ESTIMATION OF THE T_4 BINDING CAPACITY OF SERUM TBG AND TBPA BY A SINGLE T_4 LOAD ION EXCHANGE RESIN METHOD*

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Most commonly used methods for the determination of thyroxine (T_4)-binding capacity of thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA) utilize electrophoretic separation of serum proteins (*1*). The Elzinga, Carr, and Beierwaltes modification *(2)* of Robbins *(3)* reverse-flow paper electrophoresis using glycine acetate buffer (*4*) is currently the standard procedure for the quantitation of the T_4-binding capacity of TBG and TBPA. Although technically simple, this method is time consuming, requiring careful sample application, a prolonged electrophoretic run for adequate protein separation, identification of the protein bands, strip scanning, and integration (or alternatively, zone sectioning before counting). Furthermore, the number of samples which can be run in a single assay is limited not only technically but also by the number of electrophoretic units available in the laboratory. For the past 3 years we have used an ion exchange resin procedure for the routine determination of the T_4-binding capacity of serum TBG and more recently TBPA. Although the method has not been reported previously, published results from early experience with this test were in reasonable agreement with those obtained electrophoretically (*5*). Since this publication, further modification and experience with more than 3,000 determinations indicate that measurement of the T_4-binding capacity of serum proteins by this method is both simple and accurate and can be adapted for routine use in clinical laboratories on unlimited number of samples.

The purpose of this communication is to present technical details of this new and simple method for determining the T_4-binding capacity of serum proteins and to provide data regarding its accuracy, reproducibility, and the effect of other serum proteins. Values are given from a population survey and for patients with various clinical states and diseases. The T_4-binding capacity of TBG by this resin method is compared in a double blind study to results obtained electrophoretically and by radioimmunoassay in two different laboratories.

PRINCIPLE OF THE METHOD

The principle of the method is based on the observation that the sensitivity of the T_4 assay by competitive binding analysis is dependent upon the number of high affinity binding sites in serum proteins available to the ligand over a wide range of dilutions. Indeed, using this assay as described in detail by Murphy and Jachan (*6*), a progressive increase in the concentration of TBG results in a family of standard curves with progressively reduced sensitivity (Fig. 1A). Variation in the TBG concentration was achieved by diluting serum with high TBG capacity (84 $\mu g T_4$/100 ml) obtained from a patient (MF) with severe myxedema taking high doses of estrogens, with serum from a patient (MiP) with congenital TBG deficiency *(7)*. The total protein content of the mixtures remained constant. By selection of these particular sera, the amount of T_4 from endogenous sources added to the system was negligible: the total T_4 of Patient MF was 0.5 and that of MiP was 1.2 μg/100 ml. For construction of the standard curves, the serum mixtures were diluted 1:50 with barbital buffer, which effectively blocks T_4-binding to TBPA (*8*). For each serum dilution, a constant amount of T_4-^{125}I and increments of unlabeled T_4 were added.

The shift to the right of the major early component of each curve with increasing concentrations of TBG (Fig. 1A) is due to the increasing concentration of TBG. To demonstrate this, data from Fig. 1A were replotted as a function of the molar concentration of T_4 per liter of undiluted TBG containing serum (Fig. 1B). It is apparent that the percent of T_4-^{125}I

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* Comparison of two unrelated methods, survey of population, and abnormalities in various diseases.

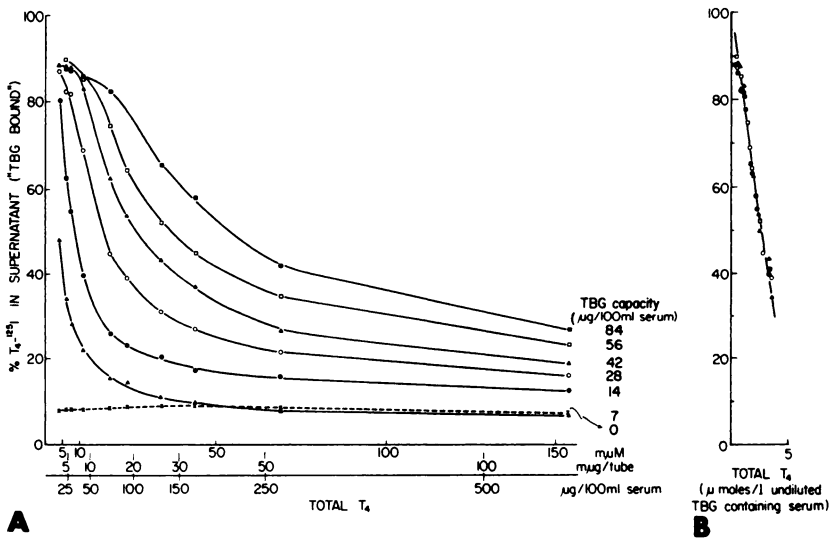

FIG. 1. A shows standard curves by competitive binding analysis of Murphy and Jachan (6) using human serum diluted 1:50. Variation in TBG capacity was achieved by mixing serum with TBG capacity of 84 μg T_4/100 ml with serum from patient with congenital TBG deficiency (7) in different proportions. B contains data from A replotted as function of T_4 content in constant amount of TBG. Absorption of T_4-^{125}I from serum (supernatant) by resin with fixed amount of T_4 is inversely proportional to TBG content irrespective of its concentration or content of other serum T_4-binding proteins in medium.

not absorbed by the resin ("TBG-bound T_4-^{125}I") is proportional to the degree of TBG saturation with T_4 irrespective of dilution over a range from 1:50 to 1:600.

MATERIALS

Equipment. Constant temperature bath (Thelco Model 83, Precision Scientific Co., Chicago, Ill.)

Horizontal Kahn shaker (180 oscillations/min, 1½-in. stroke) (Precision Scientific Co., Chicago, Ill.)

Vortex Genie mixer

Ice bath

Push-button microliter pipettes with disposable tips (100 and 1000 μl) (Eppendorf or Oxford Samplers)

Cornwall continuous pipetting outfit with 5-ml Luer-lok syringe (Becton-Dickinson Co., Rutherford, N.Y.)

Metallic 0.5-ml resin measuring scoop

Dispensing cups constructed from polystyrene microcentrifuge tubes (1-ml capacity, Scientific Products) coated with aluminum paint to prevent clinging of the resin beads and a rack capable of holding 50–60 dispensing cups

Metal funnel for addition of the resin to the tubes

Disposable polystyrene tubes 17 × 100 mm (No. 2017, Falcon plastics)

Well gamma scintillation counter

Cold room, if available

Reagents. Barbital buffer, 0.075 *M*, pH 8.6

Glycine-acetate buffer (0.2 *M* glycine, 0.12 *M* sodium acetate); pH 8.6 adjusted with NaOH

Thyroxine solution containing L-thyroxine-^{125}I* and unlabeled T_4 added to the buffer solution to known total concentration of 0.25–0.50 μg of T_4/ml as the free acid. T_4-^{125}I is added in sufficient amount to obtain approximately 4 × 10^6 cpm/ml. The usual contribution of the label to the total T_4 concentration in the solution is 0.045–0.11 μg of T_4/ml depending on the efficiency of counting and the specific activity of the radioisotope.*

Standard or reference serum with known total T_4 concentration, TBG, and TBPA capacity. Reference serum can be obtained by pooling a large amount of sera from euthyroid healthy subjects taking no drugs known to affect the serum T_4-binding capacity. If more than 200 carefully selected normal samples are pooled, the concentration of endogenous T_4 will most probably be 6.8 μg/100 ml and the TBG and TBPA capacities approximately 20 and 270 μg/T_4/100 ml, respectively. It is, however, advised that these parameters be carefully quantified by standard methods. The pool is stored frozen in small aliquots and is stable for at least 1 year.

Anionic exchange resin beads (Cl or $ClSO_4$; mesh-size 16–50)†. The resin is prepared by thoroughly washing batches of 1 lb 4–5 times with distilled water, removing small resin particles which do not readily settle. The resin is left to soak overnight, filtered with light suction in a Buchner funnel, and dried in a shallow pan at 60–70°C for approximately 12 hr. Too wet or over-dried resin beads will stick to each other and to the dispensing scoop. Optimal condition of the resin beads is tested by their ability to pour freely from a scoop.

* Obtained from Abbott Laboratories in 50% propylene glycol and used without further purification.

* Over the past 1½ years 32 lots of T_4-^{125}I were obtained from Abbott Laboratories. The specific activity varied from 37.6 to 92.1 μc/μg T_4.

† Rexyn-201 or 202 (Fisher Scientific Co., Fair Lawn, N.Y.) or Amberlite IRA-400 or 401S (Mallinckrodt Chemical Works, Saint Louis, Mo.).

The resin is stored in a screw-cap glass jar at room temperature.

METHODS

TBG capacity. For determination of the TBG capacity, all working solutions and serum dilutions are made in barbital buffer.

Standard curves (TBG isobars) are constructed as follows: Reference serum with known TBG capacity (usually 20 μg of T_4/100 ml) is diluted 1:20, 1:30, 1:35, 1:40, 1:60, 1:80, and 1:160. A constant known amount of T_4-^{125}I is added in a small volume to yield approximately 100,000 cpm/ml. For each of the seven curves, a pair of polystyrene tubes containing 0, 2.5, 5.0, 7.5, 10.0, 12.5, and 15 mμg of unlabeled T_4 are prepared, most conveniently by pipetting 0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 ml of a T_4 solution containing 50 mμg T_4/ml in absolute ethanol and evaporating to dryness. One milliliter of each of the T_4-^{125}I containing reference serum dilutions is added to the seven duplicate tubes containing increments of dried unlabeled T_4. An additional pair of tubes containing 1 ml from any of the serum reference dilutions is also prepared for the purpose of obtaining total ^{125}I counts. All tubes are incubated in the water bath for 15 min at 45°C. At the end of the incubation period the tubes are placed in the ice bath for 20 min. The 0.5 ml of resin beads carefully measured with a resin scoop are dispensed in as many cups as tubes being run. With the tubes in the ice bath, the resin is dispensed from the cups in rapid succession, facilitated by use of the metal funnel. It is preferable to add the resin first to one tube of each pair, then to the duplicate in the same order. No resin is added to the pair of tubes prepared for the estimation of the total counts. After addition of the resin, the tubes are allowed to remain in the ice bath for 2 min, then shaken in the horizontal shaker (preferably kept in a cold room) for 2 min. After this time, they are immediately replaced in the ice bath and 4 ml of distilled ice water is dispensed to each tube in rapid succession (in the same order the resin was dispensed) using a Cornwall continuous pipetting outfit. Forceful addition of the water stirs the resin and mixes the solution. The resin beads settle within seconds. Without further delay, using a push-button microliter pipette, 1 ml of solution from each tube (including the pair without resin beads) is transferred to a counting tube. All tubes should remain in the ice bath until aliquoted. Each tube is then counted in the gamma scintillation counter for 1 min.

Calculation and plotting of TBG isobars. The sum of duplicate counts, differing by less than 5%, is expressed as a percent of the total counts obtained from the sum of the pair of tubes to which no resin was added. Each standard curve is plotted with the percent counts in the supernatant (not absorbed by the resin) on the ordinate, against the total T_4 in each tube (sum of the endogenous T_4, T_4-^{125}I and unlabeled T_4 added) on the abscissa. A representative semilogarithmic plot of the TBG isobars is shown in Fig. 2. As unknown samples are usually diluted 1:40, the TBG capacity assigned to each isobar is self-explanatory. Data could also be plotted on ordinary graph paper. Reading by interpolation is, however, easier on the semilogarithmic plot.

Unknown samples. All unknown samples are run diluted 1:40 and at a single T_4 loading concentration as follows: 3.8 ml of barbital buffer are serially dispensed into as many polystyrene tubes as samples being run using the Cornwall continuous pipetting outfit. Using the push-button microliter pipette, 0.1 ml of unknown serum is added. After all unknowns have been aliquoted, the tubes are shaken gently in the rack and using the same pipette with a clean tip 0.1 ml of the thyroxine solution containing both T_4-^{125}I and a loading dose of unlabeled T_4 (see Reagents) is added to each tube. Mixing is completed by Vortex, and duplicate 1-ml aliquots are placed in polystyrene tubes using the push-button microliter pipette. From pooled excess diluted serum, a pair of 1-ml aliquots is reserved for estimation of total counts. The rack containing all tubes is then immersed in the water bath at 45°C. The procedure from this point on is that already described for the construction of the TBG isobars. A reference serum (serum used for the construction of the TBG isobars) and preferably one sample with high and one with low TBG content are run with each assay for quality control. The total T_4 per tube is estimated by summing the contribution of the endogenous T_4* [(T_4 mμg/100 ml)/4,000], and the labeled and unlabeled T_4 added to each tube. The latter two are constant for any particular assay and usually are 11–15 mμg/tube. From the total T_4 and the percent of T_4-^{125}I in the supernatant, the TBG capacity is read from the TBG isobars. Points falling between isobars are read by interpolation of parallel lines (Fig. 2). The result is read at TBG capacity intervals not smaller than 0.5 μg of T_4/100 ml. For TBG capacities greater than 40 or smaller than 5 μg of T_4/100 ml, the determination is repeated on half (0.05 ml) or double (0.2 ml) the amount of serum, respectively. The amount of endogenous

* The endogenous T_4 content is measured by the competitive binding assay on ethanol extracts of serum using the same principle and equipment as for the TBG determination or could be derived from the PBI.

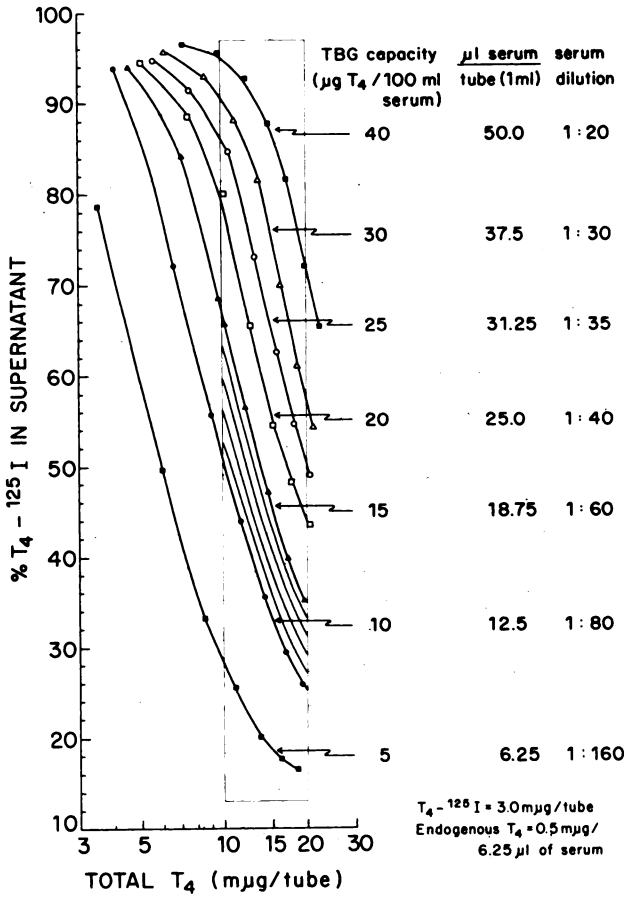

FIG. 2. TBG isobars used as standard curves for determination of TBG capacity. Dilution of normal pooled serum and its concentration of 1 ml of reaction mixture appear on right. TBG capacity in μg T_4/100 ml is read directly from TBG isobars. Area enclosed by vertical rectangle indicates more sensitive or working zone of isobars. For points falling in between isobars, TBG capacity is read by interpolation of parallel lines as indicated between 10 and 15 μg T_4/100 ml isobars.

T_4 and serum dilution should be taken in consideration in the final calculation. In rare instances, samples with TBG capacity of less than 3 μg T_4/100 ml can be repeated for greater accuracy on mixtures of the unknown with serum of known TBG capacity (most conveniently the reference standard), bringing the value within the more sensitive region of the TBG isobars.

If the assay conditions are kept constant, the same TBG isobars are satisfactory for repeated use. The incorporation of internal standards in each assay keeps a constant check on the validity of the isobars. The most common factor invalidating a set of TBG isobars is use of a lot of T_4-^{125}I or batch of resin different than the one used for their construction. With such changes, it is simpler to reconstruct the TBG isobars using the new material although a correction using a set of known samples could be applied. In a regular work day a single technician can comfortably do 60 TBG determinations, most conveniently in three batches of 20 unknowns running three internal standards each time.

TBPA capacity. For determination of the TBPA capacity, all working solutions and serum dilutions are made in glycine acetate buffer. Standard curves ("TBPA isobars") are constructed in the same manner as for the TBG, using the same serum dilutions. The only difference other than buffer is the addition of 10–20 fold more concentrated unlabeled T_4 for the construction of both "TBPA isobars" and loading of unknown samples. As the "TBPA isobars" measure the combined TBG and TBPA capacities, the binding capacity in glycine buffer minus the binding capacity in barbital buffer gives a more accurate estimate of the TBPA. The error introduced when this correction is not applied is more marked in pregnancy and in patients treated with estrogens, when the resultant TBG elevation is accompanied by a diminution in the TBPA (*5,9*), or in patients with profound idiopathic decrease in TBPA.

RESULTS

Stability of resin T_4 uptake. Four anion exchange resins were tested for their relative usefulness in the assay. It is apparent from Fig. 3 that at 0°C, stability of T_4 partition between resin and dilute

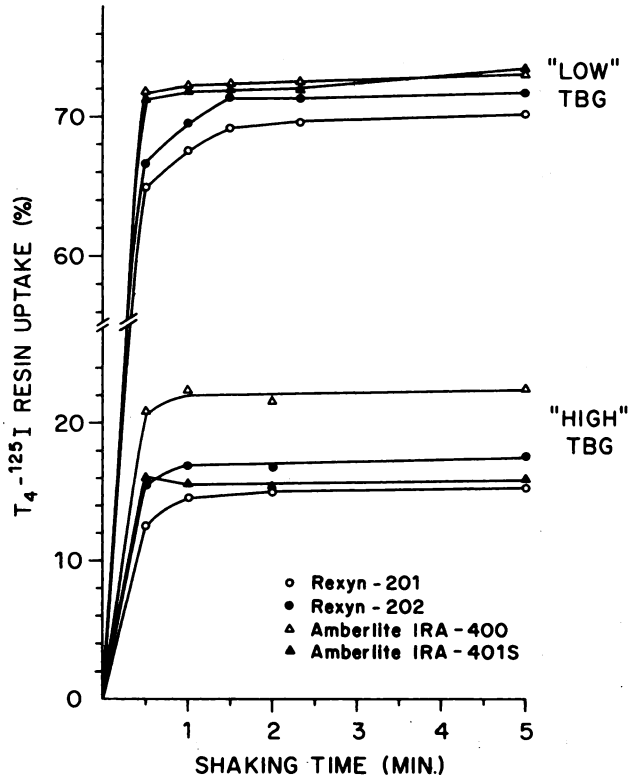

FIG. 3. Partition of T_4 between serum resin (barbital buffer at 0°C) as function of time. Reasonable stability is evident after 1½–2 min contact in presence of both low and high TBG content.

serum is reached after 1½ min and that further contact does not substantially increase the resin uptake of T_4. This observation was unaffected by changes in the percent resin uptake brought about by varying the concentration of TBG. Dilution of the system with ice water after 2 min of shaking and allowing the resin to settle to the bottom of the tube (see Methods) further reduces the tendency for continual irreversible T_4 uptake by the resin beads and allows time for aliquoting the supernatant. The same stability was observed in the system using glycine-acetate buffer.

Effect of varying the T_4 load. During our early experience with this method, sera were run at 2 or 4 different loading doses of unlabeled T_4. In all sera, including sporadic or congenital alterations in TBG capacity, after saturation of the binding proteins, all points paralleled the same isobar on the standard curve without significant cross over.

Effect of TBG dilution and total protein concentration. To determine whether serum proteins other than TBG have any effect on determination of the TBG capacity, the latter was measured in four sera with wide differences in TBG content, in the usual 1:40 dilution using 0.1 ml of serum, half or double the amount of serum, and in the presence of variable concentration of serum total protein achieved by addition of TBG deficient serum. Results of such determinations are shown in Table 1. It is apparent that the TBG values were unaffected by serum dilution or the presence of up to six fold the usual concentration of other serum proteins, including TBPA. Also, results of TBG determinations carried out on serum mixtures were the expected arithmetic sum of the TBGs in the original sera. It should be noted that in the calculation, changes in the amount of endogenous T_4 added to the system, as well as serum dilution, were taken in account.

Comparison of TBG and TBPA determinations by different assay methods. Table 2 presents data on the TBG and TBPA capacities determined by the method described in this communication (Method A), and the reverse-flow paper electrophoresis in glycine acetate buffer (Method B). TBG was also

TABLE 1. EFFECTS OF SERUM DILUTION, PROTEIN CONCENTRATION, AND SERUM MIXTURE ON DETERMINATION OF TBG CAPACITY

Serum sample	ml serum/ 4 ml buffer	Endog. T_4 (μg/100 ml)	T_4 (mμg/tube)		T_4-^{125}I in supernatant†	TBG capacity (μg/100 ml)		
			Endogenous	Total*		Observed: From std. curve†	Observed: Corrected for dilution	Theoretical
Usual 1:40 dilution								
P-4	0.1	6.5	1.6	14.8	56.5	20.0	20.0	20
MiP	0.1	1.2	0.3	13.5	9.6	<5	<5	0
PR	0.1	3.2	0.8	14.0	21.5	6.0	6.0	6.0
NV	0.1	13.8	3.5	16.7	96.2	>40	>40	54.0
Varying dilutions								
P-4	0.05		0.8	14.0	35.7	10.0	20.0	20.0
P-4	0.2		3.2	16.4	82.6	39.5	19.8	20.0
PR	0.2		1.6	14.8	37.5	11.5	5.8	6.0
NV	0.05		1.8	15.0	69.8	27.0	54.0	54.0
Serum mixtures								
P-4	0.1						20.0	20.0
+			1.9	15.1	54.6	20.0		
MiP	0.1						0	0
P-4	0.1						20.0	20.0
+			2.5	15.7	55.2.	20.5		
MiP	0.3						0.2	0
P-4	0.05						20.0	20.0
+			1.7	14.9	32.6	10.0		
MiP	0.3		2.4	15.6	65.2	26.0	0	0
P-4	0.1						20.0	20.0
PR	0.1						6.0	6.0
NV	0.05		2.2	15.4	73.1	30	54.0	54.0
PR	0.05						6.0	6.0

P-4 is normal serum pool.
MiP is congenital TBG deficiency with normal TBPA (see Table 2) (7).
PR is heterozygous female from a family with congenital TBG deficiency (see Table 2) (7).
NV is heterozygous female from a family with congenital TBG excess (see Table 2) (11).
* Exogenous T_4 load (labeled and unlabeled) = 13.2 mμg/tube.
† See Fig. 2.

determined by radioimmunoassay (Method C) and expressed as milligrams of purified human TBG standard per 100 ml of serum (*10*). The radioimmunologic determinations were performed by Richard Levy. The mean normal TBG in his series is 3.5 mg/100 ml ± 0.7 s.d. The TBG and TBPA capacities obtained electrophoretically were known only to one of the authors who selected the samples. All subsequent determinations were done by two independent workers in two different laboratories on coded samples. The code was broken by the senior author only after the final results were obtained. Sera of subjects from five families with congenital X-linked TBG abnormalities [deficiency and excess (*11*)] were included. All values agree within the methodologic error. The coefficient of correlation of plots of Method A vs B and A vs C are 0.9886 and 0.9735, respectively. It is interesting to note that the antiserum used for the human TBG immunoassay did not cross react with monkey or dog T_4 binding proteins, the former possessing a binding protein with electrophoretic mobility identical to human TBG (*12*). Because the slope of both monkey and dog binding-protein isobars differs from that of human TBG, their measurement using human TBG standard is not recommended.

Reproducibility of the assay. Data on the reproducibility of TBG determinations in a single assay and repeat determinations of the same samples by three different technicians over the period of 2 months using the same TBG isobars is given in Table 3. The coefficient of variation expressed as

TABLE 2. COMPARISON OF TBG DETERMINATIONS BY THREE AND TBPA BY TWO DIFFERENT METHODS

					TBG			TBPA	
Code	Description	TP (gm/ 100 ml)	TT_4 (μg/ 100 ml)	FTI	Method A capacity	Method B (μg T_4/ 100 ml)	Method C (mg/ 100 ml)	Method A capacity	Method B (μg/ 100 ml)
P-3	Normal serum pool #3	7.2	6.5	6.5	20.0	19.6	3.6	200	215
P-4	Normal serum pool #4	7.4	6.5	6.5	20.0	20.8	3.5	250	246
4011	Normal female	6.7	6.8	5.8	27.5	31.0	4.4	320	337
4059	Normal female	7.5	4.3	5.2	16.0	18.8	3.4	250	216
4012	Normal male	8.1	6.0	7.3	17.5	21.2	3.1	300	296
1778	Severe myxedema	7.6	0.2	0.2	15.0	16.8	2.8	370	386
1783	Thyrotoxic, Graves' Disease	7.3	26.4	41.0	17.0	18.5	3.0	210	199
	High and low TBG								
1789	Male on estrogens	8.6	8.9	3.5	33.0	32.1	6.0	250	266
1647	Female on estrogens	—	12.8	3.9	51.0	50.9	—	260	288
M-4	Pregnancy at term	6.6	11.3	9.1	61.0	58.0	8.1	175	170
2462	Terminal illness	4.7	1.3	2.7	4.1			25	24
	Inherited TBG Abnormalities								
	Family P								
5P	Deficient XO (MiP)	5.5	1.2	4.6	0	0	0	302	310
1003A	Deficient male (RR)	8.1	1.4	4.4	0	0	0	310	313
1002A	Affected female (DL)	7.7	3.8	5.3	8.0	9.8	1.4	325	321
1007A	Affected female (PR)	7.3	3.2	6.5	6.0	7.1	1.0	300	312
	Family G								
103G	Affected male (TG)	7.7	1.9	3.5	2.6	2.5	0.8		
104G	Affected female (DG)	7.5	5.3	5.1	17.5	17.5	3.8		
	Family A								
10A	Affected male (JA)	7.5	1.6	4.4	3.0	3.1	0.6		
15A	Affected female (CA)*	7.2	5.2	6.0	20.0	21.0	4.1		
11A	Normal female (EA)	6.7	4.6	5.1	20.0	20.8	3.6		
	Family L								
1L	Affected male (AL)	7.8	2.6	4.4	5.0	2.4	0.6		
2L	Affected female (RL)	9.7	5.4	5.8	17.5	12.5	2.8		
3L	Normal male (FL)	8.7	7.8	6.7	25.0	20.2	4.1		
	Family N								
	Affected female (NV)	8.1	13.8	7.2	54.0	48.0	9.9		
	Animal sera								
150-68	Pregnant Rhesus monkey	7.6	5.2	—	33.0†	—	0		
D-1	Dog	7.2	2.2	—	3.5†	—	0		

TP = Total protein; TT_4 = Total T_4; FTI = Free T_4 index.
Method A = Single T_4 load — ion exchange resin.
Method B = Reverse-flow paper electrophoresis in glycine acetate buffer, pH 8.6 (*7*).
Method C = Radioimmunoassay (*10*).
* On oral contraceptives.
† TBG-like capacity as measured in the human TBG isobar system; electrophoretic analysis has previously been reported.

TABLE 3. REPRODUCIBILITY OF TBG ASSAY

	Sample No.	N	TBG capacity (μg T_4/ml) Mean	s.d.	Range	s.d. (% of mean)
Repeated determinations in same assay	12463	10	12.61	0.17	12.3–12.9	1.4
	12465	10	25.00	0.31	24.5–25.5	1.2
Determinations in different assays up to 2 months apart by three technicians	PR	8	5.94	0.34	5.6– 6.5	5.7
	12463	12	12.33	0.31	12.0–13.0	2.5
	12465	12	24.33	0.64	23.0–25.5	2.6
	BF	5	43.48	0.82	43.0–44.5	1.9

N = Number of determinations.

the percent standard deviation of the mean value, ranged from 2 to 6% and is greater at low than high TBG capacity.

Tests of the principle of TBG determination. Preliminary data from determinations of the TBPA capacity using glycine acetate buffer in an assay identical to the TBG method were in agreement with determinations obtained electrophoretically. Because triiodothyronine (T_3) binds to the T_4-binding site on TBG but does not bind to TBPA in any buffer system (*1*), the following experiment was designed to gain some insight into the mechanism of the TBPA assay. Sera from two subjects, one with TBG deficiency (MiP) and a normal contral (SR), approximately matched for their TBPA content, were individually enriched with T_4-^{125}I and T_3-^{125}I. Displacement of the labeled hormone was achieved by adding unlabeled T_4 over a range covering both TBG and TBPA capacities. Results are presented graphically in Fig. 4. As anticipated, in the presence of T_3-^{125}I, the TBG deficient serum surrendered the isotope to the resin, and no displacement curve was observed. On the other hand, in the presence of normal serum there was evidence of initial binding of the T_3-^{125}I to serum followed by a precipitous drop in the bound fraction with the addition of a relatively small amount of unlabeled T_4. The resultant curve is reminiscent of a curve using T_4-^{125}I when binding to TBPA is inhibited with barbital buffer. With T_4-^{125}I, binding to serum was present in both the TBG deficient and normal sera. Relatively large amounts of unlabeled T_4 were necessary to displace the T_4-^{125}I. In the region of the curve where increased resin uptake is due primarily to the displacement of T_4-^{125}I from TBPA, the curves of both sera closely coincide, in keeping with their quasi-identical TBPA capacities. The discrepancy in the early portion of these two curves is due to the presence of T_4-^{125}I binding to TBG in the normal serum; this is rapidly displaced with unlabeled T_4.

Figure 4 also gives the results of displacement of T_4-^{125}I with unlabeled T_3 in normal serum. Evidence

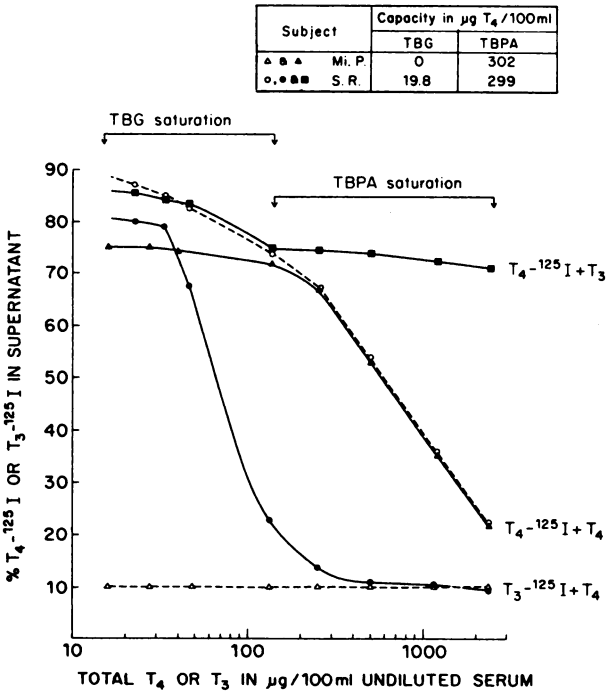

FIG. 4. Displacement analysis of T_4 and T_3 in presence of binding to two serum proteins in glycine acetate buffer. Zones for TBG and TBPA saturation and TBG and TBPA capacities of sera used are indicated. In presence of T_3-^{125}I, which does not bind to TBPA, there was no binding to TBG deficient serum (open triangles) and displacement by T_4 in normal serum (SR) was typical of binding to TBG alone (closed circles). T_4-^{125}I, which in glycine acetate buffer binds to both TBG and TBPA, produced, with addition of unlabeled T_4, displacement curves with both TBG deficient (closed triangles) and normal (open circles) sera. Note discrepancy in early portion (TBG saturation zone) of curves with superimposition in TBPA saturation zone (TBPA capacity for both sera was closely matched). Displacement of T_4-^{125}I from normal serum with unlabeled T_3 (closed squares) shows minimal displacement with saturation of TBG but no substantial drop in TBPA zone. T_3 could not displace the T_4-^{125}I from TBPA.

for displacement of T_4 from TBG is shown in the early diminution of percent of T_4-^{125}I in the supernatant. In the TBPA displacement region, however, addition of unlabeled T_3 produced little change in the T_4-^{125}I resin uptake compared with the effect of addition of unlabeled T_4. This phenomenon is due to the inability of the T_3 to displace the T_4-^{125}I,

which is now primarily bound to TBPA. Minimal diminution in the percent of bound T_4-^{125}I is probably due to contamination of the unlabeled T_3 with T_4*. The latter was clearly demonstrated on paper chromatography (*13*) of the unlabeled T_3 using a sensitive dye developer (*14*).

TBG capacity in the "normal" population. Serum samples were obtained from 110 (55 males and 55 females) healthy blood bank donors and subjects presenting for pre-employment physical examination at the University of Chicago. None were taking medications capable of altering T_4 binding to serum proteins. Mean standard deviations and range for age, serum total protein (TP)*, serum total thyroxine (TT_4) (*6*), free thyroxine index (FTI) (*15*), and TBG capacity are given in Table 4. Statistical analysis is provided in Table 5. Figure 5 illustrates the distribution of the TBG capacity in the same population. There was no significant difference in

* Mann Research Laboratory, New York, N.Y. Similar results obtained with T_3 from Sigma Chemical Co., St. Louis, Mo., and Nutritional Biochemicals Corp., Cleveland, Ohio.

* By refractometry (T. C. Refractometer, American Optical Corp., Buffalo, N.Y.).

TABLE 4. TBG CAPACITY AND OTHER PARAMETERS OF THYROID FUNCTION IN NORMAL POPULATION AND SELECTED CONDITIONS KNOWN TO ALTER T_4 TRANSPORT IN BLOOD

Group No. and description	Female/male ratio	N	Mean ± s.d. and range				
			Age	TP (gm/100 ml)	TT_4 (μg/100 ml)	FTI	TBG (μg T_4/100 ml)
1. Normal controls	55/55	110	32.7 ± 14.0 (16–74)	7.4 ± 0.5 (5.9–9.3)	6.8 ± 1.3 (3.8–9.2)	6.6 ± 1.4 (3.8–10.0)	20.4 ± 4.1 (12.0–30.5)
2. Normal males	0/55	55	33.1 ± 15.4 (18–60)	7.4 ± 0.5 (6.4–9.3)	6.6 ± 1.2 (4.5–9.2)	6.8 ± 1.4 (3.8–10.0)	19.2 ± 4.0 (12.0–30.5)
3. Normal females	55/0	55	32.0 ± 12.0 (16–74)	7.4 ± 0.5 (5.9–8.6)	7.1 ± 1.4 (3.9–8.9)	6.4 ± 1.3 (4.7–9.2)	21.6 ± 4.0 (13.0–30.5)
4. Euthyroid on estrogens	27/0	27	39.7 ± 14.8 (22–75)	7.7 ± 0.6 (6.3–8.9)	9.2 ± 2.2 (6.7–14.7)	5.3 ± 1.3 (3.1–8.2)	44.1 ± 5.9 (30.0–52.0)
5. Euthyroid pregnant	14/0	14	30.6 ± *7.0 (22–42)	6.7 ± 0.5 (5.5–7.6)	10.9 ± 1.6 (7.8–13.8)	6.4 ± 2.1 (3.1–9.8)	53.7 ± 7.1 (41.0–69.0)
6. Euthyroid in renal failure	3/6	10	52 ± 13.7 (33–71)	6.5 ± 0.3 (5.9–6.9)	5.2 ± 1.7 (2.6–8.6)	7.4 ± 2.5 (4.0–10.3)	14.1 ± 3.1 (10.8–19.0)
7. Hyperthyroid	9/6	15	42.9 ± 16.0 (23–73)	6.9 ± 0.5 (6.3–7.4)	21.9 ± 7.6 (9.8–33.7)	37.3 ± 17.0 (18.7–67.6)	15.7 ± 3.2 (8.5–21.5)
8. Hypothyroid	13/2	15	48.1 ± 18.4 (14–74)	7.8 ± 0.7 (6.6–9.0)	0.9 ± 0.8 (0.1–3.2)	0.5 ± 0.4 (<0.1–1.5)	24.6 ± 5.1 (14.0–32.0)

For abbreviations see Table 2.
N = number of determinations.

TABLE 5. COMPARISON OF MEAN VALUES OF GROUPS FROM TABLE 4 (P VALUES OF STUDENT DISTRIBUTION)

Group No.*	Age	TP	TT_4	FTI	TBG
1 & 2	NS	NS	NS	NS	NS
1 & 3	NS	NS	NS	NS	NS
1 & 4	$0.025 < p < 0.05$	$0.02 < p < 0.025$	<0.001	<0.001	<0.001
1 & 5	NS	<0.001	<0.001	NS	<0.001
1 & 6	0.001	<0.001	<0.005	NS	<0.001
1 & 7	$0.025 < p < 0.05$	<0.001	<0.001	<0.001	<0.001
1 & 8	<0.001	<0.01	<0.001	<0.001	<0.001
2 & 3	NS	NS	$0.02 < p < 0.05$	$0.05 < p < 0.1$	<0.005
3 & 4	NS	$0.025 < p < 0.05$	<0.001	<0.005	<0.001
3 & 5	NS	<0.001	<0.001	NS	<0.001
4 & 5	$0.025 < p < 0.05$	<0.001	$0.01 < p < 0.02$	$0.05 < p < 0.1$	<0.001
4 & 8	$0.01 < p < 0.02$	NS	<0.001	<0.001	<0.001
5 & 8	<0.005	<0.001	<0.001	<0.001	<0.001
6 & 7	NS	$0.05 < p < 0.1$	<0.001	<0.001	NS
7 & 8	NS	<0.005	<0.001	<0.001	<0.001

For abbreviations see Table 2.
* From Table 4.
NS = Not significant ($p = 0.1$).

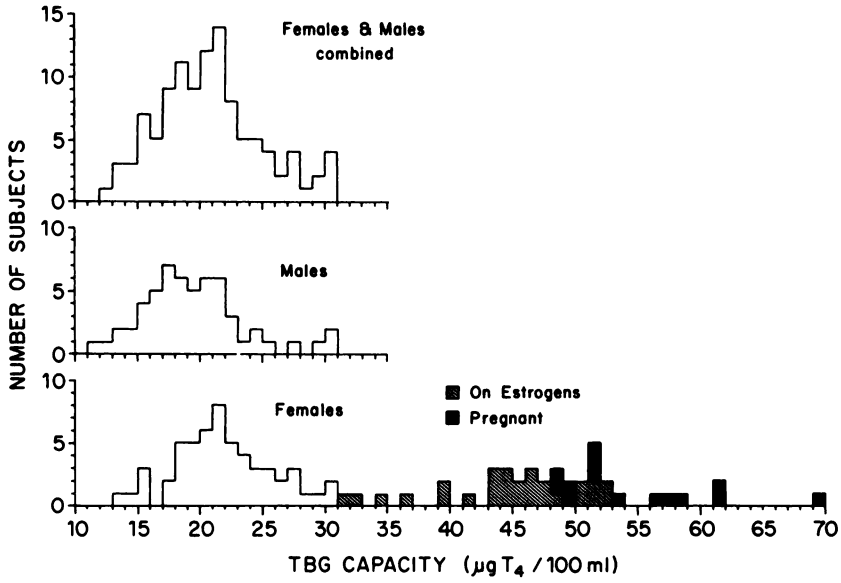

FIG. 5. TBG capacity in population groups. Normal males, normal females, females in the last trimester of pregnancy, and females on various doses of estrogen-containing drugs.

the mean age, TP, and FTI between males and females. The mean TT_4 and TBG were significantly higher in the female group with $p < 0.05$ and 0.005, respectively.

Effect of pregnancy, estrogens, and various thyroidal and nonthyroidal illnesses on the TBG capacity. Table 4 provides additional data on the following groups of patients: Group 4, 27 euthyroid females taking different doses of various estrogen-containing drugs (mostly anovulatory agents); Group 5, 14 euthyroid patients in the last trimester of pregnancy; Group 6, 10 euthyroid patients in renal failure due to a variety of renal diseases with BUN values ranging from 76 to 205 mg/100 ml (normal <20 mg/100 ml); Group 7, 15 patients with moderate to severe thyrotoxicosis; and Group 8, 15 patients with long standing primary myxedema. All samples were selected in sequence from sera sent to the University of Chicago Thyroid Function Laboratory for the determination of TT_4 and FTI over the period of 3–4 months. Data confirming each diagnosis were obtained from hospital records. Most patients with thyroid diseases were well known to one of the authors. Statistical analysis is provided in Table 5. Because no significant difference of mean values for all recorded parameters was observed between female or male normals and the combined group, it was considered legitimate to compare groups with uneven female-to-male ratio to the combined female-male normal population. Statistically significant difference in the TBG capacity was observed between the normals and all other groups. It is worth noting that, although decreased TBG capacity in the renal failure and hyperthyroid groups was associated with a significant diminution in the TP concentration, the percent of TBG decrease was out of proportion to the percent of TP fall. Also in pregnancy a significantly lower mean TP level was associated with approximately 2½-fold elevation in the mean TBG capacity.

In all euthyroid groups, mean TBG changes were always accompanied by proportional changes in the mean TT_4, resulting in a normal FTI. However, the mean TBG elevation of the estrogen treated group was slightly out of proportion to the mean TT_4 increase, resulting in a mean FTI for this group significantly lower than the normal mean, although still within the normal range.

Sporadic TBG abnormalities. Table 6 presents limited data on abnormal TBG capacity associated with a variety of unrelated illnesses, some of which (i.e. hepatitis, heart failure) are well known to temporarily affect this parameter (*9,16*).

DISCUSSION

Methods for the estimation of the T_4-binding capacity of TBG (*17–19*) and TBPA (*17*) by non-electrophoretic methods using either Dextran-coated charcoal (*18*) or ion-exchange resin (*17,19*) have been reported recently. Although all may prove useful for routine clinical use, experience with these methods has so far been limited. The method of Keane et al (*17*) uses diluted serum in phosphate buffer, ion exchange resin for the separation of "bound" from "free" T_4, and a Scatchard analysis of the data for the determination of the binding capacity. One of the disadvantages of this method is the necessity to run each individual unknown serum at eight different T_4 loading concentrations. Also, although the binding capacities reported in a

limited number of sera are within the range of those reported by electrophoretic methods, no direct comparison was made. The other two methods (*18,19*) are simpler and seem to give results comparable to those obtained electrophoretically. The method of Nicoloff et al (*19*), an adaptation of the T_3 resin sponge uptake (Abbott Triosorb), has been inaccurate in our hands, giving at best a rough estimate of the T_4-binding capacity of TBG.

The method described in this communication is simple, accurate, reproducible, and has been adapted successfully for routine hospital use. Values obtained are in agreement with electrophoretic and radioimmunologic determinations in both normal samples and in presence of altered serum T_4 binding capacity. As previously reported, significant differences in the mean TBG capacity were observed between normal males and females (Tables 4 and 5) (*20,21*), and values were not different than those obtained by electrophoresis on paper (*22*), polyacrylamide (*21*), and agar gel (*23*). Higher mean TBG capacity in normal females was accompanied by a significantly greater mean TT_4 concentration. Such a difference was observed by Murphy and Pattee using the same method for serum total T_4 determination (*24*) but not by Braverman et al using the PBI (*20*). As in the case of free T_4 measured by dialysis (*20*), no difference in the mean FTI (an estimate of the free T_4) of males and females was observed. In agreement with previous publications are the observations that mean TBG capacity in the last trimester of pregnancy is 2½-fold normal (*25,26*), high in patients on estrogen-containing drugs (*27*), diminished in thyrotoxicosis and increased in hypothyroidism to the same extent as reported by Inada and Sterling (*22*), and decreased in patients with renal failure (*26*). Also, as previously reported (*9*), nonthyroidal illnesses had variable effect on the TBG capacity (Table 6). Although differences in the TBG capacity is not due to the uneven female-to-male distribution among these groups, the effect of differences in mean age on the TBG (*28*) can not be assessed as the groups were not matched for age. Changes in TP does not seem to be responsible for the rather wide variation in the TBG capacity in various clinical groups.

Finally, no support could be found for the hypothesis that patients with congenital TBG abnormalities have abnormal TBG with altered affinity to T_4 (*29*). Indeed, in each of the five families with total or partial TBG deficiency or TBG excess, TBG content was similar whether measured by the determination of its capacity to bind T_4 or immunologically. The characteristic slope of the TBG saturation curve was identical in all patients including those with inherited and acquired changes in TBG capacities, suggesting that most abnormalities in man so far described are due to changes in the concentration of this protein in serum.

SUMMARY

The principle and method of a simple assay for the determination of the thyroxine (T_4)-binding capacity of thyroxine binding globulin (TBG) and thyroxine binding prealbumin (TBPA) are described. The procedure is accurate, reproducible, rapid, and has been adopted for routine hospital use. A single technician can perform in a regular working day 60 determinations in addition to the three internal control samples. Results obtained using this method compared favorably with those obtained electrophoretically and by radioimmunoassay. Mean and standard deviations for the TBG capacity in the normal adult population are 19.2 ± 4.0 in males, 21.6 ± 4.0 in females, 44.1 ± 5.9 in patients taking estrogen containing medications, 53.7 ± 7.1 in the last trimester of pregnancy, 14.1 ± 3.1 in patients with renal failure, 15.7 ± 3.2 in hyperthyroid,

TABLE 6. ABNORMAL TBG CAPACITY IN VARIOUS NONTHYROIDAL ILLNESSES

Diagnosis and remarks	Sex and age	TP (g/100 ml)	TT_4 (μg/100 ml)	FTI	TBG capacity (μg T_4/100 ml)
Mycosis fungoides (terminal)	M 21	4.7	1.3	2.7	4.1
Parasitic bowel disease (strongyloides stercolis) with eosinophilia	M 40	—	3.8	6.0	12.0
Severe congestive heart failure	F 85	—	6.7	10.8	12.0
Oat cell carcinoma of lung	M 53	—	8.6	5.8	27.5
Transfusion hepatitis	F 67	7.1	9.8	6.5	32.5
Infectious hepatitis	F 12	7.7	8.7	3.4	46.0
After recovery (2 months later)		7.5	6.8	4.1	34.5
Angioneurotic edema	F 41	—	7.1	3.6	42.0
Lymphosarcoma	M 72	7.0	12.6	7.4	53.5

For abbreviations see Table 2.

and 24.6 ± 5.1 μg/100 ml in hypothyroid patients. These results are in agreement with previously reported values using different assay methods. Data obtained from five families with X-chromosome linked inherited TBG abnormalities suggest that the defect is quantitative rather than due to a protein varying in its ability to bind thyroxine.

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