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Simultaneous Determination of Free Thyroxine and Capacity of Thyroxine-Binding Globulin

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A simple method is described for the simultaneous determination of capacity thyroxine-binding of globulin (TBG) and free thyroxine concentration (FT_4). The ratio of bound to free T_4 (B/F) is first determined for two total-T_4 concentrations using a Sephadex G-25 competitive-binding technique. TBG capacity and FT_4 can both be calculated assuming a known value of affinity constant of TBG. The method is linear over a twenty-fold serum dilution. FT_4 calculated is identical to that calculated using the method of Irvine. TBG capacity is shown to be linearly correlated to TBG concentration as determined by radioimmunoassay and is consistent with a molecular weight of 69,000 Daltons and one T_4 binding site per molecule. FT_4 is found to correlate with the free thyroxine index in a complicated way, depending on the degree of TBG saturation.

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Thyroid hormone (T_4) is transported in serum by proteins that bind it: thyroxine-binding globulin (TBG), thyroxine-binding pre-albumin (TBPA), and albumin. Variations in the concentrations of these proteins can lead to alteration of serum total-T_4 concentrations without causing any change in the concentration of free-thyroxine fraction. In conditions where the thyroxine-binding proteins are out of normal range, knowledge of FT_4 and TBG capacity allows the physician to interpret accurately serum total-T_4 concentration. FT_4 is usually determined by equilibrium dialysis, which is difficult for routine use. More commonly, TBG capacity is assessed by one of the triiodothyronine (T_3) uptake methods. Total T_4 is then interpreted along with T_3 uptake results and is expressed as an adjusted T_4 value, the free T_4 index (FT_4I). It has been recognized, however, that the FT_4I can be misleading in conditions where there is significant abnormality in serum protein concentrations (*1*). A competitive-binding method was used by Irvine (*2*) to determine serum free-T_4 concentration; this was later expanded by Sutherland (*3*) to determine the association constants and binding capacities of serum thyroxine-binding proteins. The technique allows accurate determination of the ratio of bound to free hormone (B/F).

Using a published value for the binding constant for TBG, the TBG capacity and FT_4 can be determined from B/F ratios for two known T_4 concentrations. We have used this two-point titration procedure to study the TBG capacity and FT_4 for a number of serum samples, and have compared these values with TBG concentrations as determined by RIA and FT_4I.

MATERIALS AND METHODS

Plasma samples from patients in various states of thyroid function were obtained from the clinical laboratory. Total T_4 was determined by radioimmunoassay, and T_3 uptake by using the Autopak Kit* (*4*). FT_4I is given by the product of T_4 and the normalized T_3U:

$$FT_4I = T_4 \times \frac{T_3U}{T_3U_{normal}}.$$

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TBG concentration was determined both by the competitive-binding technique and by radioimmunoassay.†

The competitive-binding study was performed with Sephadex G-25, medium grade, used untreated. Iodine-125-labeled T_4 was also obtained commercially. For the determination of partition coefficients (α), it was purified by ascending paper chromatography using Whatman No. 1 paper and a solvent system of butanol: acetic acid: water (4:1:1). Otherwise it was used without purification, free iodide contamination of less than 5% being tolerated. The binding study was carried out in sodium phosphate buffer (0.05 *M*) at pH 7.4, with 0.1 *M* sodium chloride. The apparatus used for measuring relative activity is a modification of that of Sutherland et al. (*3*). Instead of a flask with a sidearm holding a constant volume for repetitive activity determination, we used a lead-shielded test-tube holder with an aperture for activity measurement (Fig. 1). The geometry of the apparatus is such that only the supernatant can be seen by the detector. The detector, a NaI(Tl) crystal and photomultiplier tube, was connected to an amplifier and a single-channel analyzer set for 35 keV, the gamma energy peak of I-125. Twenty-milliliter plastic test tubes were filled with 14 ml of buffer, 100 μl of plasma, and 100 μl of a solution of buffer and I-125 T_4, resulting in an initial volume (before Sephadex) of V = 14.2 ml. The system was allowed to incubate in a water bath at 37°C for 1 hr, after which the tubes were placed in the apparatus of Fig. 1 and the first count rate (R_0) measured. Sephadex (1.5 g) was then added to each tube and the tube shaken gently for ~45 min. The Sephadex was then allowed to settle and the tubes counted again (as in Fig. 1) to obtain the second count rate (R_1). Next, a known small quantity of nonlabeled T_4 (73.5 ng) was added to each tube and the tubes were shaken gently until a new equilibrium was reached. The tubes were counted again in the apparatus to give a third count rate (R_1').

The partition coefficient (α) of Sephadex G-25 for T_4 was determined in parallel without any plasma added. Addition of unlabeled T_4 is known not to influence this coefficient. Since free iodide distributes almost equally between inclusion and exclusion volume with 9.6% bound to Sephadex, iodide contamination in the labeled T_4 can be routinely corrected for by subtracting 0.904 × % free iodide, R_0, from R_1 and R_1'. Inclusion volume for 1.5 g Sephadex is determined by the use of I-131-labeled albumin as well as I-125-labeled TBG provided in the radioimmunoassay kit.

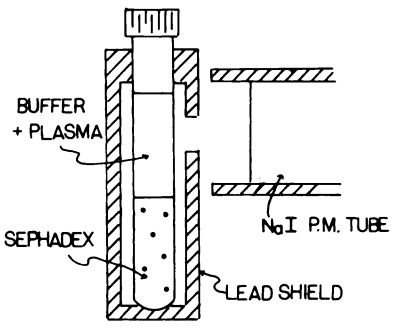

FIG. 1. Apparatus used to determine relative activity of supernatant.

Calculation of free and bound T_4. When a quantity of Sephadex is added to a solution containing T_4 and T_4-binding proteins, the Sephadex will absorb a volume of the buffer, the inclusion volume (I). Because of their large molecular weights, thyroxine-binding proteins and bound T_4 will be concentrated in the exclusion volume (EV), which is equal to V−I, the initial volume minus the inclusion volume. If this system is allowed to incubate until equilibrium is reached, the T_4 will exist in three states: T_4 bound to thyroxine-binding proteins, T_4 bound to the Sephadex, and free T_4 in the solution. The amount of T_4 in each state can be given by the concentration multiplied by the respective volume of distribution.

We introduce the following definitions: ρ_F = concentration of free T_4 in the exclusion volume; ρ_B = concentration of bound T_4 in the exclusion volume; ρ_{T_4} = initial concentration of T_4 before the addition of Sephadex (the product $\rho_{T_4} \times V$ gives the total quantity of T_4 in the system); V = total initial volume of reaction mixture; I = inclusion volume by 1.5 g of Sephadex; α = constant of proportionality having the dimensions of volume, the ratio of the quantity of T_4 bound to Sephadex over the concentration of free T_4 in the exclusion volume. Because of the large capacity of Sephadex for T_4, this value remains constant over a wide range of T_4 concentrations.

The relationship of "conservation of T_4" can be expressed as:

$$(V-I)\rho_B + (V-I)\rho_F + \alpha\rho_F = V\rho_{T_4}. \qquad (1)$$

When the Sephadex is allowed to settle, the supernatant will represent a true sample of the excluded volume. Measurement of the concentrations of free and bound T_4 in the supernatant is therefore a measure of the same concentrations in the exclusion volume. The concentration of T_4 in the supernatant, ρ_s, is given by the sum of the concentrations of free and bound T_4 in the supernatant. The identity of the concentrations in the supernatant to those in the exclusion volume allows a relationship to be written as:

$$\rho_s = \rho_F + \rho_B \qquad (2)$$

When a radiotracer is used to represent the stable thyroxine, ρ_{T_4} is proportional to the count rate of the solution seen through the aperture before Sephadex is added (R_0), and $\rho_{T_4} = \epsilon R_0$; ρ_s is proportional to the count rate of the supernatant after the Sephadex is added (R_1), and $\rho_s = \epsilon R_1$. V, I, and α are characteristic of the system and can be predetermined. Since R_0 and R_1 are measurable,

Eqs. 1 and 2 represent a solvable system of two simultaneous equations, with two unknowns, ρ_F and ρ_B.

If one assumes that I-125-labeled albumin distributes in the exclusion volume in the same way as T_4 bound to the TBP, I-125-labeled albumin can be used to determine the inclusion and exclusion volumes. Since there is no free I-125, $\rho_F = 0$ and $\rho_s = \rho_B$. Making these substitutions into Eq. 1:

$$(V-I)\rho_s = V\rho_{T_4}, \text{ or } V-I = V\frac{\rho_{T4}}{\rho_s} = \frac{V\,R_0}{R_1}$$

α can be determined using radioactive T_4 without the addition of any TBP. When only radioactive T_4 is added to the buffer, $\rho_B = 0$ and $\rho_s = \rho_F$. Making these substitutions into Eq. 1:

$$[(V - I) + \alpha]\rho_s = V\rho_{T_4},$$

$$\therefore \alpha = \frac{V\rho_{T_4}}{\rho_s} - (V - I) = \frac{VR_0}{R_1} - (V - I).$$

After α and I are determined, the free T_4 fraction (ρ_F/ρ_{T_4}) and the bound-to-free ratio (B/F) can be solved in terms of ρ_s and ρ_{T_4}, and therefore of R_1 and R_0.

$$\text{Free } T_4 \text{ fraction} = \frac{\rho_F}{\rho_{T_4}} = \left[V + \frac{\rho_s}{\rho_{T_4}}(I - V)\right] \div \alpha$$

$$= \left[V + \frac{R_1}{R_0}(I - V)\right] \div \alpha \quad (3)$$

$$(B/F) = \rho_B/\rho_F = \alpha\left[V + \frac{\rho_s}{\rho_{T_4}}(I - V)\right]^{-1} - 1$$

$$= \alpha\left[V + \frac{R_1}{R_0}(I - V)\right]^{-1} - 1 \quad (4)$$

These ratios are true for the radiotracer as well as the stable T_4. To obtain the free-T_4 concentration one would substitute the concentration of stable T_4 (ρ_{T_4}) into Eq. 3. The free-T_4 concentration is then given by:

$$\rho_F = \rho_{T_4} \times \text{free-}T_4 \text{ fraction.}$$

Calculation of TBG capacity and serum free T_4. The free and bound concentrations of T_4 are related to the capacities and affinities of the binding proteins by the equation:

$$\frac{\rho_B}{\rho_F} = \sum_{i=1}^{3} \frac{C_i}{k_i + \rho_F}, \quad (5)$$

where C_i and k_i (i = 1, 2, 3) are the capacities and affinities of TBG, TBPA, and albumin, respectively. In writing Eq. 5 we are assuming a model in which one protein molecule has one T_4 binding site. Although evidence for multiple binding sites on TBPA and albumin have appeared in the literature (*5,6*), these apparently do not affect our calculation at physiological levels of T_4. Sutherland et al., using the same Sephadex partition method, have determined the values of the k_i to be: $k_1 = 8.2 \times 10^{-5}$ μg/ml, $k_2 = 4.85 \times 10^{-3}$ μg/ml, and $k_3 = 2.18$ μg/ml (*3*). In situations where $\rho_F \ll k_2$, Eq. 5 may be simplified to:

$$\rho_B/\rho_F = \frac{C_1}{8.2 \times 10^{-5} + \rho_F} + G, \quad (6)$$

where $G = C_2/k_2 + C_3/k_3$. Equation 6 can be thought of as $y = C_1x + G$, where $y = \rho_B/\rho_F$ and $x = (8.2 \times 10^{-5} + \rho_F)^{-1}$. When x and y are known at two points, C_1 and G can be solved: $C_1 = y_2 - y_1/x_2 - x_1$, and $G = y_1 - C_1x$.

The first pair of x and y is obtained by the ratio of R_1/R_0 and the initial quantity of T_4 in the plasma volume added. The second pair of x and y is obtained by the ratio of R_1'/R_0 after 73.5 ng of T_4 is added to the same mixture.

The values of C_1 and G determined are the TBG capacities and the weighted capacities of TBPA and albumin in the exclusion volume. To obtain the corresponding plasma capacities, these values must be multiplied by a dilution factor that is equal to the exclusion volume divided by the volume of plasma added to the test tube. Under physiological conditions, the concentration of bound T_4 (ρ_B) is equal to the concentration of total T_4(ρ_{T_4}) minus the concentration of free T_4(ρ_F). We can therefore write an expression relating ρ_F and ρ_{T_4} in vivo:

$$\frac{\rho_{T_4} - \rho_F}{\rho_F} = \frac{C_1}{8.2 \times 10^{-5} + \rho_F} + G. \quad (7)$$

Since C_1, G, and ρ_{T_4} are known for the undiluted plasma, the free-T_4 concentration in the undiluted plasma may be determined by solving Eq. 7 for ρ_F.

A sample calculation is given in the Appendix.

RESULTS

The inclusion volume of 1.5 g of Sephadex G-25 was determined to be I = 3.403 ml using I-131 HSA, which is essentially the same as that obtained using I-125 TBG. We had no reason to assume that the included volume for TBPA would be significantly different, and thus used the value I = 3.403 for all calculations. The value of α was determined to be 62.97 ml using I-125 T_4 purified by paper chromatography.

In order to study the characteristic response to different TBG concentrations, the two-point titration method was used to calculate FT_4 and TBG capacities for 11 dilutions of a normal plasma (T_4 = 7.0 μg/dl, FT_4 = 2.2 ng/dl, TBG = 16 μg/dl). The quantities of plasma used ranged from 5 to 200 μl per tube and the results were normalized to our standard plasma volume of 100 μl. This represented a dilution range of from 1/20 to 2/1. The results are shown in Fig. 2. Clearly, the method is linear over a wide range of TBG capacities and T_4 concentrations.

TBG concentrations for a number of serum samples were determined by radioimmunoassay and were com-

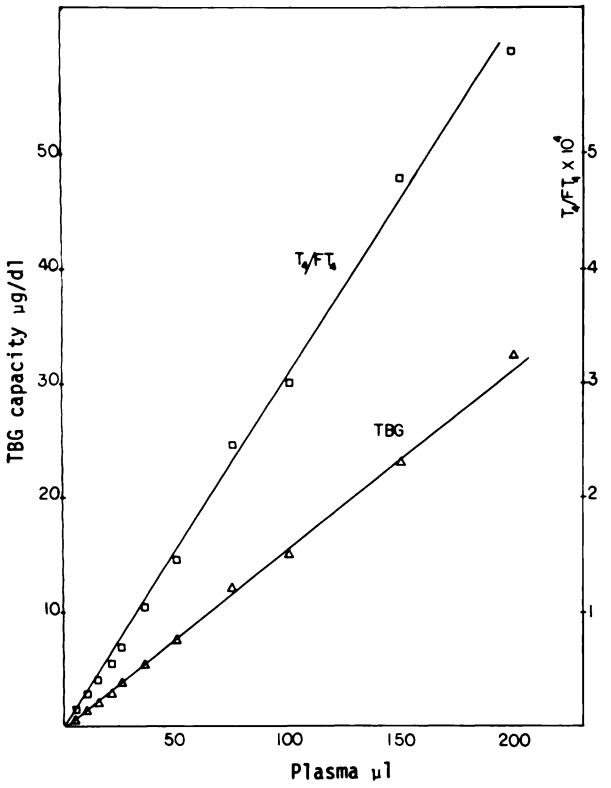

FIG. 2. Results of two-point titration analysis performed on 11 dilutions of normal plasma sample.

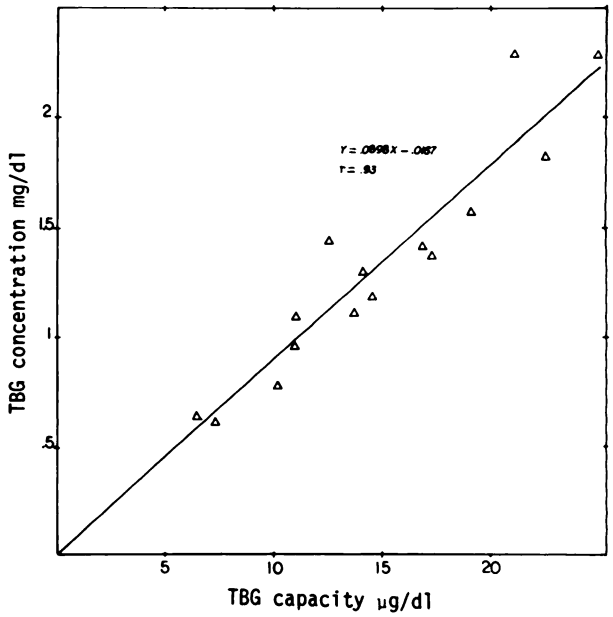

FIG. 3. Comparison of TBG capacity as determined by two-point titration analysis and TBG concentration as determined by radioimmunoassay.

pared with those obtained by the two-point titration method. The results are shown in Fig. 3. The slope of the line, m = 89.9, gives a molecular weight of TBG of 69,000 Daltons (89.9 g of TBG per g of T_4 × 777 g/mole of T_4 = 69,000 Daltons). This is in reasonable agreement with the published value of 65,000 Daltons (*7*).

FT_4 values were calculated by both the method of Irvine and the two-point titration method. Both use Sephadex to amplify the free-T_4 fraction. The method of Irvine uses interpolation of free T_4 between two total-T_4 concentrations, whereas our method depends on extrapolation, using calculated values of TBG capacity and a weighted capacity for TBPA and albumin. The FT_4 calculated by both methods yields the same result. In three plasma samples, our FT_4 values were in good agreement with FT_4 as determined by a commercial laboratory using equilibrium dialysis.

Some representative values of TBG capacity and FT_4 are given in Table 1. In a group of sera in which T_4 concentrations were low and TSH values were not obtained, TBG capacity and FT_4 values are all significantly different from normal. This observation suggests that a deficiency of thyroxine-binding protein is not infrequent in this group of patients, consisting of eight with nonthyroidal illness and three with suspected hypothyroidism whose TSH were not available.

A comparison between FT_4I and FT_4 for 40 serum

TABLE 1. REPRESENTATIVE VALUES OF TBG CAPACITY, G AND FT_4

	T_4(µg/dl)	TBG capacity (µgT_4/dl)	G	FT_4(µg/dl)	Comment
Normal (n = 11)	7.9 ± 1.74	23.4 ± 4.96	1107 ± 473	2.67 ± 0.9	T_4 = 5–10 µg/dl
Hyperthyroid (n = 5)	13.9 ± 3.25	17.3 ± 4.6*	869 ± 273	6.78 ± 1.66†	All have elevated T_3(RIA)
Hypothyroid (n = 3)	3.7 (2.4–5.4)	21 (11–23)	878 (707–1200)	1.24 (0.7–2.1)	All have elevated TSH
Low T_4 (n = 11)	3.06 ± 0.86	12.6 ± 5.2*	739 ± 267*	1.62 ± 0.62†	TSH unknown

* Different from normal, $p < 0.05$.
† Different from normal, $p < 0.01$.

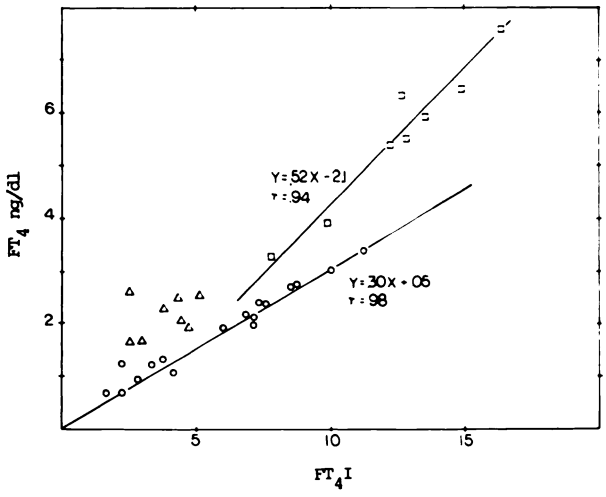

FIG. 4. Plot of FT_4 as determined by two-point titration and FT_4I for three groups of patients.

samples is shown in Fig. 4. As has been reported by other investigators (*8,9*), one of two possible correlations is observed, depending on the degree of saturation of TBG. For the group whose TBG is not saturated, FT_4I and FT_4 correlate with a line passing through the origin. This group had normal values of TBG binding capacity (23 ± 5 μg/dl) with an average value of G (from Eq. 7) of 900 ± 50. The total-T_4 values ranged from 2.4 to 11.9 μg/dl. For sera with relatively saturated TBG (open squares) FT_4I increases less rapidly for the same increment of FT_4 than for the unsaturated sera. These sera had both TBG and G values in the normal range (TBG = 17.5 ± 4 μg/dl, G = 950 ± 100), and the total-T_4 values ranged from 11 to 19 μg/dl. A third group of sera was also definable (open triangles). It was characterized by low TBG (7 ± 2 μg/dl), low G (400 ± 100), and T_4 values that ranged from 2 to 4 μg/dl. This group is made up of eight patients with nonthyroidal illness: chronic renal failure (one), congestive heart failure (two), rheumatoid arthritis (two), Cushing's syndrome (one), hypoplastic anemia (one), and systemic lupus erythematosus (one). FT_4I does not correlate well with FT_4, and frequently FT_4I gives a false diagnosis of hypothyroidism.

DISCUSSION

Thyroxine-binding proteins (TBP) are known to vary under different metabolic, hormonal, or genetic conditions (*10–12*). Depressed levels of TBG and TBPA in malnutrition and elevated levels of TBG during pregnancy are well-known examples. Associated with changes in thyroxine-binding protein concentrations are parallel changes in serum T_4 concentrations without alteration of serum free-T_4 concentrations. Since factors affecting TBP concentrations are independent of the factors affecting thyroid function status as measured by FT_4, evaluation of TBP concentration is a very important consideration in the assessment of serum-T_4 concentrations. A clinically useful test of thyroid function should readily provide information on free T_4 and the TBP concentrations. Direct measurement of FT_4 has commonly been found to be useful as part of thyroid-function tests (*13*). Free T_4 concentration is usually determined by equilibrium dialysis, which is very sensitive to free iodide and organic iodide contamination. Recently immunoassay has been used to determine FT_4, based on the kinetic consideration of the interaction between free T_4 and the antithyroxine antibody (*14*). Irvine determined FT_4 using a Sephadex method, but it has not gained the popularity it deserves.

TBG concentration is most often estimated indirectly by a T_3U method. Strictly speaking it estimates the unsaturated capacity of TBG. Many methods have been developed to combine T_3U and T_4 to give some index of free-thyroxine concentration. These are expressed as FT_4I, or adjusted T_4, or the ratio of T_4 to TBG (*9,15,16*). Though these methods have been justified on empirical grounds, they all have weak theoretical bases. The lack of correlation between FT_4 and FT_4I for the group of patients with nonthyroidal illness further demonstrates the weakness of the T_3U method, which provides much less detailed information than our titration method. The ability to determine TBG and FT_4 concentrations exactly will, we hope, provide new insight into the thyroid status in these patients. Our finding of low TBG and low TBPA capacities in these patients is consistent with the finding of Helenius and Liewendahl (*17*).

Specific measurement of TBG is provided either by electrophoresis (*18*) or radioimmunoassay (*19*). TBG capacity estimated by electrophoresis is probably more useful in evaluating abnormalities of TBG binding. TBG radioimmunoassay is easier to perform, but note that immunoreactive TBG may not bind T_4 (*20*), and a TBG abnormality should still be considered if the clinical picture indicates it. The two-point titration method provides information on both FT_4 and TBG capacity. It is a modification of the method of Sutherland and Simpson-Morgan, by which the binding affinities and capacities of a mixture of serum thyroxine-binding proteins can be determined. The method is a general one and was successfully applied to study an *abnormal* thyroxine-binding protein (*1*). When the method is applied in a limited way—such as for determining TBG capacity and FT_4—two data points are sufficient and necessary, and the assumption that $\rho_F \ll K_2$ must be carefully checked. The present study has demonstrated that TBG capacity and FT_4 can be measured accurately, as the theory predicts. The factor G is a measure of the low-affinity binding property of the serum that is contributed by TBPA and albumin.

The sources of error of the two-point titration method are (a) from the weighting of Sephadex, (b) from counting statistics, and (c) from pipetting. Errors from

TABLE 2. RANDOM VARIATIONS IN THE DETERMINATION OF TBG CAPACITY AND FREE T_4 CONCENTRATION FOR VARIOUS PLASMA SAMPLES

Sample no.	TBG μg/dl	FT_4 ng/dl	Total T_4 μg/dl
1	2.15 (2.12–2.20)	2.55 (2.49–2.63)	1.05
2	5.35 (5.31–5.39)	2.42 (2.32–2.54)	2.45
3	15.9 (15.7–16.0)	2.23 (2.13–2.33)	7.0
4	31.8 (31.2–32.5)	2.28 (2.19–2.37)	14.1
average CV%	1.6%	3.6%	

weighting of Sephadex and from the counting of tracer can be minimized to less than 1%. The error arising from pipetting is usually in the range of 1–3%. To estimate how these errors affect our calculation of the TBG binding capacity and free-T_4 concentration, the two-point titration procedure of the last section was performed in triplicate on four plasma samples with various levels of total T_4 and TBG. The results are summarized in Table 2. Random fluctuations in the TBG determinations amounted, on the average, to 1.6% of the calculated value of TBG, and fluctuations in the free-T_4 determination amounted to 3.6% of the calculated value of free T_4. The total T_4 for the plasma sample determined by RIA is also used in the calculation. The error in the determination of total T_4 is propagated systematically through our calculation, and may therefore be a hidden source of major error not included in the coefficient of variation.

Compared with other available methods, the two-point titration is probably the simplest and most direct method for simultaneous determination of FT_4 and TBG capacity. Unlike T_3U methods, it gives FT_4 and TBG capacity directly. It is better than a radioimmuno method in that it does not require special reagents, such as purified antibodies and antigens, and is a direct calculation not requiring a calibration curve. Though we have used a special apparatus designed for the full titration of serum T_4 binding property, the method can easily be adapted to sampling by pipetting and should prove to be useful as part of routine laboratory evaluation of thyroid function.

FOOTNOTES

* Rohm and Haas, Hursham, PA.
† CIS, Sorin.
‡ Industrial Nuclear Co., St. Louis, MO.

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APPENDIX

Sample calculation. One-tenth milliliter of normal plasma (T_4 = 7.0 μg/dl = 7 ng/0.1 ml, T_3U = 42%) and 0.1 ml of a tracer I-125 T_4 solution were added to 14 ml of buffer in a plastic test tube. The tube was placed in the apparatus of Fig. 1 and an initial count rate recorded, R_0 = 18617 cpm. Sephadex, 1.5 g, was added to the tube and the tube shaken gently for ~45 min. The Sephadex was then allowed to settle, the tube was replaced in the counting apparatus, and the count rate from the supernatant measured, R_1 = 20219 cpm. The concentration of free T_4 and the bound/free ratio for T_4 in the supernatant was determined using Eqs. 3 and 4 by making the following substitutions: $R_1/R_0 = 20219/18617 = 1.0861$; $\rho_{T_4} = 0.007\ \mu g \div 14.2$ ml = 0.5 ng/ml, $\alpha = 62.97$, V = 14.2 ml, I = 3.403 ml. The result was:

$$\rho_F = 0.0183 \text{ ng/ml}, \rho_B/\rho_F = 28.26.$$

Unlabeled T_4, 73.5 ng, was then added to the tube, and again it was shaken and counted in the apparatus (R_1' = 17966 cpm). Equations 3 and 4 were used again with the substitutions: $R_1'/R_0 = 17966/18617 = 0.965$, $\rho_{T_4} = (0.007 + 0.0735) \div 14.2 = 5.67$ ng/ml. The result was:

$$\rho_F = 0.624 \text{ ng/ml}; \rho_B/\rho_F = 15.86.$$

With ρ_F and ρ_B/ρ_F known for two values of total T_4 C_1 and G in Eq. 6 were determined by substituting for x and y in the linear regression: $x_1 = 10^5/(8.2 + 1.83) = 9970$, $y_1 = 28.26$; $x_2 = 10^5/(8.2 + 62.4) = 1416$, $y_2 = 15.86$. C_1 and G were found to be $C_1 = (y_2 - y_1)/(x_2 - x_1) = 1.46$ ng/ml, $G = y_1 - C_1x_1 = 13.80$.

C_1 is the T_4 binding capacity of TBG of 0.1 ml of plasma diluted to the exclusion volume (14.2 − 3.4 = 10.8 ml). To obtain the capacity of the undiluted plasma, we must multiply C_1 by the dilution factor 108, thus: TBG capacity = 1.46 ng/ml × 108 = 157 ng/ml = 15.76 μg/dl. The concentration of free T_4 in the undiluted plasma is obtained by making the following substitutions in Eq. 7: C_1 = 157 ng/ml, G = 13.7 × 108 = 1480, ρ_{T_4} = 7 ng/ml, and then solving the equation for ρ_F; ρ_F = 23.3 pg/ml.

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