Differential Binding of Thyroxine and Triiodothyronine to Acidic Isoforms of Thyroid Hormone Binding Globulin in Human Serum†

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ABSTRACT: The differential availability of thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) to liver from the circulating thyroid hormone binding globulin (TBG)-bound pool suggests that the two thyroid hormones may bind to different TBG isoforms in human serum. In the present study, the binding of [^{125}I]T_4 and [^{125}I]T_3 to human serum proteins was investigated by using slab gel isoelectric focusing and chromatofocusing. In normal human male serum, [^{125}I]T_4 was localized to four isoforms of TBG called TBG-I, -II, -III, and -IV, with isoelectric points (p*I*'s) of 4.30, 4.35, 4.45, and 4.55, respectively. [^{125}I]T_3 was localized to only two isoforms of TBG, TBG-III and -IV, with p*I*'s that were identical with those for [^{125}I]T_4. In normal female serum, [^{125}I]T_4 was localized to the same four isoforms of TBG as those of normal male serum, while [^{125}I]T_3 was localized to TBG-II, -III, -IV, and -V (p*I* = 4.65). In pregnant female serum, [^{125}I]T_4 was localized to five isoforms, whereas [^{125}I]T_3 was localized to four. IEF was also performed with male serum loaded with various concentrations of unlabeled T_3. The K_i values of T_3 binding to TBG-I, -II, -III, and -IV were 5.0, 2.4, 0.86, and 0.46 nM, respectively. The TBG isoforms in normal male serum were also separated by sequential concanavalin A–Sepharose affinity chromatography and chromatofocusing (pH range of 3.5–5.0). T_4 preferentially bound to the most acidic isoforms of TBG in the p*I* range of 3.8–4.0, whereas the less acidic fractions (pH 4.0–4.2) bound both T_4 and T_3. In conclusion, this study shows that T_4 and T_3 do not bind to a single competitive binding site on TBG. Instead, T_4 is preferentially bound by the most acidic TBG isoforms owing to a 10-fold lower affinity of T_3 for these proteins.

The thyroid hormone binding site on thyroid hormone binding globulin (TBG)[1] has been, until recently, viewed as a single competitive site for T_4 and for T_3 (Robbins & Rall, 1955; Hao & Tabachnick, 1971; Gershengorn et al., 1980). However, this concept has been recently questioned by Maberly et al. (1985) on the basis of a competitive inhibition analysis. TBG is known to circulate in human blood as multiple isoforms (Marshall et al., 1973; Gartner et al., 1981; Grimaldi et al., 1983). TBG is a glycoprotein that is composed of a single polypeptide and four carbohydrate side chains (Flink et al., 1986). Therefore, it is possible that the heterogeneous T_4/T_3 binding sites on TBG (Maberly et al., 1985) reflect differential binding of T_4 and T_3 to TBG isoforms. However, to date there has apparently been no comparative study of T_4 and T_3 binding to TBG isoforms, except for the studies by Gartner et al. (1981), who showed that T_4 and T_3 bind to the same isoforms of TBG. In these studies, however, TBG was purified by T_3 affinity chromatography (Gartner et al., 1981). Therefore, it is possible that T_4-specific isoforms of TBG were lost during the T_3 affinity chromatography purification process.

Physiologic evidence also suggests differential binding of T_4 and T_3 to TBG in human serum. Following portal vein injection, T_3 is nearly freely transportable into rat liver in vivo from the circulating human TBG-bound pool, while T_4 bound to human TBG is not transportable after portal injection in the rat (Pardridge & Mietus, 1980). Similarly, studies in humans have shown that TBG-bound T_3 is selectively available for uptake by liver (Zaninovich et al., 1966).

The purpose of the present study is to clarify the binding characteristics of T_4 and T_3 to TBG isoforms. We examined the binding of [^{125}I]T_4 and [^{125}I]T_3 to the isoforms of thyroid hormone binding proteins in human serum using isoelectric focusing (IEF) and autoradiography. Since IEF measures binding at acid pH, we also used chromatofocusing to separate TBG isoforms prior to measuring the binding of [^{125}I]T_4 and [^{125}I]T_3 at neutral pH. The results indicate that the most acidic TBG isoforms selectively bind T_4 but not T_3.

Experimental Procedures

Materials. Radiolabeled L-T_4 ([^{125}I]T_4; 1250 μCi/μg) and L-T_3 ([^{125}I]T_3; 1200 μCi/μg) were purchased from New England Nuclear Corp. (Boston, MA). The radiochemical purity of the labeled T_4 and T_3 was at least 97%, as judged by cellulose (250 μm) thin-layer chromatography with radiochromatogram scanning (Packard Model 7230 radiochromatogram scanner; Packard, Downer's Grove, IL) using a solvent system of chloroform/*tert*-butyl alcohol/2 N NH_4OH (60:376:70). Acrylamide, *N*,*N*′-methylenebis(acrylamide) (BIS), and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), glycerol, dextran (molecular weight 71 500), and activated charcoal were purchased from Sigma Chemical Co. (St. Louis, MO). Pharmalyte (pH 3–10 and pH 4.0–6.5), concanavalin A–Sepharose, polybuffer 74, and polybuffer exchanger 94 (PBE 94) were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Ultrofilm

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[1] Abbreviations: IEF, isoelectric focusing; T_4, thyroxine; T_3, 3,5,3′-triiodothyronine; TBG, thyroid hormone binding globulin; RHB, Ringer's–Hepes buffer; DDC, dextran-coated charcoal; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BIS, *N*,*N*′-methylenebis(acrylamide); TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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^3H was purchased from LKB (Gaithersburg, MD). Kodak GBX developer and fixer were purchased from Eastman Kodak Co. (Rochester, NY). All other chemicals were reagent grade. Pooled human serum was obtained from three healthy normal male subjects, three cirrhotic male patients, three pregnant subjects, and three healthy normal female subjects.

Isoelectric Focusing and Autoradiography. Isoelectric focusing (IEF) was carried out on prefocused, horizontal polyacrylamide slab gels over a pH range of 3.8–6.5 at 4 °C using a flatbed electrophoresis apparatus (C.B.S. Scientific Co., Del Mar, CA), an electrophoresis constant-power supply (ECPS 3000/150, Pharmacia Fine Chemicals), and a volt-hour integrator (VH-1, Pharmacia Fine Chemicals). Twenty-five milliliters of gel solution containing 4.875% acrylamide, 0.125% BIS, 8% glycerol, 1.06 mL of Pharmalyte (pH 4.0–6.5), and 0.6 mL of Pharmalyte (pH 3–10) was prepared (Murata et al., 1985). After degassing, polymerization was carried out in a casting mold (thickness 1 mm, width 140 mm, length 160 mm) in the presence of 250 μL of 3% ammonium persulfate and 28 μL of TEMED. Prefocusing was done with an anode solution (25 mM DL-aspartic acid and 25 mM L-glutamic acid) and a cathode solution (250 mM glycine) at a constant power of 9 W for 60 min, reaching a maximum of 2000 V. Then the serum sample was preincubated with 10 μCi/mL $[^{125}I]T_3$ or $[^{125}I]T_4$ for 30 min at 4 °C, and 7 μL of the sample was applied on the center of the gel. IEF was carried out for 5 h at a constant power of 13 W, reaching a maximum of 2300 V. Immediately after the current was turned off, the pH gradient in the gel was determined with a pH meter (pH meter 140; Corning Science Products, Medfield, MA); 0.5-cm slices of gel were eluted in 2 mL of 50 mM KCl. The gel was then transferred to filter paper, covered with plastic film, and dried for 1 h at 60 °C on a gel slab dryer (Bio-Rad Model 224, Richmond, CA). The dried gel was then exposed to LKB ultrofilm for 3–8 days at room temperature. The film was developed with Kodak GBX developer for 4 min. The development was stopped by washing in 2% acetic acid for 30 s followed by a 30-s wash with water. The film was then fixed with Kodak GBX fixer for 5 min, washed in water for 20 min, and dried completely. Each band on the film was quantitated by a densitometer (E-C Apparatus Corp.).

In Vitro Equilibrium Dialysis. The in vitro percentage of free $[^{125}I]T_3$ in the presence of normal male human serum in Ringer's–Hepes buffer (RHB), pH 7.4, which contains 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, and 10 mM Hepes, was determined by equilibrium dialysis (Pardridge & Mietus, 1980); 1.5 mL of 10% human serum containing 0.5 μCi/mL $[^{125}I]T_3$ was placed in a dialysis bag made of a 1.5 × 15 cm strip of dialysis tubing (molecular weight cutoff of 12 000–14 000; Spectrum Medical Industries, Inc., Los Angeles, CA) and was predialyzed against 15 mL of RHB containing 0.05% sodium azide at 4 °C for 12 h. The serum was then dialyzed against another 15 mL of RHB for 72 h at 4 °C. The in vitro percentage of free T_3 in 100% human serum was calculated from the ratio of dpm per milliliter in the dialysis buffer to that in the dialysis bag times the dilution factor of 10.

Separation of TBG Isoforms by Concanavalin A Affinity Chromatography and Chromatofocusing. TBG isoforms in healthy normal male serum were separated by sequential concanavalin A–Sepharose affinity chromatography (Gartner et al., 1981) and chromatofocusing (Keenan & Holmes, 1985); 100 mL of concanavalin A–Sepharose was applied to a 5 × 30 cm column and washed with 500 mL of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl at 4 °C; 4 mL of pooled human male serum was applied to the concanavalin A–Sepharose column and then washed with 300 mL of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl at 4 °C. Glycoproteins were eluted at room temperature with 200 mL of 0.06 M methyl α-D-mannoside in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl (Gartner et al., 1981); 2 mL of each eluate was collected by a fraction collector (Frac-100, Pharmacia Fine Chemicals), and A_{280} was measured by a spectrophotometer (Model 34, Beckman). The peak fractions were pooled and concentrated to 3 mL by a stirred ultrafiltration cell (Model 8010; Amicon Corp., Danvers, MA) with a PM-10 Diaflo ultrafilter (molecular weight cutoff 10 000, Amicon Corp.). The concentrated glycoprotein solution was then dialyzed against 4 L of 25 mM piperazine hydrochloride buffer (pH 5.0) for 16 h at 4 °C. Chromatofocusing was performed with a 1 × 35 cm column containing 27 mL of PBE 94. The chromatofocusing column was equilibrated with 150 mL of 25 mM piperazine hydrochloride buffer (pH 5.0), and the dialyzed protein solution was applied to the column. The column was eluted with 250 mL of polybuffer 74 which was diluted 1:10 with distilled water and adjusted to a pH of 3.5 with HCl at a flow rate of 0.5 mL/min, thereby generating a pH gradient between 5.0 and 3.5; 1.25 mL of each eluate was collected for pH and A_{280} readings. An aliquot was removed and immediately neutralized to pH 7.4 prior to measurement of $[^{125}I]T_3$ and $[^{125}I]T_4$ using the dextran-coated charcoal assay.

Dextran-Coated Charcoal (DCC) Assay. The binding of $[^{125}I]T_3$ or $[^{125}I]T_4$ to TBG in the chromatofocusing fractions was determined by a dextran-coated charcoal (DCC) assay; 100 μL of TBG isoform solution was mixed with 400 μL of 0.15 M Tris-HCl buffer (pH 7.4) containing 0.2% gelatin in a plastic tube; 10 μL of $[^{125}I]T_3$ or $[^{125}I]T_4$ solution containing 20 000 cpm per 10 μL of 0.01 N NaOH was added to the tube. After a 60-min incubation at 4 °C, ice-cold DCC solution containing 10 mM Tris-HCl (pH 7.4), 0.025% dextran (molecular weight 71 500), and 0.25% activated charcoal was added to each tube while vortexing. The charcoal-containing tubes were centrifuged for 10 min at 2000 rpm (4 °C), and the TBG-bound radioactivity in the supernatant was decanted and counted for ^{125}I (Gamma 5500, Beckman Instruments, Fullerton, CA).

RESULTS

Isoelectric Point of Thyroid Hormone Binding Serum Proteins Bound to $[^{125}I]T_4$ or $[^{125}I]T_3$. The isoelectric points (p*I*'s) of thyroid hormone binding serum proteins were determined by slab gel isoelectric focusing (IEF) and with consecutive autoradiography in the presence of $[^{125}I]T_4$ or $[^{125}I]T_3$. Four different types of human sera, i.e., normal male, cirrhotic male, pregnant female, and normal female, were used. The results of a typical autoradiogram are shown in Figure 1. In normal human male serum, $[^{125}I]T_4$ was localized to four isoforms of thyroid hormone binding globulin (TBG) called TBG-I, -II, -III, and -IV, with isoelectric points (p*I*'s) of 4.30, 4.35, 4.45, and 4.55, respectively. $[^{125}I]T_4$ was also localized to prealbumin (p*I* = 5.15) and to one albumin isoform (p*I* = 5.10). In contrast, $[^{125}I]T_3$ was localized in normal male serum to only two isoforms of TBG, TBG-III and -IV, with p*I*'s that were identical with those for $[^{125}I]T_4$ in normal male serum, and was also localized to an acidic isoform of albumin (p*I* = 4.90). In cirrhotic human male serum, $[^{125}I]T_4$ was localized to four isoforms of TBG, TBG-I, -II, -III, and -IV, with p*I*'s that were identical with those for $[^{125}I]T_4$ in normal male serum. $[^{125}I]T_4$ binding to prealbumin was diminished in cirrhosis. $[^{125}I]T_3$ was localized in cirrhotic male

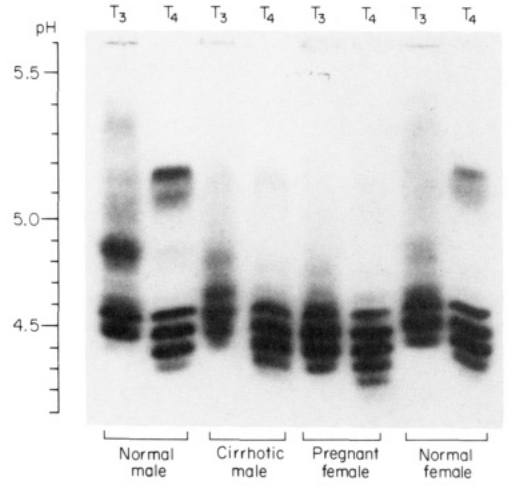

FIGURE 1: Autoradiogram of [^{125}I]T_3 or [^{125}I]T_4 bound to TBG isoforms, albumin isoforms, and prealbumin in serum from normal male subjects, cirrhotic male patients, pregnant subjects, and normal female subjects following separation by isoelectric focusing. The pH values of the gel are shown in the ordinate of the figure.

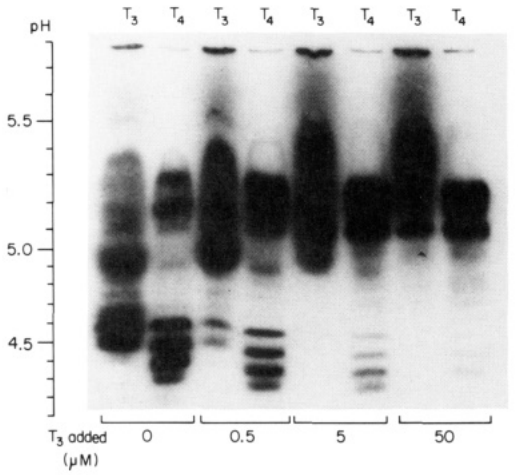

FIGURE 2: Autoradiogram of [^{125}I]T_3 or [^{125}I]T_4 bound to TBG isoforms, albumin isoforms, and prealbumin separated from normal male serum by isoelectric focusing at varying concentrations of unlabeled T_3 added to the serum. The pH values of the gel and the total T_3 concentrations are shown in the ordinate and the abscissa of the figure, respectively. The diffuse [^{125}I]T_3 band at pH 5.4 is free T_3.

serum to three isoforms of TBG, TBG-III and -IV, with p*I*'s that were identical with those for [^{125}I]T_4 in normal male serum and to TBG-V, with a p*I* of 4.65. In pregnant human serum, [^{125}I]T_4 was localized to at least five isoforms of TBG, TBG-I, -II, -III, and -IV, with p*I*'s that were identical with those for [^{125}I]T_4 in normal male serum and to TBG-I′, with a p*I* of 4.25. Similar to cirrhosis, [^{125}I]T_4 binding to prealbumin was decreased in pregnancy. [^{125}I]T_3 was localized in pregnant serum to four isoforms of TBG, TBG-I, -II, -III, and -IV, with p*I*'s that were identical with those for [^{125}I]T_4 in normal male serum. In normal human female serum, [^{125}I]T_4 was localized to four isoforms of TBG, TBG-I, -II, -III, -IV, and to prealbumin, with p*I*'s that were identical with those for [^{125}I]T_4 in normal male serum. [^{125}I]T_3 was localized in normal female serum to four isoforms of TBG, TBG-II, -III, and -IV, with p*I*'s that were identical with those for normal male serum and to TBG-V, with a p*I* that was identical with that for [^{125}I]T_3 in cirrhotic male serum, and was slightly localized to albumin. As shown in Figure 1, isoforms of thyroid hormone binding serum protein bound with [^{125}I]T_4 or with [^{125}I]T_3 were different, not only among the four human sera examined but also between [^{125}I]T_4 and [^{125}I]T_3 in the same human serum.

Inhibition by T_3 of the Binding of [^{125}I]T_4 or [^{125}I]T_3 to Thyroid Hormone Binding Serum Proteins in Normal Male Human Serum. The inhibitory effects of unlabeled T_3 on the binding of [^{125}I]T_4 or [^{125}I]T_3 to thyroid hormone binding serum proteins were also examined by IEF and autoradiography for normal male human serum loaded with various concentrations of unlabeled T_3 (final serum concentrations of added T_3 were 0, 0.5, 5.0, and 50 μM). As shown in Figure 2, the binding of [^{125}I]T_4 or [^{125}I]T_3 to TBG was displaced to albumin and prealbumin as the concentration of the loaded T_3 was increased. At 5 μM T_3, no binding of [^{125}I]T_3 to TBG-III and -IV was observed, whereas comparatively stronger binding of [^{125}I]T_4 to TBG-I and -II was found. Essentially no binding of [^{125}I]T_3 and [^{125}I]T_4 to TBG isoforms was observed in the presence of 50 μM T_3. Regarding albumin isoforms bound with [^{125}I]T_4 or [^{125}I]T_3, three p*I*'s (4.95, 5.10, and 5.20) were observed for T_3-loaded serum. In serum with no added T_3, [^{125}I]T_3 bound to the most acidic isoform (p*I* = 4.95), and [^{125}I]T_4 bound to the least acidic isoform (p*I* = 5.20) of albumin (Figures 1 and 2). In the presence of 50 μM T_3, both [^{125}I]T_3 and [^{125}I]T_4 bound to the intermediate albumin isoform, p*I* = 5.10 (Figure 2). Only one p*I* (5.30) was observed for [^{125}I]T_4-bound prealbumin in either the absence or the presence of unlabeled T_3 in serum. The distribution of [^{125}I]T_4 or [^{125}I]T_3 to serum isoforms was quantitated by densitometric scanning of the autoradiograms; the scan for the first two lanes of Figure 2, i.e., no T_3 added, is shown in Figure 3. The relative peaks on each scan were calculated by the trapezoid method, and these values were plotted against

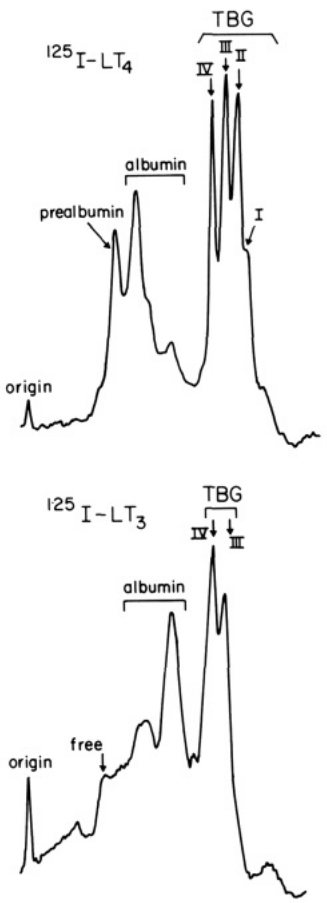

FIGURE 3: Typical densitometric scans of autoradiographs of [^{125}I]T_3 and of [^{125}I]T_4 bound to TBG isoforms, albumin isoforms, and prealbumin separated from normal male human serum by isoelectric focusing. The upper panel shows bound forms of [^{125}I]T_4, and the lower panel shows bound forms of [^{125}I]T_3.

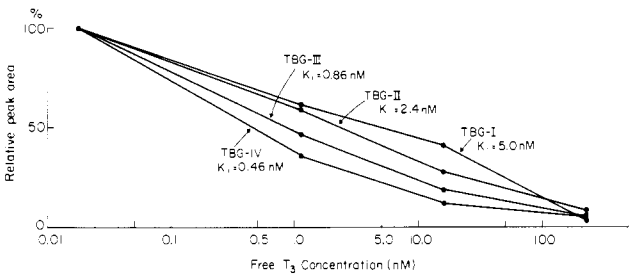

FIGURE 4: Inhibition of [^{125}I]T_4 binding to TBG isoforms in serum of normal male subjects in the presence of varying concentrations of T_3. The relative peak areas were plotted against the free T_3 concentrations in serum. Each peak area was measured by the trapezoid method on the densitometric scan of the autoradiographs of [^{125}I]T_4 bound to TBG isoforms at varying concentrations of T_3 (e.g., Figure 3). Free concentrations of T_3 in serum were determined by in vitro equilibrium dialysis at 4 °C. The inhibition constants (K_i's) of T_3 inhibition of [^{125}I]T_4 binding to TBG isoforms were determined by eye as the ED_{50}.

Table I: Binding Constants of T_3 Bound to TBG Isoforms in Normal Male Human Serum

isoform	p*I*	dissociation constant of T_3 (nM)[a]	inhibition constant of T_3 (nM)[b]
I	4.30		5.0
II	4.35		2.4
III	4.45	0.46	0.86
IV	4.55	0.33	0.46

[a] Values were determined by plotting (Figure 4) the peak areas (e.g., Figure 3) for each [^{125}I]T_3-bound TBG isoform in Figure 2 versus the concentration of free T_3 as determined by equilibrium dialysis; these plots (not shown) were similar to that shown in Figure 4. [b] Values were determined by plotting (Figure 4) the peak areas (e.g., Figure 3) for each [^{125}I]T_4-bound TBG isoform in Figure 2 versus the concentration of free T_3 as determined by equilibrium dialysis.

the respective serum concentration of free T_3 (Figure 4). Serum-free T_3 was determined by equilibrium dialysis (Experimental Procedures). In the presence of tracer concentrations of unlabeled T_4 (0.8 μg/100 mL, i.e., the same concentration of [^{125}I]T_4 as in the IEF study; Figure 2), the in vitro free percentages of [^{125}I]T_3 were 0.177%, 0.225%, 0.320%, and 0.452% (mean of duplicates that varied <10%) at 0, 0.5, 5, and 50 μM concentrations of unlabeled T_3. These values were used to compute the free T_3 concentrations for the saturation analysis of [^{125}I]T_4 binding to TBG isoforms inhibited by T_3, as shown in Figure 4. The T_3 inhibition constants (K_i's) for the respective TBG isoforms were determined by eye as the ED_{50} and are listed in Table I. The T_3 K_i value for TBG-I was 10 times higher than that for TBG-IV (Table I). The dissociation constants (K_d's) of T_3 binding to TBG-III and -IV were also determined. In these studies, the in vitro free percentages of [^{125}I]T_3 were 0.105%, 0.233%, 0.363%, and 0.450% (mean of duplicates that varied 10%) at 0, 0.5, 5, and 50 μM concentrations of loaded T_3. The K_d values for T_3 are listed in Table I and were found to be nearly similar to the respective T_3 K_i values for TBG-III and -IV (Table I).

[^{125}I]T_4 and [^{125}I]T_3 Binding to Chromatofocused TBG Isoforms in Normal Male Human Serum. The TBG isoforms in normal male serum were separated by sequential concanavalin A–Sepharose affinity chromatography and chromatofocusing (pH range of 3.5–5.0). The elution profiles of protein, pH, and binding activity of T_3 or T_4 are shown in Figure 5. TBG isoforms were eluted at the pH range of 3.8–4.2. In the pH range of 3.8–4.0, selective binding of T_4 to the most acidic isoforms of TBG was observed (Figure 5). In contrast, both T_4 and T_3 were bound to the less acidic

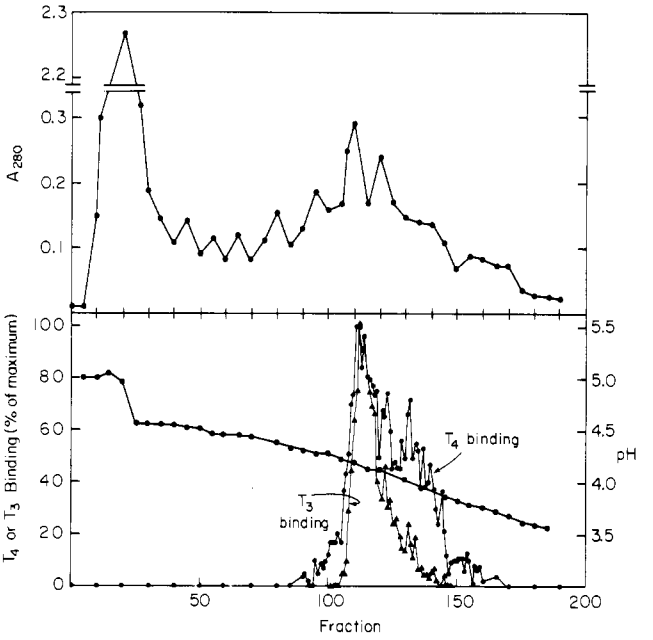

FIGURE 5: Chromatofocusing elution profile of TBG in pooled male serum. A_{280} and pH values for each fraction are shown in the upper panel and in the right ordinate of the lower panel, respectively. The binding affinity of TBG isoforms to [^{125}I]T_3 and to [^{125}I]T_4 was determined by a dextran-coated charcoal assay and is shown as relative percentages of the maximum value in the left ordinate of the lower panel.

isoforms of TBG in the p*I* range of 4.0–4.2.

Discussion

The present studies assess the differential binding of [^{125}I]T_4 and [^{125}I]T_3 to thyroid hormone binding proteins in human serum. These studies are consistent with the following conclusions. First, there is a preferential binding of T_4 to acidic isoforms (TBG-I, p*I* = 4.30; and TBG-II, p*I* = 4.35) in human male serum. Second, there is isoform switching that is associated with increased estrogens and that causes increased binding of T_3 to the acidic isoforms, e.g., to TBG-II in female serum and to TBG-I and to TBG-II in pregnancy serum. Third, there is differential binding of T_4 and T_3 to albumin isoforms with preferential binding of T_4 to the less acidic isoform (p*I* = 5.10) and preferential T_3 binding to the more acidic albumin isoform (p*I* = 4.90). Fourth, the preferential binding of T_4 to the acidic TBG isoforms arises from a 10-fold lower affinity of T_3 binding to TBG-I (p*I* = 4.30) as compared to TBG-IV (p*I* = 4.55) (Table I). Fifth, sequential concanavalin A affinity chromatography and chromatofocusing provides a strategy for partial purification of the T_4-specific TBG isoforms (Figure 5). The method of sequential IEF-autoradiography of human serum labeled with [^{125}I]T_4 or [^{125}I]T_3 is an approach to detect TBG isoform switching under physiologic conditions that is advantageous over immunologic detection of TBG isoforms (Gartner et al., 1981). The use of labeled thyroid hormones in autoradiography allows for a functional assessment of thyroid hormone binding to the various albumin and TBG isoforms.

The interpretation of the IEF–autoradiography data regarding selective binding of T_4 to certain TBG isoforms assumes that acid conditions (pH 4–5) do not significantly alter the distribution of [^{125}I]T_4 and [^{125}I]T_3 to plasma proteins that occurs at the physiologic pH of the sample prior to application to the gel. Although Gershengorn et al. (1977) show that TBG does not bind thyroid hormones at pH <6 in the presence of 2 M guanidinium chloride, this effect appears to be due primarily to the chaotropic agent. For example, the K_D of TBG

binding of T_3 at pH 4.5 is 0.33 nM (Table I), which is not significantly different from the K_D measured at physiologic pH (Gershengorn et al., 1980). In addition, the chromatofocusing experiments (Figure 5) involve measurement of thyroid hormone binding to TBG at the physiologic pH, and these data confirm the IEF results. A second potential caveat in interpretation of the data is the possibility that the unlabeled T_4 in human serum may inhibit the binding of [^{125}I]T_3 to TBG. However, the concentration of free T_4 in human serum, about 0.03 nM (Gershengorn et al., 1980), is negligible compared to the K_D of T_3 binding to TBG, i.e., $\geq$0.33 nM (Table I).

The present results, showing heterogeneity of TBG–thyroid hormone binding sites with preferential binding of T_4 to acidic isoforms, provide evidence that the traditional view of a single competitive binding site on TBG for T_4 and T_3 must be reevaluated. The data shown in Table I and Figure 4 indicate that T_4 and T_3 bind to multiple TBG isoforms that differ over a 10-fold range in terms of TBG affinity for T_3. The gene for TBG is a single-copy gene on the X chromosome which encodes for a 45-kilodalton polypeptide that contains four carbohydrte binding sites (Flink et al., 1986). The basis for the TBG microheterogeneity lies in differential posttranslational glycosylation of the TBG isoforms. Therefore, the differential glycosylation of the protein apparently influences the characteristics of T_4 and T_3 binding to the polypeptide.

The functional significance of differential binding of T_4 and T3 to TBG isoforms is not clear at present. On the basis of previous in vivo physiological studies of T_4 and T_3 delivery to rat liver following portal vein injection of human serum (Pardridge & Mietus, 1980), it is believed that one function of TBG in human serum is the selective amplification of T_3 delivery to liver as compared to T_4 (Pardridge, 1987). That is, T_3 is readily available for uptake by liver from the circulating human TBG-bound pool, whereas T_4 is not (Zaninovich et al., 1966; Pardridge & Mietus, 1980). The selective delivery of T_3, but not T_4, to liver by TBG is hypothesized to involve a mechanism of enhanced hormone dissociation from the plasma protein binding site that occurs in vivo within the organ microcirculation (Pardridge, 1987). The enhanced dissociation mechanism is believed to involve conformational changes about the hormone binding site that are triggered by transient interactions between the surface of the hepatocyte plasma membrane and the surface of the plasma protein (Mizuma et al., 1986; Terasaki & Pardridge, 1987). If the T_4-specific isoforms of TBG do not interact with the hepatocyte cell membrane to the same extent as do the isoforms carrying T_3, then differential delivery of T_4 and T_3 to liver from the circulating TBG-bound pool may occur. This hypothesis attempts to correlate the physiologic results, showing differential delivery of T_4 and T_3 to liver by TBG, with the biochemical results, showing differential binding of T_4 and T_3 to TBG isoforms. The sequential concanavalin A affinity chromatography and chromatofocusing protocol allows for separation of the T_4-specific TBG isoforms (Figure 5) and offers an approach for large-scale partial purification of these isoforms that may be used in future physiologic studies of T_4 and T_3 delivery to liver in vivo.

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