Interactions of Thyroxine with Thyroxine-Binding Globulin of Low Binding Capacity

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The physico-chemical interactions between thyroxine (T_4) and thyroxine-binding globulin (TBG) of low binding capacity were investigated by radioimmunoassay, equilibrium dialysis, and reverse flow electrophoresis. Knowledge of total and free T_4 (FT_4) concentrations in serum, and of the total binding capacity of the protein carrier (TTBG), allowed the determination of the association constant ruling these interactions (K_{thg}). Correlation between T_4 and FT_4 varies with TTBG, shifting the normal range for T_4. The level of FT_4 in serum is a function of the fractional saturation of TBG by endogenous hormones, which depends on T_4 and TTBG. Data on T_4, TTBG, and K_{thg} were integrated into the general equation of the law of mass action and the results showed a very significant linear correlation with the values of FT_4 measured by equilibrium dialysis. It is concluded that the misleading results of serum T_4 measurements and of the free T_4 index, obtained in euthyroid individuals with low TTBG, cannot be ascribed to a reduction of the intrinsic sensitivity of the assay due to oversaturation of TBG by endogenous T_4, as previously postulated by others, but to a shift of their normal ranges produced by abnormal variations of TTBG. These results stress the need for data regarding TTBG for the proper interpretation of T_4, and for the calculation of the fractional saturation of TBG and the FT_4 concentration in serum. We have solved this problem by using an empirical equation relating TTBG to T_4 and triiodothyronine (T_3) uptake, which was previously derived by other workers.

The misleading results of the in vitro thyroid function tests (measurement of thyroxine concentration in serum [T_4] and uptake of radioactive triiodothyronine by a secondary binder [T_3U] and the corresponding free thyroxine index [FT_4I]—calculated as the product of T_4 and T_3U), obtained in some clinical circumstances characterized by serious alterations in the major thyroxine binding globulin (TBG) in serum (*1–4*), has made endocrinologists uneasy for many years.

These limitations of in vitro thyroid function testing have been ascribed to a reduction of the intrinsic sensitivities of the T_3U (*5*) and T_4 (*5,6*) assays when TBG is oversaturated by endogenous T_4, as in sera from euthyroid individuals with abnormally reduced total TBG binding capacity (TTBG). We have recently shown, however, that the misleading FT_4I is not the outcome of reduced intrinsic sensitivities of these assay systems, but a consequence of a shift of their normal ranges related with variations in TTBG (*7*). In addition, the interactions between T_4 and its protein carrier should be in compliance with the strict rule of the physico-chemical law of mass action, which means that on a theoretical basis the oversaturation of TBG is not possible without a corresponding increase in serum free thyroxine (FT_4) concentration and the consequent appearance of the characteristic signs and symptoms of thyrotoxicosis.

Our present investigation was designed to test the last hypothesis, and to explore the means to obtain a more accurate indicator of thyroid function than the usual FT_4I from the results of the in vitro assays.

MATERIALS AND METHODS

Patients

This study was performed with 65 sera samples from seven hypothyroid patients, selected because their low T_4 concentration (less than 1.3 μg/dl; 1.5×10^{-8} M), and their decreased TTBG, which varied from 7.1 to 21.8 μg T_4/dl ($9.14–28.06 \times 10^{-8}$ M). Diagnosis was made upon integrating the clinical findings and course with data on FT_4, as measured by equilibrium dialysis, and on TTBG, as measured by reverse flow electrophoresis. All sera were collected from seriously ill cardiac patients.

Methods

Aliquots from all samples were loaded with graded amounts of exogenous T_4 (0–12 μg/dl; $0–1.54 \times 10^{-7}$ M). To avoid the effects of dilution, the hormone solution was added to the test tube before the sample and was kept at room temperature until totally dry. The serum aliquots were then added and maintained overnight under constant shaking at room temperature, to assure a proper distribution of the added T_4 in the sample. Results obtained in those aliquots with oversaturated TBG (more T_4 than TTBG) were excluded from the graphic analysis.

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Serum aliquots were then assayed in triplicate for the following parameters:

Total T_4 concentration. This was measured by radioimmunoassay by using a commercial kit* (normal range: 4.5–11.5 μg/dl; 5.8–14.8 × 10^{-8} M).

Free T_4 concentration. Three milliliters of undiluted sera were added with a tracer amount of ^{125}I-T_4 and incubated with constant shaking overnight at 4°C to reach complete equilibration with nonradioactive T_4 in the sample. The labeled sera were dialyzed against 5 ml of 0.04 M veronal (barbital) buffer at 37°C for 24 hr with constant shaking. The free T_4 fraction (FT_4F) was determined from the percentage of the total ^{125}I-T_4 (measured previously in a scintillation well counter) that was able to pass through the membrane in 24 hr, as measured by eluting the dialyzate through a Sephadex G-25 column with the same buffer system. The radioactivity eluted in this way corresponds to the free ^{125}I-iodide present as a contaminant and was used to correct the total radioactivity of ^{125}I-T_4 in the sample. The free ^{125}I-T_4 absorbed to the gel was eluted from the column with pooled normal sera and measured in a scintillation well counter. The absolute concentration of FT_4 was then calculated as the product of the FT_4F and the concentration of total T_4 in the sample (*8*) (normal range: 1.2–3.6 ng/dl; 1.54–4.63 × 10^{-11} M).

Total TBG binding capacity. Electrophoretic distribution of T_4 was determined by adding tracer amounts of ^{125}I-T_4 to the sample. Total TBG binding capacity was measured by loading the sample with 200 μg T_4/dl before electrophoresis and by calculating the product of the fraction of ^{125}I-T_4 present at the TBG electrophoretic band (between alpha-1 and alpha-2 globulins) and the total T_4 concentration in the sample (including the 200 μg/dl added). Reverse flow electrophoresis was carried out by using 0.04 M veronal (barbital) buffer, after complete saturation of the paper strips with the buffer over 6 hr (normal range: 15–28 μg T_4/dl; 1.93–3.60 × 10^{-7} M).

Free TBG binding capacity (FTBG). The concentrations of unbound sites in TBG were calculated by subtracting the amounts of endogenous T_4 bound to this carrier from its total binding capacity.

Fractional saturation of TBG by T_4. This was calculated

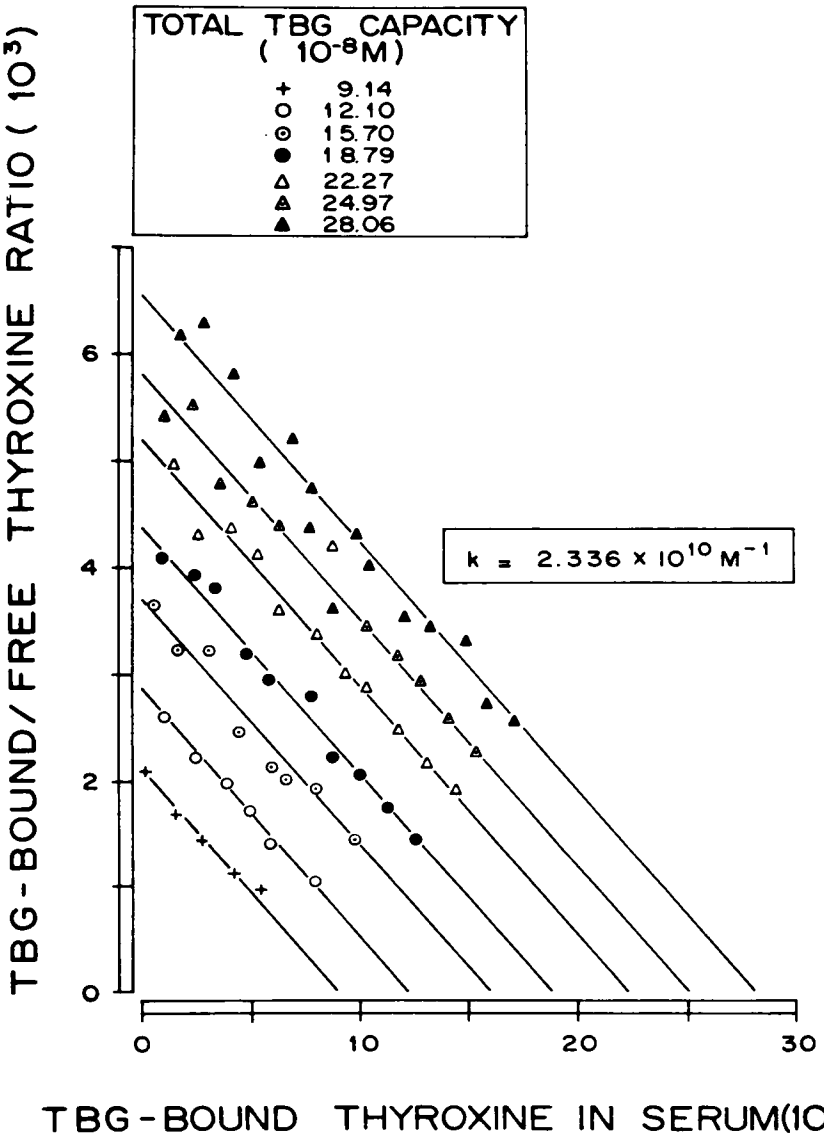

FIG. 1. Scatchard plot correlating the ratio bound thyroxine/free thyroxine in the samples with the corresponding number of hormone molecules bound to its protein carrier. Identical slope of the extrapolated lines through the experimental points obtained in sera with the same total thryoxine-binding globulin capacity, means that each binding site has the same intrinsic association constant (K_{tbg} = 2.336 × 10^{10} M^{-1}). *K* was calculated by dividing the intercepts on the ordinate by the corresponding intercepts on the abscissa.

as the product of T_4 and 100 divided by total TBG binding capacity.

RESULTS

Calculation of the Association Constant between Serum Thyroxine and Thyroxine-Binding Globulin

The association constants for the binding of T_4 by TBG (K_{tbg}) and albumin (K_{alb}) were calculated individually for each sample by using the mass action expression for equilibrium between bound and unbound T_4 (*9*):

$$K_{prot} = \frac{(\text{Prot } T_4)}{(F_{prot})\ (FT_4)} \qquad \text{Eq. 1}$$

where: K_{prot} = association constant for the binding of T_4 to the carrier protein

Prot T_4 = concentration of binding sites occupied by T_4 in the carrier protein

F_{prot} = concentration of unoccupied binding sites in the carrier protein

FT_4 = concentration of FT_4.

As FT_4 concentration is three orders of magnitude lower than the concentration of total T_4, the latter was taken as Prot T_4.

The calculated value of K_{tbg} on the 65 triplicate samples was $2.336 \pm 0.075 \times 10^{10}\ M^{-1}$, with a coefficient of variability of 3.21%. Figure 1 shows the Scatchard plot of the experimental data obtained in the 65 samples, which follow distinctive straight parallel lines for each of the seven subsets of sera with different TTBG.

The association constant between T_4 and albumin (K_{alb}) was much lower: $0.85 \pm 0.06 \times 10^{6}\ M^{-1}$, with a coefficient of variability of 7.06%.

Relationship between serum T_4, TTBG, and FT_4. This relationship can be derived by substituting the terms of equation 1 by specific data:

$$K_{tbg} = \frac{(\text{TBG } T_4)}{(\text{FTBG})\ (FT_4)} \qquad \text{Eq. 2}$$

and rearranging them to solve the equation for FT_4:

$$FT_4 = \frac{(\text{TBG } T_4)}{(\text{FTBG})\ (K_{tbg})} \qquad \text{Eq. 3}$$

The effect of increasing amounts of T_4 on the FT_4 concentration showed a significant dependency on TTBG, as dictated by equation 3 (Fig. 2).

Estimation of FT_4 from FTBG and total T_4 concentration. Equation 3 was used to calculate FT_4 on each sample. Figure 3 indicates that a close relationship exists between the

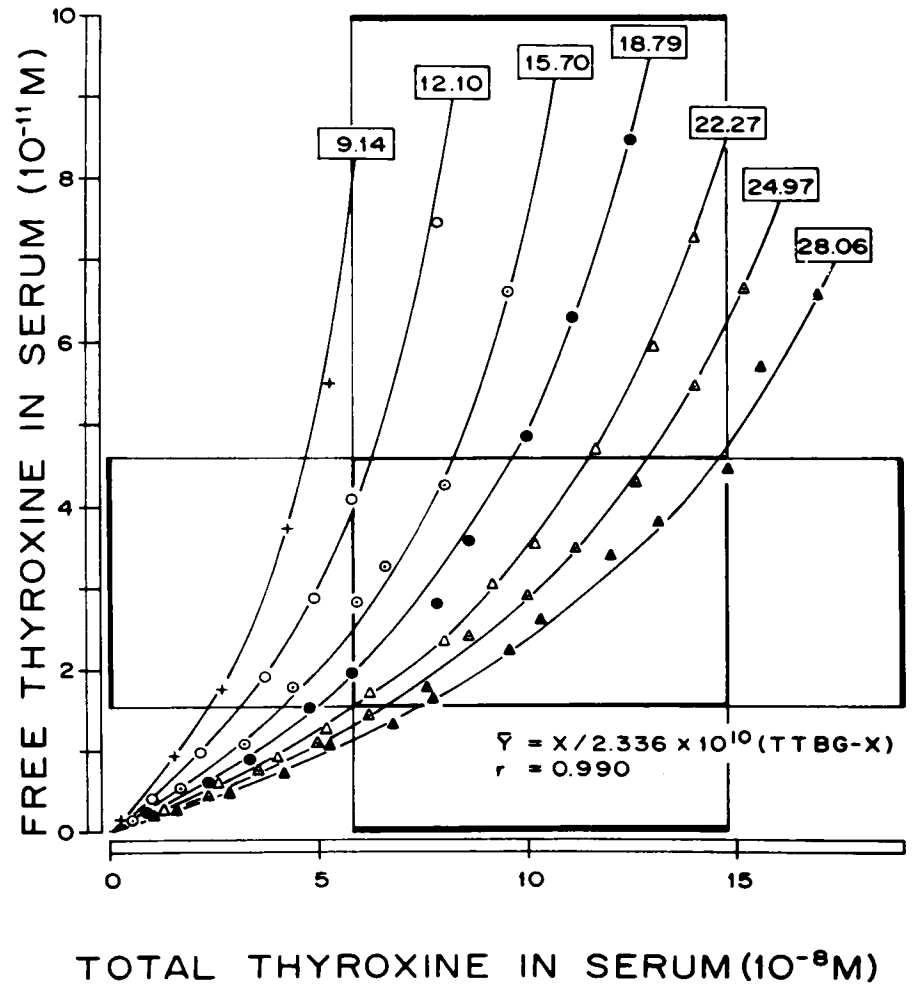

FIG. 2. Correlation between total and free thyroxine concentrations in serum. The value in the square heading each line on the graph identifies the total thyroxine-binding globulin capacity on each set of samples ($\times 10^{-8}$ M). These lines were constructed by using the mass action expression (equation 2). TTBG = total thyroxine-binding globulin capacity.

estimated FT_4 and its actual values measured by equilibrium dialysis, with all data points following a single straight regression line that approaches the identity line of the graph, independently of TTBG (r = 0.999).

Relationship between total T_4, TTBG, and fractional saturation of TBG by T_4. The fractional saturation of TBG by T_4 gradually increased by adding graded amounts of T_4, but each subset of samples followed a different positive linear regression line, depending on TTBG in the sera (Fig. 4).

Relationship between fractional saturation of TBG and FT_4 concentration. The free T_4 concentration in serum appeared to be closely related to the fractional saturation of TBG by T_4 (Sat TBG), according to a positive nonlinear function (Fig. 5):

$$(FT_4) = (\text{Sat TBG}) / (K_{tbg}) (100 - \text{Sat TBG}) \quad \text{Eq. 4}$$

It seems that FT_4 concentration reaches thyrotoxic levels when more than 51% of the binding sites in TBG are saturated by T_4, while FT_4 becomes defective when less than 25% of the total TBG binding capacity is occupied by the hormone, independently of the particular TTBG in the sample.

Estimation of FT_4 concentration from the fractional saturation of TBG. Equation 4 was used to calculate FT_4 in each sample. The results showed a close linear correlation with the actual values of FT_4 measured by equilibrium dialysis, with a regression line approaching the identity line of the graph (Fig. 6) and a correlation coefficient identically significant to that obtained by using equation 3 (Fig. 3).

DISCUSSION

Three major thyroxine-binding proteins have been characterized in serum: the inter-alpha type of thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA), and albumin (alb). At normal levels of thyroid hormones in serum, TBG binds most of the circulating hormone. Thyroxine-binding prealbumin is next in importance for T_4 (*10*), although it

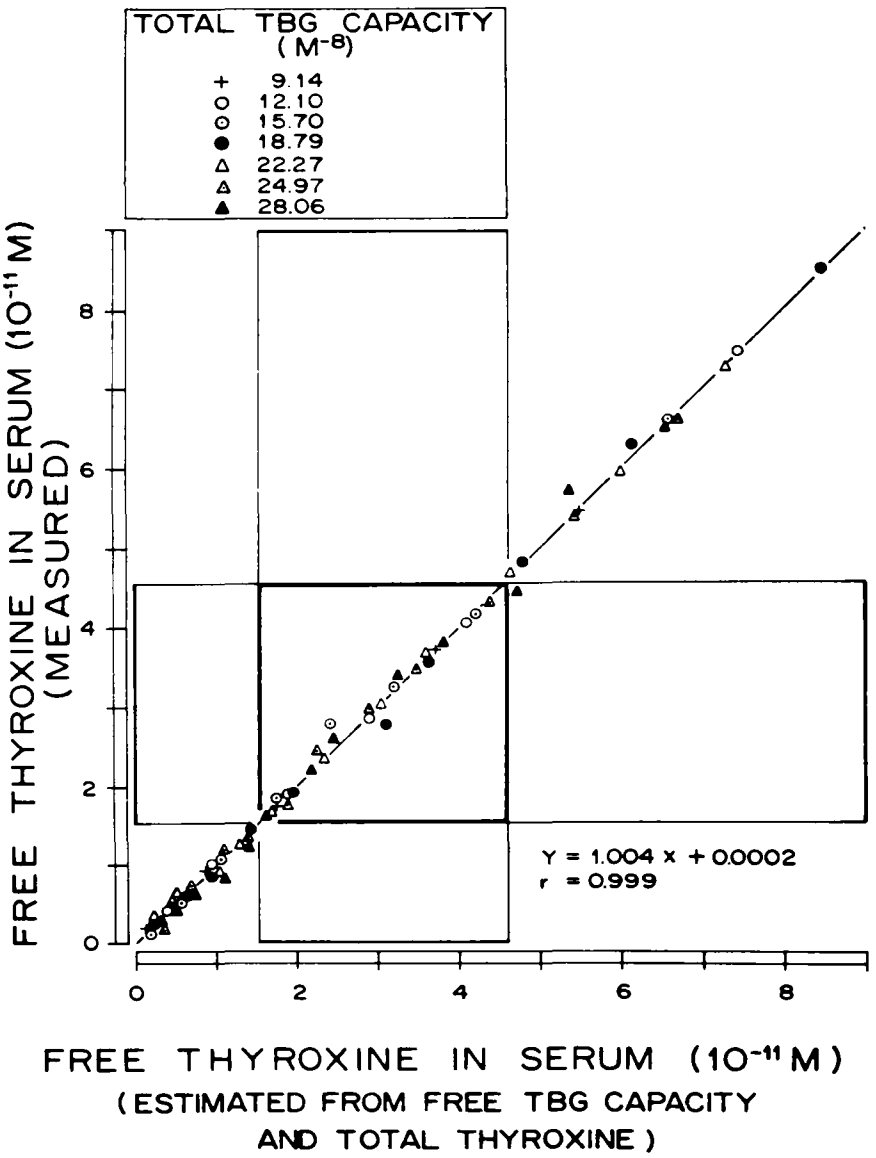

FIG. 3. Relationship between free thyroxine concentration in serum estimated by using the mass action expression (equation 2) and its actual values measured by equilibrium dialysis.

is unable to bind triiodothyronine and some workers believe that the binding of T_4 to TBPA may be artifactually created by certain buffer systems (*11-13*). In any case, this binding is selectively inhibited by veronal (barbital) ions, which were basic in the buffer solutions we used (*10,14*). During this study we were unable to detect any degree of T_4 binding to TBPA.

Albumin is the most abundant of the three proteins and has the highest binding capacity, but its affinity for the thyroid hormones is the lowest. The association constant between T_4 and albumin (K_{alb}) estimated during this investigation (0.85×10^6 M^{-1}) was very similar to the values reported by other workers (*1,15*) and resulted four orders of magnitude lower than K_{tbg} (2.336×10^{10} M^{-1}). The product of the ratio K_{alb}/K_{tbg} and the free albumin binding capacity resulted sufficiently small and was discarded as an influential factor on the interactions between T_4 and TBG. These observations show that TBG is by far the most prominent carrier protein for T_4 in our assay systems.

Precise knowledge of total T_4, FT_4, and TTBG on every single sample assayed, allowed the individual calculation of K_{tbg} by following the general equation of the physicochemical law of mass action (equation 1). These individual results were further confirmed by the Scatchard plot, correlating the bound T_4/FT_4 ratio on each sample with the corresponding number of T_4 molecules bound to TBG (Fig. 1). The experimental points obtained on each subset of samples with distinctive TTBG followed a particular straight line. All these lines were parallel and had identical slopes, indicating that each of the binding sites in TBG has the same intrinsic association constant (*16*), which resulted on the same order of magnitude of the values previously reported (*1*). The small individual variations between experimental points and a particular line could be explained by the additive error in measuring T_4 and FT_4 concentrations (3.21%).

Graphic correlation of total T_4, FT_4, and TTBG rendered clear evidence of the compliance of the law of mass action in the interactions between these molecules, and further information on the cause of the apparent decrease of specificity for hypothyroidism, and of sensitivity for thyrotoxicosis, of the measurements of T_4 in sera with very low TTBG, when the results are interpreted according to its normal range determined in sera with normal TTBG ($5.8 - 14.8 \times 10^{-8}$ M) Fig. 2). Each subset of samples with different TTBG followed a distinct nonlinear positive function depending on T_4 and TTBG. The slopes of the resulting curves were increased as TTBG was reduced in the samples. Consequently, FT_4 concentration in serum reaches its lower limit of normality (1.54×10^{-11}) and the border with thyrotoxicosis (4.63×10^{-11} M) with lower total T_4 concentrations in sera with reduced TTBG, than in sera with normal TTBG (higher than 19.3×10^{-8} M).

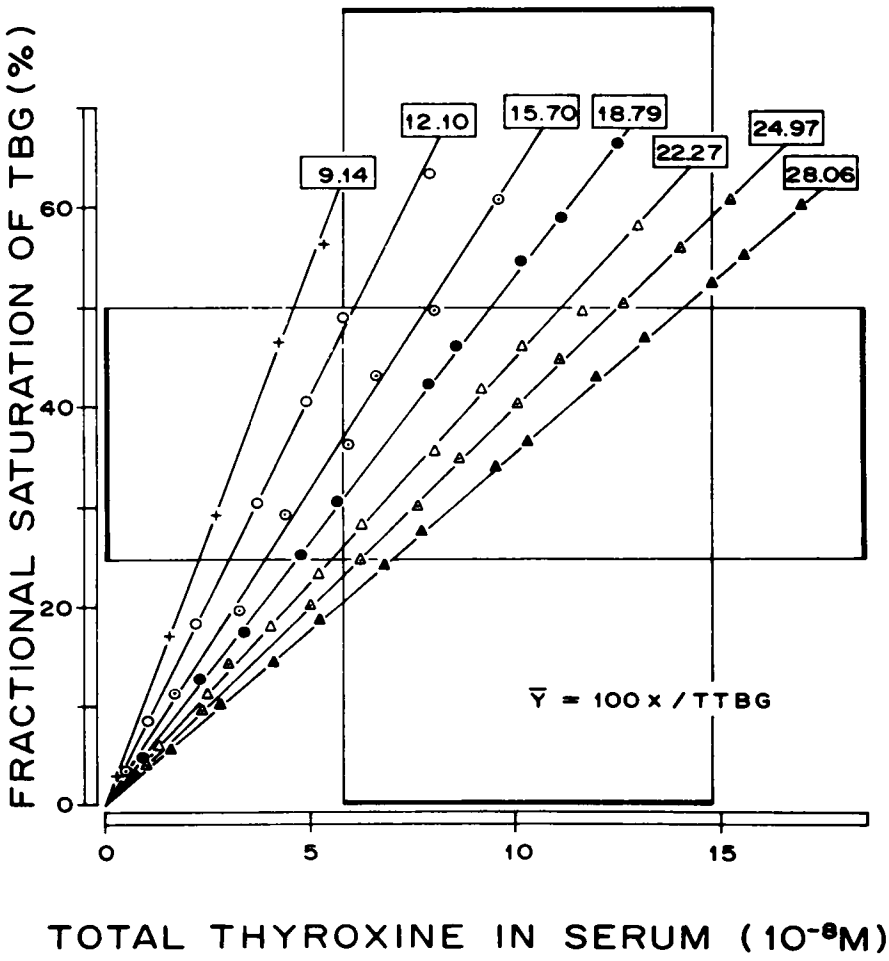

FIG. 4. Realtionship between total thyroxine concentration in serum and the fractional saturation of thyroxine-binding globulin by the hormone. The value in the square heading each line on the graph identifies the total thyroxine-binding globulin capacity on each set of samples ($\times 10^{-8}$ M) (fractional saturation = total thyroxine x 100/total binding capacity).

Another proof of the submission of T_4 interactions with TBG to the law of mass action was the very significant accuracy with which we were able to calculate FT_4 from the data on total T_4, TTBG, and K_{tbg} by using the general equation of this important physico-chemical law (Fig. 3).

All this evidence pointed toward the well-known importance of TBG as a regulator of the FT_4 (*17,18*), the only hormonal fraction able to diffuse into the tissues and hence be available for their metabolism and action (*19*). That means that FT_4 in serum should be dependent on the degree of saturation of TBG by endogenous T_4.

We tested this hypothesis by correlating total T_4, TTBG, and the fractional saturation of the carrier protein by the hormone. Figure 4 shows that TBG stauration depends on its total binding capacity and on the magnitude of total T_4 concentration in the sample; figure 5 demonstrates that FT_4 concentration is directly related to the degree of saturation of the binding globulin and hence, inversely proportional to its unsaturated fraction (100 − Sat). By combining these two factors with K_{tbg}, a new equation (equation 4) was derived to calculate FT_4 from the information on T_4 and TTBG, by which we obtained results (Fig. 6) similar to those calculated through the general equation of mass action (equation 3, Fig. 3).

It was confirmed, then, that the misleading results of serum T_4 measurements in euthyroid patients with nonthyroidal illness affecting TTBG cannot be ascribed to a reduction of the intrinsic sensitivity of the assay due to oversaturation of the protein by endogenous hormones, as postulated by other workers (*5,6*), but to a shift of its normal range when TTBG is decreased, as previously described (*7*). Interactions between T_4 and TBG are strictly ruled by the law of mass action and, hence, any decrease in TTBG is followed by a reduction of T_4 secretion by the thyroid and of its concentration in serum, so that the euthyroid state is maintained.

Calculation of the FT_4I, as originally derived by Clark and Horn (*20*), is based on the observation that free TBG capacity (TTBG-T_4) is inversely related to T_3U, and on the fact that T_4 concentrations and T_3U show opposite behavior when

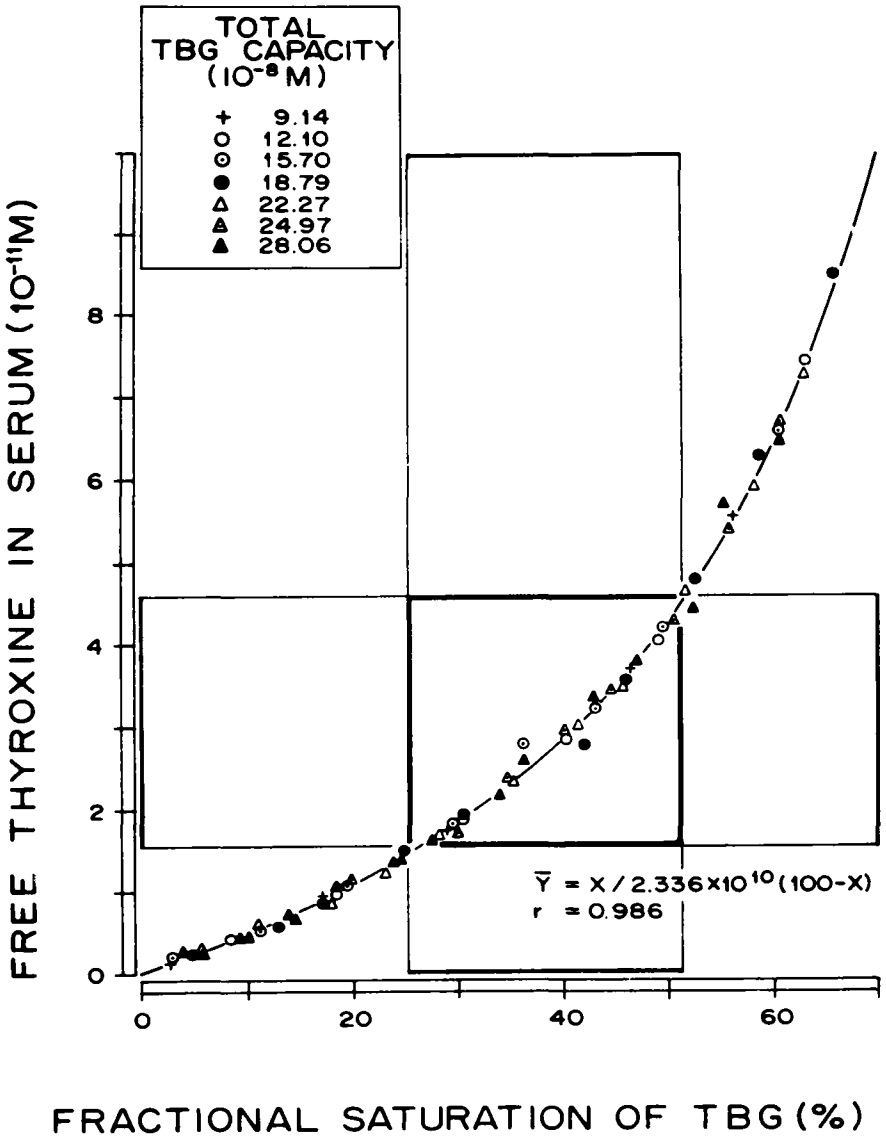

FIG. 5. Correlation between the fractional saturation of thyroxine-binding globulin by thyroxine and the concentration of the free hormone in serum.

measured in sera with altered TTBG. It is supposed that a low value for T_4 in sera with reduced TTBG will be compensated by a high value for T_3U, and that their product will render a normal value (*21*). These relationships can be described by the following equations:

$$FT_4 = \frac{(TTBG\ T_4)}{(K_{tbg})\ (FTBG)} = \frac{(T_4\ tot)}{(K_{tbg})\ (1/T_3)} = \frac{(T_4 tot)\ (T_3U)}{(K_{tbg})}$$

Eq. 5

As can be observed in these equations, the calculation of the FT_4I as the product of T_4 and T_3U oversimplifies the relationship between FTBG and T_3U and does not take into account the needed K_{tbg}, thereby introducing an error that becomes significant when there are wide variations in TTBG. This will be the subject of another paper.

Our present results stress the need for data regarding TTBG for the proper interpretation of serum T_4 concentration, either by setting its normal ranges for different levels of TTBG, as previously described (*7*), or by using the value of TTBG for the calculation of both the fractional saturation of TBG by endogenous T_4 and FT_4 concentration, through the equations described here.

It is important to note that the fractional saturation of TBG by T_4 should not be mistaken with the ratio between total T_4 and the serum TBG concentration measured by radioimmunoassay, previously suggested by Szpunar et al. (*6*) as another free thyroxine index. In order to be comparable with the fractional saturation of TBG, this ratio should be calculated by using the molar concentrations of the two molecules in serum. Otherwise, this ratio could yield meaningless data, as those reported by Szpunar et al. (*6*) with TBG saturations higher than 100% in euthyroid sera.

Specific measurement of TTBG by electrophoresis is time consuming and not practical for a daily routine, but we have overcome this problem by using the empirical equation derived by Nusynowitz and Benedetto (*22*) to estimate TTBG from the values of T_4 and T_3U, by which we have found a highly significant linear positive correlation between the estimated and the actual values of TTBG measured by electrophoresis.

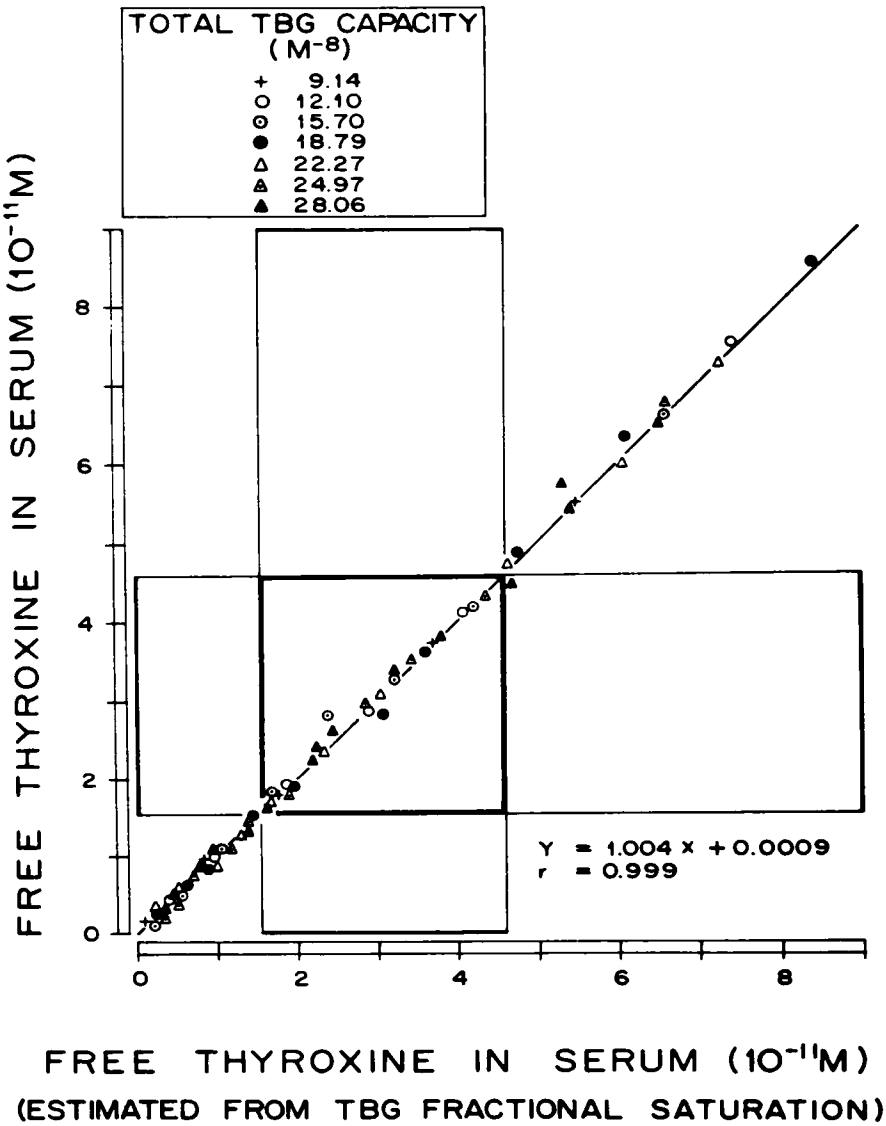

FIG. 6. Correlation between free thyroxine concentration in serum estimated from the fractional saturation of the thyroxine-binding globulin by the hormone (equation 4) and its actual values measured by equilibrium dialysis.

Unfortunately, that equation only applies to the particular in vitro testing systems employed by the authors, and only if the normal ranges for T_4 and T_3U are the same as those reported by them. Otherwise the equation must be appropriately revised and corrected with experimental values arising from other kits.

NOTE

*Tetra-Tab RIA, Nuclear Medical Laboratories, Division of Warner-Lambert Technologies, Inc., Irving, TX.

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