma disappearance $t_{\frac{1}{2}}$ ranged from 5.3 to 6.0 days in three normal subjects. They estimated the distribution volume to be approximately twice the plasma volume. Thus, TBG and transcortin exhibit similar in vivo kinetic properties.

An abnormal level of serum TBG, assessed by maximal T_4-binding capacity, has been observed in some patients with thyroid dysfunction. Decreased TBG has been described in thyrotoxicosis (28, 29). TBG maximal T_4-binding capacity is increased above normal to a variable degree in a large proportion of patients with hypothyroidism (29-32). We anticipated finding a slower than normal removal rate of injected labeled TBG in hypothyroidism, but only one of the four patients in this category showed a significant prolongation of the $t_{\frac{1}{2}}$. In this patient (L. H.) a low catabolic clearance rate was counterbalanced by a high serum TBG concentration, so that the absolute turnover rate was normal. In none of the hypothyroid cases studied was the TBG turnover rate (presumably equal to production rate) reduced below the control range. Moreover, for all of the subjects in this study, there was no correlation between the serum TBG level and the absolute turnover rate. Because of the wide range of values for the kinetics parameters obtained in this small group of hypothyroid cases, no conclusions can be drawn regarding the pathogenetic mechanism of the elevated serum TBG level found so often in this condition.

Studies of albumin kinetics in hypothyroid patients have shown a decrease in fractional removal rate (33, 34) which is reversed toward normal after treatment with thyroid hormone (34). In contrast to our findings, albumin synthesis is reduced as well in hypothyroidism; the serum level of albumin is either normal or subnormal in this condition. This differs from the case of TBG; elevations in TBG are commonly found in hypothyroidism as noted above.

The mechanisms by which TBG and other plasma glycoproteins are cleared from the circulation are not understood, but available evidence (summarized in reference 35) indicates that enzymatic removal of one or more residues of sialic acid may be an initial step in the normal catabolic process. The liver has been implicated as an important site of uptake of desialylated glycoproteins and may also be the organ where desialylation occurs (35). In fact, a partially desialylated TBG has been detected in some patients with severe hepatocellular damage (36). The question arises, whether the hypothyroid patient (L. H.) who exhibited an abnormally slow catabolic clearance of TBG suffered from a specific defect in one or another of the steps of TBG degradation. Our patient had no clinical or laboratory evidence of hepatic disease. Unfortunately, it was not possible to repeat the TBG turnover study after he had been rendered eumetabolic.

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