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Studies on human thyroxine-binding globulin (TBG). IX. Some physical, chemical, and biological properties of radioiodinated TBG and partially desialylated TBG.

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Research Article

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Studies on Human Thyroxine-Binding Globulin (TBG)

IX. SOME PHYSICAL, CHEMICAL, AND BIOLOGICAL PROPERTIES OF RADIOIODINATED TBG AND PARTIALLY DESIALYLATED TBG

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ABSTRACT Thyroxine-binding globulin (TBG) and partially desialylated or slow TBG (STBG) were purified from human serum by affinity chromatography. Purified TBG was identical to TBG present in serum by the criteria of electrophoretic mobility, affinity for thyroxine (T_4), and heat-inactivation response. Purified STBG had slower electrophoretic mobility and lower affinity for T_4. Both bound T_4 in an equimolar ratio, were immunoprecipitable, and had similar inactivation $t_{\frac{1}{2}}$ at 61°C.

TBG and STBG were iodinated by the chloramine-T-catalyzed reaction. An average of from 0.02 to 6 atoms I could be incorporated per molecule of the protein by adjusting the conditions of the reaction (time, protein and iodide concentrations). ^{125}I, ^{131}I, and ^{127}I were used. Iodination increased the anodal mobility of TBG but did not affect the reversible T_4-binding, precipitation by antiserum, or the heat-inactivation properties. "Heavily" and "lightly" iodinated TBG had identical disappearance half-times from serum in the rabbit.

15 min after the intravenous administration of [^{131}I]-STBG and [^{125}I]TBG mixture to rats, more than 90% of the injected ^{131}I dose was in the liver, and the liver $^{131}I/^{125}I$ ratio was 32-fold that of serum. Selective uptake of STBG by the liver was also observed in the rabbit and in man. The serum [^{125}I]STBG/[^{131}I]TBG ratio declined from 1 to 0.2 in 10 min in the intact rabbit but remained unchanged for 1 h in the acutely hepatectomized animal. In the rabbit, $t_{\frac{1}{2}}$ was approximately 3 min for STBG and 0.8–3.4 days for TBG. The radioiodine derived from the iodinated proteins is partly excreted in bile but the bulk was precipitable with specific antibodies. Some isotope in the form of iodide appeared in blood and was excreted in the urine.

Since radioiodinated TBG and STBG preserve their biologic and immunologic properties they are useful as tracer materials for metabolic studies. In rat, rabbit, and man STBG is rapidly cleared from serum by the liver. Conversion of TBG to STBG may be the limiting step in the regulation of TBG metabolism.

INTRODUCTION

More than 99% of circulating thyroxine (T_4)[1] in vertebrates is bound to serum proteins (1, 2). In man three major serum T_4-binding proteins have been identified. These are: thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA), and albumin (1). However, for a given level of serum T_4, the concentration of TBG is the chief determinant of the amount of free T_4 available to body tissues (3, 4). A fourth serum protein with electrophoretic mobility slightly cathodal to TBG has been infrequently detected in some sera (5–9). Initially believed to represent an artifact (5, 6), it has been recently shown that as earlier postulated by Premachandra, Perlstein, and Blumenthal (8) this slow TBG (STBG) is composed of TBG desialylated to varying degrees (9).

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[1] *Abbreviations used in this paper:* DPH, diphenylhydantoin Na; PBS-G, phosphate-buffered saline (0.01 M phosphate, 0.145 M NaCl), pH 7.5, containing 0.1% NaN_3 and 0.2% gelatin; STBG, partially desialylated TBG (slow TBG); T_3, triiodothyronine; T_4, thyronine; TBG, thyroxine-binding globulin; TBPA, thyroxine-binding prealbumin; TCA, trichloroacetic acid.

By affinity chromatography and DEAE-Sephadex, TBG and partially desialylated TBG (STBG) have been purified from human serum (9, 10). Their purity, homogeneity and some physical and chemical properties have been extensively studied (9–14). The availability of highly purified human TBG and STBG preparations, which might lend themselves to radioiodine substitution, constituted an attractive tool for the study of their metabolism in vivo and ultimately for the elucidation of the mechanism of inherited (15, 16) and acquired (1, 8, 9) TBG and possibly STBG abnormalities in man.

This communication describes the iodination reaction of TBG and STBG with ^{125}I, ^{131}I, and ^{127}I allowing the addition of an average of from 0.02 to 6 mol I/mol protein. Some physical and chemical properties of the purified TBG and STBG were studied and compared to those of unpurified, native serum TBG and to iodinated TBG and STBG. The biological properties of the radioiodinated proteins were studied in the rat, rabbit, and man. As shown by Morell, Gregoriadis, Scheinberg, Hickman, and Ashwell for other desialylated glycoproteins (17), the liver appears to rapidly and selectively clear STBG from serum.

METHODS

Materials. Human TBG was purified from serum by affinity chromatography and STBG was prepared by desialylation of purified TBG as previously described (9, 10). The sialic acid content of STBG was about ⅓–¼ that of TBG. Material used was from four different batches, two for each TBG and STBG. Protein concentration was determined spectrophotometrically by using an extinction coefficient of 6.95 (11). The purified proteins were stored in 0.06 M Tris-HCl buffer, pH 8.6, at −50°C in aliquots of 10–50 μl containing 325–1,240 μg protein per ml. A new sample was thawed each time just before use.

For radioiodination, "carrier-free" ^{125}I and ^{131}I were obtained from New England Nuclear, Boston, Mass., as the sodium salt in concentrations of from 224 to 465 mCi/ml for ^{125}I and from 129 to 635 mCi/ml for ^{131}I. ^{125}I- and ^{131}I-labeled T_4, sp act 40–90 μCi/μg, were from Abbott Laboratories, North Chicago, Ill., or Industrial Nuclear Co., Inc., St. Louis, Mo. At least 95% of the ^{125}I and ^{131}I activity of these preparations was in the form of T_4 when analyzed by paper chromatography (18).

Sephadex G-75 (medium) obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J., was prepared by repeated washing with phosphate-buffered saline (0.01 M phosphate, 0.145 M NaCl), pH 7.5, containing 0.1% NaN_3 and 0.2% gelatin (PBS-G). Gelatin was a product of Difco Laboratories, Detroit, Mich. Agarose (electrophoresis grade) was obtained from General Biochemicals, Chagrin Falls, Ohio. Chloramine-T was purchased from Eastman Kodak Co., Rochester, N. Y.; sodium metabisulfite ($Na_2S_2O_5$) from Fisher Scientific Co., Chicago, Ill.; Na T_4 pentahydrate and diphenylhydantoin Na (DPH) from Sigma Chemical Co., St. Louis, Mo.

Antibody preparation. A single adult male New Zealand albino rabbit was immunized with 65 μg of purified human TBG (0.2 ml), emulsified in 0.5 ml of complete Freund's adjuvant. Injections were given subcutaneously in multiple sites at monthly intervals for 5 mo. Blood was collected before immunization and a week before each booster injection. Antibody titer was determined as previously described (15). The highest titer of antibodies was obtained 3 wk after the first booster injection. The antisera precipitated at least 90% of freshly radioiodinated TBG and STBG.

Electrophoretic techniques. Reverse flow paper electrophoresis was carried out by Elzinga, Carr, and Beierwaltes' modification (19) of Robbins' method (20) in glycine acetate buffer, pH 8.6 (21). Agarose-gel electrophoresis was carried out by the method of Laurell and Nilehn (22) in an apparatus of their design, at 4°C, with Tris-maleate buffer, pH 8.6, as previously described (2). Polyacrylamide-gel electrophoresis was carried out in vertical gel slabs (Ortec Inc., Oak Ridge, Tenn.). The gel was 10%, polymerized with ammonium persulfate, and the buffer was Tris-borate, pH 8.4. After a pre-run for 1 h at 40 V, 8.5 mA, the samples were applied and run at 2–4°C, 180 V, 30 mA for about 3 h until the albumin marker reached a position 6 cm anodal to the origin. Gels were sliced and counted (4).

Paper chromatography. Paper chromatography was performed in the dark on Whatman no. 3 MM paper with a descending tertiary amyl alcohol: hexane: 2 M ammonia system (18). Conditions of the chromatographic run, identification of the position of carrier, and determination of the distribution of the isotope were as previously described in detail (4).

Radioiodination. TBG and STBG were labeled with either ^{125}I or ^{131}I by a modification of the method of Greenwood, Hunter, and Glover (23). In a typical reaction 1–10 μl of ^{125}I or ^{131}I (0.5–2.0 mCi) was added to 25 μl of 0.5 M Na-phosphate buffer, pH 7.5, in a small glass vial. 3.25–20 μg of TBG or STBG in 8–20 μl of the storage buffer were then added, followed by 20 μl of a freshly prepared chloramine-T solution (1 mg/ml). The reaction was usually allowed to proceed for 60 s, while the vial was being gently rotated. The reaction was stopped with 20 μl of $Na_2S_2O_5$ (10 mg/ml), followed by the addition of 50 μl of KI (4 mg/ml) in PBS-G. The final volume was from 126 to 163 μl. A sample of 1 μl was removed and counted before and after trichloroacetic acid (TCA) precipitation. The radioactivity remaining in the precipitate was determined. These data were used for the calculation of the iodination yield and the average number of iodine atoms incorporated per molecule of protein. The bulk of the iodination mixture was applied to a 12 × 0.9-cm Sephadex G-75 column and eluted with PBS-G. 15-drop (0.4-ml) fractions were collected. Radioactivity was contained in two major peaks. The first peak eluted in tubes 6 through 8 was at least 95% TCA precipitable and the second peak eluted in tubes 14 through 18 was less than 2% TCA precipitable.

The effect of variables, such as reaction time, iodine isotope (^{125}I or ^{131}I), total iodide concentration, and protein concentration were systematically examined. At constant TBG and iodide concentrations, the amount of iodine incorporated into the protein augmented with increasing time of the iodination reaction (5, 30, 60, and 120 s from the addition of chloramine-T to the addition of $Na_2S_2O_5$). A fivefold difference in the percent of iodine incorporation occurred at the extremes of reaction time. At a constant protein concentration (5.16×10^{-3} nmol) and reaction time (5 and 30 s), increasing concentrations of radioactive or stable iodide (from 0.9×10^{-3} to 9.2×10^{-2} nmol) increased proportionally the absolute amount of iodine incorporated into TBG. However, the percent of the total iodide added incorporated into the protein remained constant for each of

the two reaction times. Dependence of the iodination reaction on iodide concentration has been previously observed for nucleotides (24). Increasing concentration of the protein also increased the percent incorporation of iodine. Under identical reaction conditions the yield of the radioiodination reaction was reproducible and there was no significant difference in the efficiency with ^{125}I or ^{131}I. With variations in the above-described reaction conditions an average of as little as 0.02 to as many as 6 atoms of iodine could be incorporated per molecule of TBG or STBG. Iodine could be incorporated into TBG with a slightly greater efficiency than into STBG.

To ascertain the effect of ligand bound to TBG on the efficiency of the iodination reaction, reactions were carried out under identical conditions in the presence of various amounts of T_4 and DPH. Purified TBG was incubated for 1 h at 40°C with various amount of T_4 and with 2 times the molar amount of DPH. An aliquot from each mixture was iodinated and the remainder, after addition of [^{125}I]T_4, used for determination of the T_4-binding capacity by agarose gel electrophoresis. At the termination of the iodination reactions aliquots were immediately removed for TCA precipitation, reaction with excess TBG antiserum, and paper chromatography. As shown in Table I, addition of T_4 had little, if any, effect on the incorporation of iodine into TBG. TCA and antibody precipitability increased significantly only with the addition of T_4 at 3 and 4 times the molar amount of TBG. As shown by paper chromatography, this was associated with the formation of significant amounts of [^{125}I]T_4. Radioactivity was detected only at the origin ([^{125}I]TBG) and migrating as T_4. It is therefore apparent that T_4 bound to TBG does not affect the efficiency of the iodination reaction and that under conditions of excess T_4 a greater proportion of the hormone is labeled presumably by iodine exchange.

The stability of iodo-TBG during storage in PBS-G at −20°C was examined by TCA precipitation at various intervals. The radioactivity was 96.2, 95.4, 97.2, 94.7, 95.3, 87.7, and 81.1% precipitable at 0 h, 1 h, 12 h, 1½, 3½, 9, and 29 days after iodination, respectively. The stability was almost identical at 37°C in the presence of sterile serum from rabbits or from humans (normal or TBG-deficient). The electrophoretic mobility also remained unaltered.

Studies on the distribution, metabolism, and excretion of radioiodinated TBG and STBG in the rat. Overnight fasted, adult male Charles River albino rats weighing 300–400 g were each given intravenously a mixture of 0.5 μCi (7.1 ng) [^{125}I]TBG and 1.0 μCi (26.2 ng) [^{131}I]STBG. The proteins were, respectively, 99.1 and 101.2% TCA precipitable and were homogeneous on agarose-gel electrophoresis. 2 h before injection, 3 drops of a saturated solution of KI were given orally and the penile urethra was securely tied. Rats in groups of two were sacrificed by exsanguination through cardiac puncture at 15, 30, 60, 120, and 240 min.

The following organs were immediately dissected and weighed: heart, liver, kidney, spleen, testes, and brain. Weighed portions of these organs and portions of the thigh muscle and subcutaneous fat were counted in a Nuclear Chicago two-channel well-type scintillation spectrometer. Corrections were made for the contribution of ^{131}I in the ^{125}I channel. Error due to decay during the interval of counting was eliminated by repeated counting in reverse order. The volume of urine accumulated in the bladder was measured. Aliquots of urine and serum as well as a 10% standard of the administered dose were counted before and after TCA precipitation in the presence of carrier serum. The total radioactivity in the stomach and intestinal contents was determined separately in sealed 30-ml vials. Results were expressed as percent dose per gram or milliliter and as percent dose per organ or total urine volume or intestinal content. The ratio of $^{131}I/^{125}I$ activity was also calculated.

TABLE I

Effect of Ligand on the Efficiency of the TBG Iodination Reaction

	Percent of total isotope			
			Chromatography	
$\frac{\text{mol } T_4}{\text{mol TBG}}$	TCA	Antibody	Origin	T_4
0	10.6	9.4	9.4	0
0.5	10.4	10.1	10.1	0.2
1.0	10.9	8.8	11.0	0.8
2.0	10.1	10.2	8.9	1.6
3.0	12.0	11.9	10.6	2.1
4.0	18.0	12.0	14.7	3.3
2.0*	10.9	12.0	12.8	0

The reaction mixture contained in addition to the indicated amounts of T_4, 5.16×10^{-2} nmol TBG, 20 μCi of ^{125}I (0.92×10^{-2} nmol), 44.3×10^{-2} nmol ^{127}I (total iodide 45.2×10^{-2} nmol), and the standard amounts of chloramine-T, $Na_2S_2O_5$, and buffer.

* Moles DPH/mole TBG.

Studies on the metabolism and excretion in urine and bile of radioiodinated TBG and STBG in rabbits. Three adult male New Zealand albino rabbits weighing 3.8–4.0 kg were anesthetized with urethane; an i.v. drip of 0.9% NaCl was begun through the marginal ear vein; an 18 gauge catheter was introduced into the abdominal vena cava through a femoral vein cut down and kept open with a 0.9% NaCl drip; and a 14 gauge catheter was inserted into the bladder and secured tightly at the base of the penile urethra. The saline drip was maintained at a flow of 25–30 ml/h. Blood samples were obtained from the inferior vena cava catheter and isotope was given through the marginal ear vein. For complete urine collection 5 ml of saline was introduced into the bladder before withdrawal of each sample. The animals were kept warm and given 20 mg of KI i.v. 30 min before injection of the labeled proteins.

A total hepatectomy was performed on one rabbit. Another rabbit had cholecystectomy and cannulation of the common hepatic duct. Bile was collected by free flow and the time and volumes recorded. Bile flow was steady throughout the 5½ h of the experiment. The last rabbit was sham operated.

Each of the sham-operated and the hepatectomized rabbits received a mixture of 100×10^6 cpm [^{125}I]STBG and 25×10^6 cpm [^{131}I]TBG in carrier normal rabbit serum. The proteins were, respectively, 95.1 and 98.3% TCA precipitable and had a sp act of 10^6 cpm/18.2 ng and 10^6 cpm/82.7 ng. Blood samples were collected at frequent intervals during the first hour. The radioactivity in serum before and after TCA precipitation was determined and the $^{125}I/^{131}I$ ratios, expressed as percent of the total and TCA-precipitable dose, were calculated. All serum samples were subjected to agarose-gel electrophoresis to determine the relative amounts of isotope migrating as TBG and STBG and to examine the possibility of interconversion between these

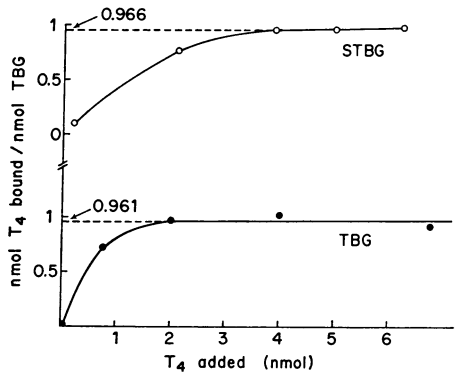

FIGURE 1 T_4-binding capacities of purified TBG and STBG. Increasing amounts of unlabeled T_4 were added to a mixture of purified TBG or STBG and tracer [^{125}I]T_4. The amount of T_4 bound to TBG or STBG in each sample was determined after separation of free T_4 from T_4 bound to protein on agarose-gel electrophoresis.

proteins. Excretion of the isotopes, in the urine, and isotopic content in liver at 2½ h (intact rabbit) were also determined.

The rabbit with the common hepatic duct cannula was given 8×10^6 cpm each of [^{125}I]STBG and [^{131}I]TBG mixed in 0.5 ml of normal rabbit serum. The sp act were 10^6 cpm/17.2 ng and 10^6 cpm/101.6 ng, respectively, and the TCA precipitability 97.4 and 95.8%, respectively. Samples of blood, bile, and urine were collected at frequent intervals during the subsequent 5½ h. The radioactivity was determined in all samples before and after TCA precipitation; carrier protein was added to urine and bile. All samples were also incubated with TBG antiserum and precipitable counts were determined after the addition of second antibody. Possible interference of serum, urine, and bile in the antibody precipitation reactions was determined by incubation of samples obtained before injection of the isotope with the injection mixture and antibodies. Selected samples of serum, urine, and bile were also subjected to paper electrophoresis and chromatography.

Long-term TBG turnover studies were performed in four additional unanesthetized male New Zealand albino rabbits. KI was added to the drinking water.

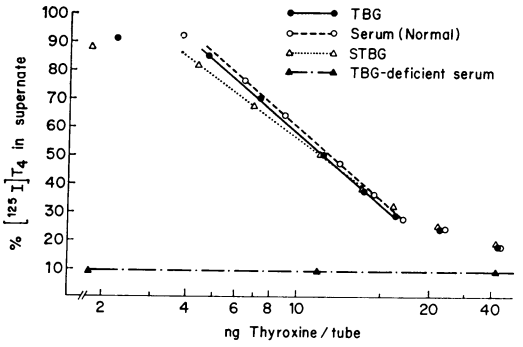

FIGURE 2 Competitive binding analysis of purified TBG and STBG, TBG in normal serum, and a TBG-deficient serum. Equimolar amounts of TBG and STBG were added to the TBG-deficient serum to match the predetermined capacity of TBG in the normal serum. Samples containing 25 µl of serum were diluted 1:40 in barbital buffer, pH 8.6. Each symbol covers the range of triplicate determinations. The slope is proportional to the association constant and the position of the curve at 50% saturation to the capacity. Purified STBG has a lower association constant than both purified and native TBG in serum. The latter two are identical. Note the lack of binding and displacement in the TBG-deficient serum, which had normal concentrations of albumin and TBPA.

RESULTS

Physical and chemical properties of purified TBG and STBG. Fig. 1 shows the T_4 saturation curves of TBG and STBG with agarose electrophoresis. With this particular batch of agarose, free T_4 migrated in the position of albumin and the total amount of labeled T_4 applied could be recovered. Trace amounts of [^{125}I]T_4 were added along with various amounts of unlabeled T_4. In this and subsequent studies results are calculated on the basis of a mol wt of 63,000 for both purified TBG and STBG (9). Average T_4-binding capacities were 0.961 and 0.966 mol of T_4 per mol of TBG and STBG, respectively. Saturation of TBG was achieved with molar levels of T_4 twice that of TBG, but T_4 in quantities fourfold the molarity of STBG was required to saturate STBG, suggesting lower affinity of T_4 for STBG than TBG (see below). Values at saturation of 0.95–0.98 mol of T_4 per mol of TBG were also obtained with acrylamide-gel electrophoresis. Saturation

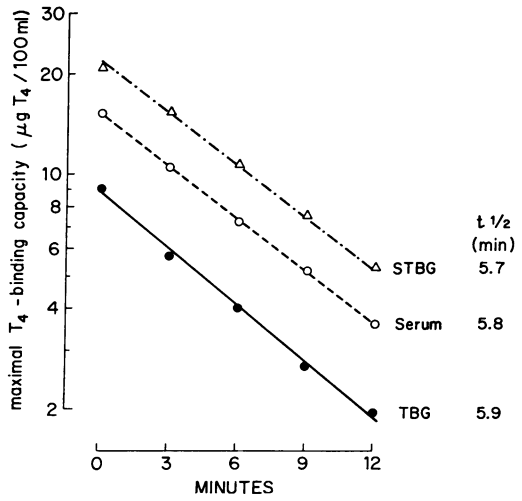

FIGURE 3 Rate of inactivation of T_4 binding to purified TBG and STBG and to native TBG in normal serum at 61±0.5°C. Aliquots of purified TBG and STBG were added to TBG-deficient serum, diluted 1:20, and heated along with identical dilutions of normal serum in a constant-temperature bath for various periods of time as indicated in the abscissa. The samples were immediately cooled in an ice bath and TBG capacity determined. Values are expressed as of micrograms T_4 bound at saturation per 100 ml of undiluted sample.

analysis on paper electrophoresis yielded much higher results. However, addition of human serum totally deficient in TBG (25) as carrier gave values of equimolar T_4 binding to TBG.

Binding capacity and affinity of TBG and STBG were also estimated by the competitive binding technique as previously described (15). Normal human serum with known TBG content was used as control and the purified proteins were added in equimolar amounts to TBG-deficient serum. As shown in Fig. 2, the capacities were similar as shown by the position of the curves at 50% saturation. As indicated by the slope of the curves, while the affinity of purified TBG for T_4 was identical to that of native TBG in normal serum, that of STBG was lower. A similar observation has been reported utilizing the fluorescence quenching technique (9).

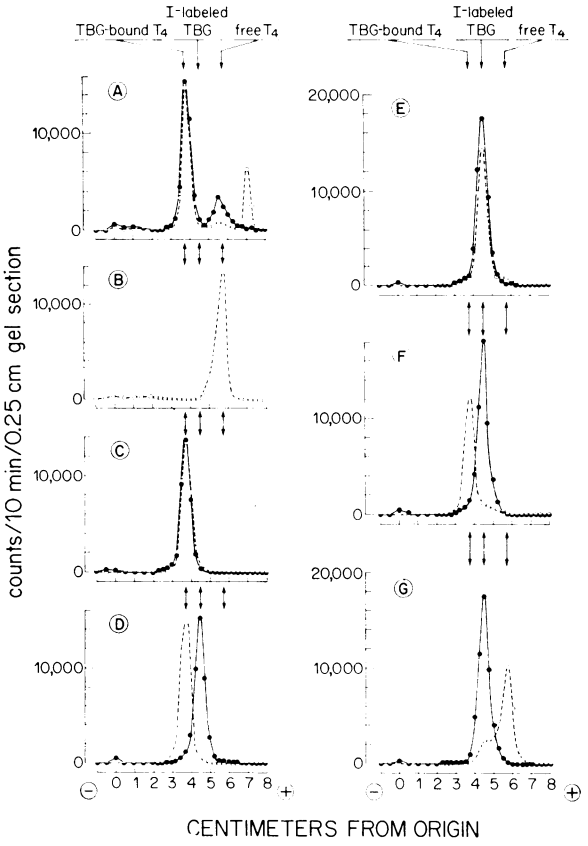

FIGURE 4 Behavior of TBG and iodo-TBG on agarose-gel electrophoresis. (A) Normal serum after addition of equal cpm of [^{131}I]T_4 (○---○) and [^{125}I]T_3 (●——●). From left to right the major peaks correspond to the positions of TBG, albumin, and TBPA. (B) Electrophoretic mobility of [^{131}I]T_4 in buffer alone. (C) Purified TBG after addition of equal cpm of [^{131}I]T_4 (○---○) and [^{125}I]T_3 (●——●). The mobility is identical to that of native TBG in serum. (D) "Lightly iodinated" [^{125}I]TBG (●——●) (in average of 0.05 atom I/molecule TBG) with added [^{131}I]T_4 (○---○). Note the anodal shift of [^{125}I]TBG. (E) "Heavily iodinated" [^{125}I]TBG (●——●) (in average of 6 atoms I/molecule TBG) with added [^{131}I]T_4 (○---○). To keep the ^{125}I activity proportional to the preparation in D, iodination was carried out in the presence of ^{127}I. Note the anodal shift of both [^{125}I]TBG and [^{131}I]T_4. (F) Addition of excess unlabeled TBG to the preparation in E before electrophoresis. [^{131}I]T_4 migrates with the bulk of noniodinated TBG. (G) Addition of excess unlabeled T_4 (approximately fourfold the molar amount of TBG) to the preparation in E before electrophoresis. Approximately ¾ of the [^{131}I]T_4 is in the position of free T_4.

TABLE II
Inactivation of Iodo-TBG at 60°C

Time	Percent of value before heat treatment		
	T_4-binding capacity	TCA precipitability	Antibody precipitability
min			
0	100	100	100
3	76.3	98.2	95.6
6	48.8	100.6	100.1
9	42.5	98.8	101.5
12	31.3	99.3	85.0

Values are means of duplicate determinations. TBG was iodinated with an average of 1.8 atoms I/molecule TBG with a combination of ^{125}I and ^{127}I. The iodo-TBG was added to TBG-deficient serum before heating. In the absence of TBG antibody the precipitate with the second antibody contained from 4.0 to 3.6% of the activity in both the unheated samples and samples heated for 12 min.

The heat lability of nonpurified TBG has been previously demonstrated by the rapid loss of its capacity to bind T_4. The $t_{\frac{1}{2}}$ at 60°C was from 6.4 (26) to 6.9 (15) min. The heat lability of purified TBG and STBG added to TBG-deficient serum was compared to that of native TBG in normal human serum as previously described (15). Fig. 3 shows that TBG from normal serum as well as purified TBG and STBG lost their capacity to bind T_4 at 61°C with a $t_{\frac{1}{2}}$ of 5.7–5.9 min.

The electrophoretic mobility of purified TBG on agarose-gel, acrylamide-gel, and paper electrophoresis, as identified by the location of bound labeled T_4, was identical to that of native TBG in serum. STBG migrated as a broader band cathodal to TBG in the three electrophoretic systems (see below).

Physical and chemical properties of iodinated TBG and STBG. As shown in Fig. 4C, tracer from [^{131}I]T_4 and [^{125}I]triiodothyronine (T_3) added in equal cpm amounts to purified TBG migrated in a single narrow band with electrophoretic mobility identical to TBG present in serum to which the same isotopes were added before electrophoresis (Fig. 4A). In this system [^{131}I]T_4 in buffer migrated with a mobility identical to human serum albumin (Fig. 4B). [^{131}I]T_4 added to "lightly iodinated" [^{125}I]TBG showed unaltered mobility al-

TABLE III

Metabolism of Labeled Human TBG in Rabbits

Rabbit I.D.	Body wt	Labeled TBG		DS	$t_{\frac{1}{2}}$
		Isotope	Sp act		
			atoms I/ molecule TBG	*ml*	*days*
1773	4,670	[^{125}I]TBG	1.0	595	3.40
B*	3,855	[^{125}I]TBG	4.8	238	0.86
B*	3,890	[^{125}I]TBG	1.8	274	1.05
C‡	3,295	[^{125}I]TBG	1.8	393	2.33
C‡	3,295	[^{131}I]TBG	0.04	383	2.28
A	3,818	[^{125}I]TBG	1.8	323	2.12

DS, distribution space.

* The same rabbit was used twice 3 mo apart and with two different [^{125}I]TBG preparations.

‡ [^{125}I]TBG and [^{131}I]TBG were injected together.

though the bulk of ^{125}I was anodal (Fig. 4D). In contrast, the position of [^{131}I]T_4 added to "heavily iodinated" [^{125}I]TBG was anodal and coincided with the ^{125}I (Fig. 4E). Thus, iodo-TBG has an electrophoretic mobility anodal to native or purified TBG. Reversible binding of T_4 to the iodo-TBG could be demonstrated by addition of noniodinated TBG (Fig. 4F) or cold T_4 (Fig. 4G) to the preparation from Fig. 4E.

Since TBG rapidly loses its ability to bind T_4 when exposed to 60°C, the effect of temperature on iodo-TBG was studied. Iodo-TBG was added to TBG-deficient serum and heated for various periods of time at 60°C. Aliquots were removed and analyzed by TCA and antibody precipitation, and after addition of [^{131}I]T_4, for TBG capacity. As shown in Table II, despite the progressive loss of ability of heated iodo-TBG to bind T_4, TCA precipitability remained unchanged and there was little loss in the immunoprecipitability after 12 min. On electrophoresis heat-denatured iodo-TBG and iodo-STBG showed greater diffusion and a significant anodal shift.

The effect of iodination on the metabolism of TBG in vivo. Before undertaking turnover studies utilizing radioiodinated TBG it was important to determine whether the level of iodination might influence its metabolism in vivo. [^{125}I]TBG with an average of 1.8 atoms I/molecule TBG (1.5 μCi/24.6 ng) and [^{131}I]-TBG with an average of 0.04 atom I/molecule TBG (1.5 μCi/152 ng) were mixed and injected i.v. into rabbit C. The total and TCA-precipitable ^{125}I/^{131}I ratios, corrected for decay, remained constant in all serum samples over the period of 6 days. The distribution space and $t_{\frac{1}{2}}$ were similar (Table III). Also, ^{125}I/^{131}I ratios in organs (brain, fat, muscle, heart, liver, kidney, lung, spleen, testis, and thyroid) obtained at the termination of the experiment remained constant and were identical to those in serum. The reproducibility was good also when the turnover study was performed at different times in the same rabbit. Considerable variation from animal to animal was noted (Table III).

Studies on the distribution, metabolism, and excretion of radioiodinated TBG and STBG in the rat. Rats were given a mixture of [^{131}I]STBG and [^{125}I]-TBG i.v. (average iodine-to-protein ratio 2.3 and 3.8, respectively), sacrificed at various times after injection, and the ^{131}I and ^{125}I activities were measured in various tissues, serum, and urine as described in the section on Methods. Data on liver and serum are shown in Fig. 5. At 15 min the liver ^{131}I/^{125}I ratio was 30–32-fold that of serum. Over the initial 30 min, the most rapid decline in the ^{131}I activity occurred in liver. During the same period of time the serum ^{131}I activity increased while its TCA precipitability rapidly declined. Although, at 4 h, about 15% of the total ^{131}I dose was still in tissues and blood, it should be noted as shown below, that [^{131}I]STBG was contaminated with approximately 10% of [^{131}I]TBG. By 4 h 14.5% of ^{131}I and 4.8% of ^{125}I were recovered in urine in 97.5% TCA-soluble form. The intestinal content of isotope also increased, with 26.2% of ^{131}I and 9.1% of ^{125}I recovered at 4 h. Except for liver

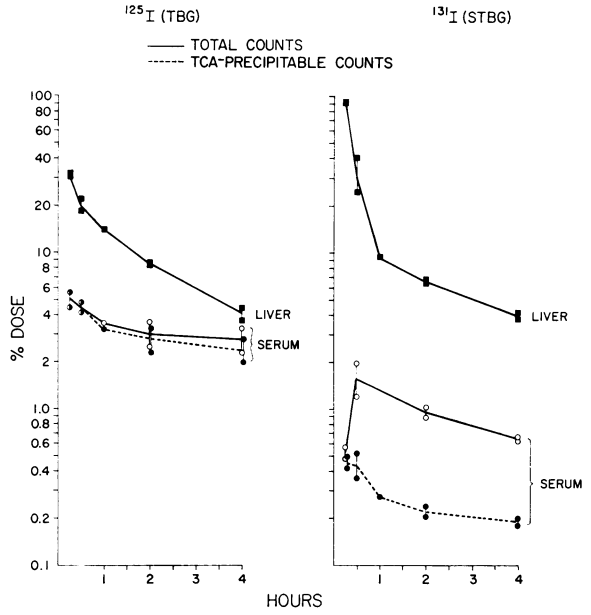

FIGURE 5 Distribution and metabolism of TBG and STBG in the rat. Rats were injected i.v. with a mixture of [^{125}I]-TBG and [^{131}I]STBG containing an average of 3.8 and 2.3 atoms I/molecule of protein, respectively. Two animals were sacrificed at the times indicated. Total recovery of the isotopes in liver (percent dose) and serum total TCA-precipitable activity (percent dose/milliliter) are plotted. Only liver had an ^{131}I/^{125}I ratio greater than 1 during the first 30 min after injection. For distribution in other organs, see text.

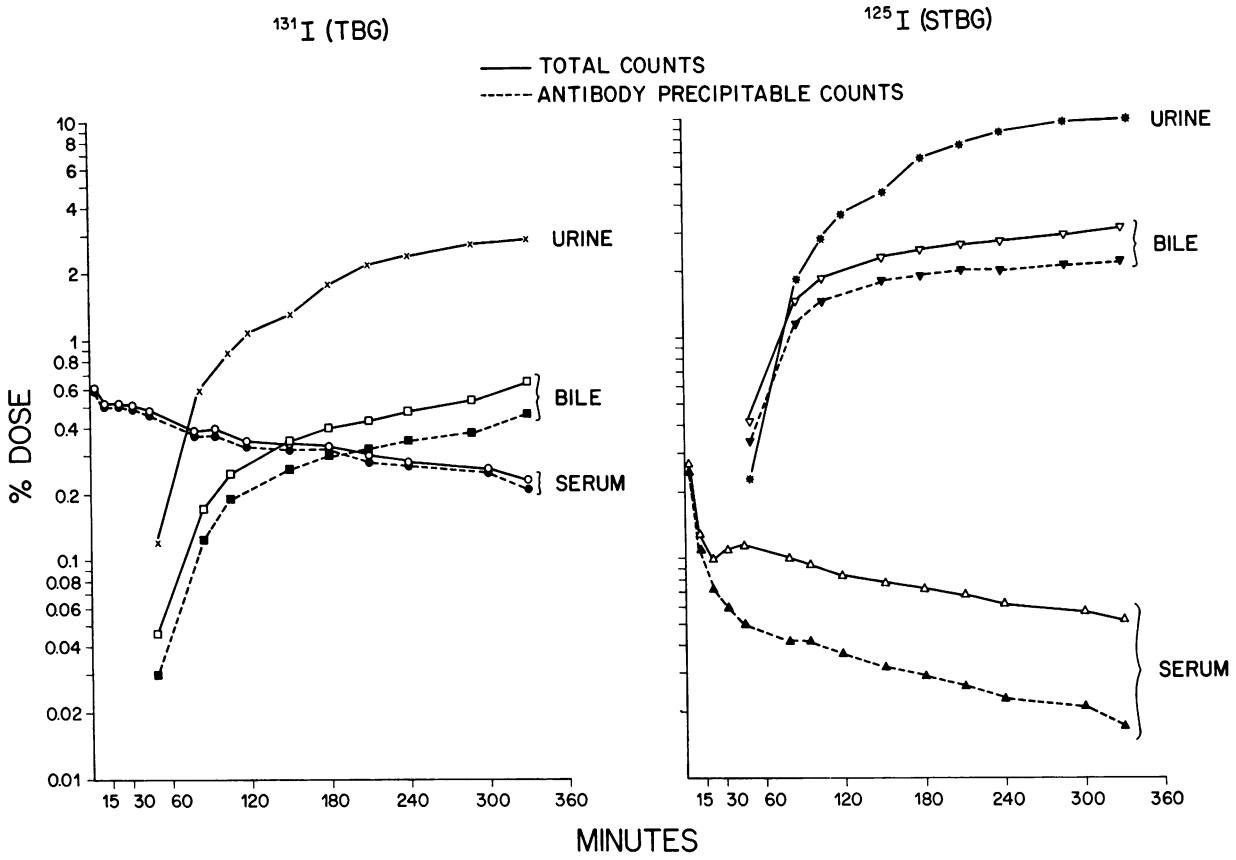

FIGURE 6 Metabolism and excretion of TBG and STBG in the rabbit. A rabbit bearing the common hepatic duct, inferior vena cava, and bladder catheters was injected i.v. with a mixture of [^{131}I]TBG and [^{125}I]STBG. Serum, bile, and urine were collected at the times indicated. Data are expressed as percent dose/milliliter for serum and cumulative percent dose excreted for bile and urine. Both total and antibody-precipitable radioactivities are shown, except for urinary radioactivity which was non-antibody precipitable. Note that loss of serum ^{125}I precipitability, as in the rat (Fig. 5), coincided with the increase in total serum ^{125}I activity 20 min after injection.

and kidneys all other organs maintained at all times an ^{131}I/^{125}I ratio similar to that of serum. These results suggest that STBG is very rapidly and selectively taken up by liver, metabolized, and partially excreted into the gut. Some of the released iodide returned to serum and was then excreted in urine. There was no evidence that any organ other than liver concentrated STBG or contributed to its metabolism. Some TBG was also taken up by liver, metabolized, and excreted. However, it was primarily distributed in serum since at no time was the ^{125}I activity in a gram of any organ, including liver, higher than that in 1 ml of serum.

Metabolism and excretion of TBG and STBG by the intact rabbit. A rabbit bearing a common hepatic duct cannula was injected i.v. with a mixture of [^{131}I]TBG and [^{125}I]STBG. Fig. 6 shows the total and antibody-precipitable radioactivity in serum, bile, and urine collected over a period of 5½ h. ^{131}I activity in serum was at all times more than 95% antibody precipitable. However, the antibody precipitability of ^{125}I rapidly declined, to only 39.5% at 5½ h. As in the rat experiment, a noticeable increase in the serum total radioactivity derived from iodo-STBG occurred between 22 and 45 min after injection. ^{131}I and ^{125}I were excreted in bile with 0.64% and 3.3% of the dose, respectively, recovered at the end of the experiment. Though only 76–68% of the radioactivity in bile was antibody precipitable, it should be noted that bile interfered with the immune reaction since 68% of ^{131}I and 65% of ^{125}I in the injected standard were antibody precipitable when added to bile obtained before injection of the isotopes. Urine and serum did not interfere with the antibody precipitation reaction. Less than 5% of the total isotope excreted in urine was both antibody and TCA precipitable. On chromatography more than 90% of both ^{131}I and ^{125}I in urine behaved as iodide. Electrophoresis confirmed the existence

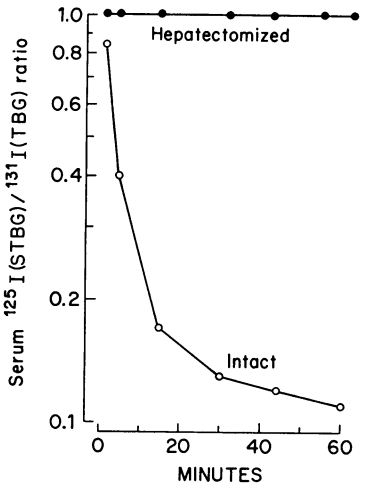

FIGURE 7 Role of the liver in the clearance of STBG. An intact (sham-operated) and a hepatectomized rabbit were both given i.v. a mixture of [^{125}I]STBG and [^{131}I]TBG. Serum samples were obtained at the times indicated and data expressed in terms of TCA-precipitable percent dose/liter ^{125}I/^{131}I ratios. Note the absence of selective clearance of STBG in the hepatectomized animal. The change in ^{125}I/^{131}I ratio slope in the intact animal is due to contamination of the [^{125}I]STBG with [^{125}I]TBG.

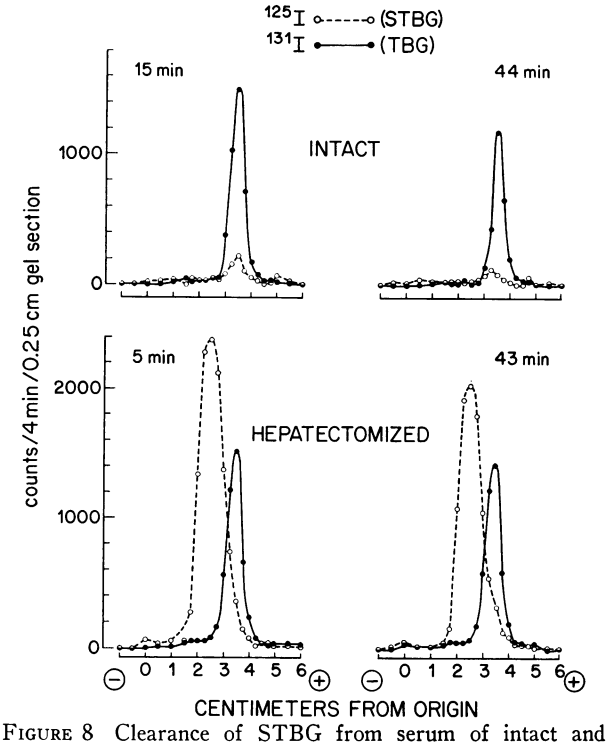

FIGURE 8 Clearance of STBG from serum of intact and hepatectomized rabbits. Agarose-gel electrophoresis of serum samples from rabbits described in Fig. 7. Note the disappearance of [^{125}I]STBG from the serum of the intact rabbit except for 5–10% [^{125}I]TBG contaminant. [^{125}I]STBG persisted in serum of the hepatectomized rabbit in amount identical to the relative content of [^{131}I]TBG in the standard.

of intact [^{131}I]TBG in serum. ^{125}I activity in serum samples collected 5 and 10 min after injection migrated as STBG but, because of presence of excess ^{125}I$^-$, was smeared on electrophoresis of samples drawn subsequently. ^{125}I activity in bile migrated primarily as a broad band similar to STBG although 17–28% of the activity was at, or just anodal to, the origin. ^{131}I in bile also migrated as a broader band cathodal to the zone normally occupied by TBG.

The role of liver in the metabolism of STBG. Fig. 7 depicts the serum ^{125}I/^{131}I ratios, expressed as percent TCA-precipitable injected dose/liter, in hepatectomized and sham-operated rabbits given a mixture of [^{125}I]-STBG and [^{131}I]TBG i.v. In the intact rabbit the ratio declined with an initial $t_{\frac{1}{2}}$ of 3 min, but in the hepatectomized rabbit it remained unchanged. The electrophoretic analyses of serum obtained at different times are shown in Fig. 8. In sera from the hepatectomized rabbit at 5 and 43 min after injection, ^{125}I migrated as STBG and ^{131}I as TBG in proportions equal to the standard. In contrast, mostly ^{131}I migrating as TBG remained in serum of the intact rabbit both at 15 and 44 min after injection of the isotopes. The [^{125}I]STBG peak was absent. The small amount (5–10%) of ^{125}I activity in these samples also migrated as TBG, suggesting that the partially desialylated STBG actually contained up to 10% of intact TBG. This observation explains the presence of 1/10 of the isotope derived from iodinated STBG in serum obtained at 60 min from the intact rabbit (Fig. 7) and the persistence of ^{131}I in rat serum and tissues obtained 1–4 h after injection of the isotopes (Fig. 5). Thus the liver appears to be responsible for the selective and more rapid removal of STBG from serum.

The role of liver in the metabolism of STBG in man. A mixture of [^{125}I]STBG (3 μCi) and [^{131}I]TBG (6 μCi), Australia antigen free and iodinated under sterile conditions,[2] was given i.v. to a single man undergoing routine diagnostic open liver biopsy during cholecystectomy. Blood samples were drawn at 3, 15, 30, and 40 min. Liver biopsy was obtained approximately 30 min after injection and showed microscopically normal liver. ^{125}I/^{131}I ratios expressed as percent dose/gram liver or milliliter serum were calculated. At the time of the biopsy, ratios were 4.7 in liver and 0.52 in serum. The liver/serum (gram/milliliter) isotope ratio for ^{131}I (TBG) was 0.23 and for ^{125}I (STBG) 1.91. If we assume a normal average liver weight of 1,500 g, 75% of ^{125}I derived from STBG was in the liver 30 min after

[2] Refetoff, S., V. S. Fang, J. S. Marshall, and N. I. Robin. Metabolism of thyroxine-binding globulin (TBG) in man. Abnormal rate of synthesis in inherited TBG deficiency and excess. Submitted for publication.

injection. The bile also contained threefold more ^{125}I than serum.

DISCUSSION

Earlier attempts to determine some of the physicochemical properties of purified TBG have yielded conflicting results, with estimates for mol wt ranging from 36,000 to 59,000 and binding capacity from 0.25 to 0.7 mol T_4/mol TBG (27–30). Although the purification methods employed varied, they had in common the use of multiple fractionation steps with low recovery and the likelihood of damage. The application of affinity chromatography for the purification of human serum TBG (10) simplified the procedure, decreased the handling, and increased the yield, making available sufficient amounts of purified material for its further characterization. Studies on such TBG preparations indicated that the mol wt is 63,000, that the molecule exists most likely in a monomeric form, and that it binds approximately 1 mol of either T_4, T_3, or 8-anilinonaphthalene-1-sulfonic acid per mol of TBG as determined by ultrafiltration and fluorescence quenching (11, 12). Affinity chromatography also allowed purification of another serum protein capable of binding T_4 and exhibiting a slower electrophoretic mobility than TBG, thus named "slow thyroxine-binding globulin" (9). This material, formerly believed to represent an artifact (5, 6) has been shown to be a partially desialylated TBG, as postulated by Premachandra et al. (8). Since at the onset of this work, our primary interest was to isotopically label purified TBG and STBG for further biological work, it was important to confirm their purity and homogeneity, and to obtain further information on their physicochemical properties for comparison with the iodinated derivatives. Techniques not previously used in the characterization of these purified proteins were employed. Using agarose, acrylamide, and paper electrophoresis, we have shown that on the basis of a mol wt of 63,000, both TBG and STBG bound T_4 mole per mole. The affinity of T_4 for the purified TBG and for TBG in its native unpurified state in serum was identical. Using the saturation analysis technique, we showed that as with the ultrafiltration technique, T_4 had a lower affinity for STBG than TBG, albeit equal capacity. The heat labilities of purified TBG and STBG, as measured by the loss of their ability to bind T_4, were undistinguishable and similar to that previously shown for unpurified TBG in serum (15, 31).

By using the chloramine-T method of Greenwood et al. (23), TBG and STBG could be iodinated with an average of as few as 0.02 atom of I per molecule to as many as 6 atoms of I per molecule. Since no attempt was made to iodinate the proteins to their maximal capacity, no conclusions can be drawn on the number of available tyrosyl or histidyl residues per molecule. The efficiency of the iodine addition reaction was dependent upon the time allowed for the iodination to proceed and the concentration of protein. Though increasing the concentration of iodide resulted in its greater incorporation into protein, the efficiency of the reaction, as defined by the percent of the total iodide incorporated into protein, did not change over the 100-fold concentration range of iodide used.

The presence of bound ligand, in the form of T_4 or DPH, did not interfere with the iodination reaction or the final yield. A portion of the T_4, however, when present in excess, was labeled by presumably an iodine exchange reaction (32).

Iodo-TBG behaved in a manner similar to TBG except during electrophoresis. It precipitated with the specific antibodies, bound T_4 reversibly with a capacity identical to that of unlabeled TBG, and lost its ability to bind the ligand at 61°C at a rate identical to unlabeled TBG. During heat inactivation, however, the TCA and antibody precipitability were by and large retained, suggesting that the biologic and immunologic sites on the TBG molecule are different. Since TBG concentrations measured by T_4-binding capacity and immunologically are similar in patients with congenital TBG deficiency or excess, the observation of different biologic and immunogenic sites on the TBG molecule is consistent with our earlier contention that inherited abnormalities of TBG capacity in man are due to variations in the concentration of a normal TBG rather than due to alterations in the T_4 affinity for an abnormal protein (15). The anodal shift of iodo-TBG is probably a common characteristic of iodine addition to proteins and polypeptides and has been shown to occur with iodoinsulin (33).

The final indication that iodo-TBG may behave identically to noniodinated native TBG and therefore constitutes a suitable tracer for metabolic studies came from data obtained through the simultaneous injection into a rabbit of two preparations of TBG iodinated to different extents by using two isotopes. Both disappeared with a similar $t_{\frac{1}{2}}$ and had an identical distribution volume (Table III).

Although STBG is normally undetectable in serum, substantial amounts have been found in some patients with obesity (8), in severe liver disease (9), or for other unknown reasons. Evidence has been given that this material, migrating as a wide band cathodally to the more ubiquitous TBG, is partially desialylated TBG (9). Its function and site of origin or formation are unknown. The slower electrophoretic mobility of desialylated glycoproteins has been previously demonstrated (34). The earlier belief that glycosylation is a means of recognition by the cell that the protein is ready for

secretion (35) has been replaced by the hypothesis that this carbohydrate residue codes the protein for its extracellular fate such as target organ specificity (17, 36) or more likely, for the control of the rate of removal from the circulation (17, 36–38). Indeed, with the exception of transferrin, all glycoproteins tested have much shorter half-life when desialylated (17). It is also apparent that the failure to observe biological activity in some desialylated proteins when injected in vivo was due to their rapid removal rather than to inactivation with the loss of the carbohydrate moiety (38).

Since we have demonstrated that STBG bound T_4 and therefore preserved its bioactivity, its metabolism in vivo was studied together with that of TBG. STBG was very rapidly cleared from blood in the rat, the rabbit, and in man. Over 90% of the administered dose was in the liver of the rat 15 min after injection. STBG in the rabbit had a half-life of approximately 3 min, as compared to 0.8–3.4 days for TBG. In a single experiment in man, ninefold more TBG than STBG remained in the circulation 30 min after their simultaneous injection, and the liver at the same time was estimated to contain at least 75% of the injected STBG. The liver appears to be the predominant if not sole organ responsible for the clearance of STBG from the circulation. No selective disappearance of STBG occurred in an acutely hepatectomized rabbit, and in the rat and rabbit all other organs analyzed contained an STBG/TBG ratio similar to that of serum. The electrophoretic analysis of serum samples 15–45 min after injection of a mixture of STBG and TBG to intact rabbits permitted us to demonstrate the contamination of our STBG preparation with approximately 10% TBG (Fig. 8). This explains our observation that after an initial rapid disappearance of the isotope associated with STBG, a residual fraction of approximately 0.1 the dose remains in serum and decays at a slower rate. Thus STBG behaves like the majority of other common serum asialoglycoproteins.

The exact fate and mode of excretion of iodo-STBG after its localization in liver is less understood. A portion of the protein is deiodinated. The iodide is returned to blood and eventually recovered in urine. Iodo-STBG is also excreted in the bile and recovered in the intestinal contents. The metabolic fate of TBG is even less clear. It is possible that TBG is desialylated before its clearance from blood. The observation that STBG is found in some sera from patients with severe hepatic failure seemed to support such a notion. Unfortunately we failed to demonstrate that TBG is converted to STBG before its removal from the circulation by the liver, perhaps because the latter process is too rapid. The presence in bile of radioactive material derived from iodo-TBG and migrating anodally to TBG may represent in part STBG but also some other form of TBG metabolites. At least 50% of the activity in bile migrated in the position of intact TBG. Although we assume that at least a portion of the TBG is desialylated before further breakdown, the site of this reaction is unknown. Whether TBG desialylation is the limiting step in TBG metabolism is currently under investigation.

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