IN VITRO NUCLEAR MEDICINE

An Assessment of Methods for the Estimation of Free Thyroxine

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Three commercial kit methods for the estimation of free thyroxine (FT_4) (Clinical Assays, Corning Medical, Damon Diagnostics) were evaluated. The effects of changes in thyroxine-binding globulin (TBG) and FT_4 concentrations in these systems were assessed. Measurements were made in serum samples from hyperthyroid, hypothyroid, and euthyroid patients with different TBG concentrations, and results were correlated with functional thyroid status.

The Clinical Assays and Damon Diagnostics assays were found to be essentially independent of binding-protein concentration effects and responded appropriately to changes in FT_4 concentration. The method of Corning Medical does not measure FT_4 directly but yields an FT_4 index calculated from a TBG-dependent T_4 uptake and the total T_4 concentration. This Corning index yields falsely elevated results in patients with marked elevation in TBG concentration.

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The total serum thyroxine concentration (TT_4) correlates well with functional thyroid status in most individuals. However, altered concentrations of thyroxine-binding proteins affect this measurement. Since free (unbound) thyroxine (FT_4) is the metabolically available thyroxine, some estimate of FT_4 concentration is of greatest clinical value for determining thyrometabolic status.

Classically, the fraction of nonprotein-bound thyroxine has been estimated by means of equilibrium dialysis (*1,2*). More simply, a free thyroxine index (FTI) has been used by relating the TT_4 concentration to saturation of available serum protein-binding sites (*3–6*). More recently, other methods for the estimation of FT_4 have become commercially available. These include relating FT_4 concentration to T_4-antibody binding in the presence and absence of endogenous binding proteins* (*7,8*); microencapsulation of I-125-labeled anti-thyroxine antibody to permit diffusion-controlled dialysis of FT_4† (*9–11*); and sequential direct radioimmunoassay using a solid-phase antibody‡ (*12*). We examined each of these methods to determine whether they indeed measure FT_4, and looked for effects attributable to endogenous thyroxine-binding proteins. Finally, we assessed the clinical utility of each of these procedures.

METHODS

Sera from 25 hyperthyroid, 25 hypothyroid, and 80 euthyroid patients—including patients with elevated or low thyroxine-binding globulin (TBG) concentrations—were studied with three FT_4 kits*,†,‡. Functional thyroid status was determined from the results of clinical evaluation and measurement of TT_4 and silicate triiodothyronine uptake (RT_3U), FT_4 by equilibrium dialysis, and (when indicated) levels of thyroid-stimulating hormone (TSH) or of total T_3, thyroidal iodine uptake, or pituitary response to thyrotropin-releasing hormone (500 μg i.v.)‖ TBG concentration in sera from all patients was estimated by immunoradiometric assay.* We observed concentrations of 10–20 μg/ml in euthyroid male and nonpregnant female patients whose total serum protein concentrations were normal and who were not receiving drugs known to alter concentrations of thyroxine-binding proteins.

The accuracy, sensitivity, and between-run precision of each FT_4 kit were evaluated. Information regarding

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possible interferring substances and antibody cross-reactivity is provided by the manufacturers, and these were not further examined by us.

Each manufacturer provides FT_4 standards that are calibrated by the manufacturer's equilibrium dialysis. The concentrations of all commercial standards were measured with our equilibrium dialysis method (*2*). In addition, concentrations of all manufacturers' standards were estimated using each of the three commercial assays. During the course of our investigations, two different lots of Corning standards were available and both were examined.

The FT_4 standard curves provided by Damon and Clinical Assays were examined and the usefulness of all assays in distinguishing normal from hypothyroid patients was assessed.

FT_4 was measured in an in-house control pool in most assay runs, and controls provided by Corning and Damon were measured in all assay runs. Between-run variability in these results was expressed as the coefficient of variation (100 × standard deviation/mean value).

We considered that FT_4 and thyroxine-binding protein concentrations, principally TBG, would both influence an assay response. The contribution of FT_4 was evaluated by examining sera from hyperthyroid, hypothyroid, and euthyroid individuals who had normal TBG concentrations. The effects of TBG relatively independent of FT_4 concentration were determined by examining sera from euthyroid individuals who had abnormal TBG concentrations.

Corning Medical. FT_4 was estimated following the manufacturer's protocol. Two sets of tubes were needed for each sample analyzed. In the "A" tubes, I-125-labeled thyroxine was added to patients' sera and equilibrated for 20 min. In the "B" tubes, patients' sera were incubated for 20 min with I-125-labeled thyroxine in the presence of thimerosal, which inhibits the binding of endogenous thyroxine by endogenous proteins. After equilibration, solid-phase antithyroxine antibody was added to both sets of tubes, which were then incubated for an additional 30 min. In the "A" tubes, all T_4 present (I-125-labeled, free and endogenously bound) was distributed among all the binders present (antibody, endogenous binding proteins) depending upon the affinities and concentrations of the binders. In the "B" tubes, there was competition between I-125-labeled and unlabeled TT_4 for a limited number of anitbody binding sites. After incubation, the solid-phase antibody-bound fractions were segregated by centrifugation. The "B" tube binding was related to TT_4 by reference to a standard curve.

Initially, Corning's suggestion for calculation was to relate the ratio of "A" tube counts over "B" tube counts (A/B) to apparent FT_4 by reference to a similarly prepared standard curve. However, because these results have been shown to be dependent upon the concentrations of endogenous thyroxine-binding proteins, the current recommendation for calculation is to relate TT_4 times "A" tube counts over total counts ($TT_4 \times A/TC$) to apparent FT_4 concentration by reference to a similarly prepared standard curve (*13*).

The dependence of Corning A/TC on TBG concentration was examined and these results were compared with those obtained with RT_3U and percent dialyzed fraction ($\%FT_4$).

The effects of FT_4 and TBG on the rate of association of I-125 thyroxine with solid-phase antibody in the "A" tubes, and on the association observed after 30 min of incubation, were ascertained.

The mass of T_4 bound by the solid-phase antibody in 30 min was determined by incubating sera with I-125 thyroxine[§] for 30 min before its addition to tubes containing only solid-phase antibody in buffer. The percentage of added I-125 thyroxine bound times TT_4 present in the tube (derived from the TT_4 concentration of the sera incubated) is the mass of T_4 bound by the antibody. Determination of the mass of T_4 bound from sera of various FT_4 and TBG concentrations provides a means of ascertaining whether the product $TT_4 \times A/TC$ provides a valid representation of FT_4.

Damon Diagnostics. FT_4 was estimated according to the manufacturer's protocol. Patients' sera were incubated for 2 hr at 37°C with suspensions of thin-membraned nylon microcapsules having large surface areas containing an antithyroxine antibody presaturated with I-125 thyroxine. During this time, labeled and unlabeled FT_4 pass through the microcapsule's membranes and equilibrate, whereas larger molecules such as protein-bound thyroxine are excluded from interaction with the antibody. At the end of this incubation period, a solution of polyethylenimine (PEI) was added to all the tubes to wash unbound labeled T_4 from the capsules and to facilitate formation of a stable centrifugal pellet. FT_4 concentration was estimated by comparison of activity present in the pellet with activity in a similarly prepared standard curve for FT_4.

The rates of microcapsule equilibration with T_4 from sera of various FT_4 and TBG concentrations were determined. The effect of varying microcapsule concentration on estimates obtained for three sera from euthyroid patients with low, normal, and elevated TBG concentrations was examined. Aliquots of the microcapsule solution were gently centrifuged and resuspended to provide microcapsule concentrations 0.25, 0.5, and 3 times the concentration provided by the manufacturer.

Clinical Assays. FT_4 concentration was estimated following the manufacturer's protocol. Patients' sera diluted in 1.0-ml assay buffer were added to tubes coated with antithyroxine antibody and incubated for 10 min at 37°C. (Currently the manufacturer recommends a 20-min incubation.) After this initial partitioning of the T_4 pool between the solid-phase antibody and endoge-

nous binding proteins, the tubes were decanted and rinsed. I-125 thyroxine was added to the tubes, which were then incubated for 1 hr at 37°C. After decantation, activity in the tubes was quantitated and FT_4 concentration measured by comparison with a similarly prepared FT_4 standard curve. The same antibody-coated tubes and I-125 thyroxine are used in the manufacturer's TT_4 assay, with differences in reagent and sample concentrations, incubation times, and temperatures.

The mass of T_4 bound by the antibody-coated tube during incubation with patients' sera was determined. I-125 thyroxine[§] was added to patients sera and incubated for 30 min at room temperature. Sera were then added to the antibody-coated tubes. After 10 min of incubation the tubes were decanted, washed, and counted. The percentage of added activity found in the tubes, times the TT_4 mass present, is the mass of T_4 extracted by the antibody. The masses of T_4 extracted from sera of various FT_4 and TBG concentrations were examined.

Equilibrium dialysis. The fraction of free dialyzable T_4 was estimated using modifications of the method described by Herrmann and Kruskemper (*2*). 3′,5′-[^{125}I]thyroxine (50 μCi/mg, >97% thyroxine)[§] was used without further purification and was diluted to a final concentration of 0.2 μg/ml. One milliliter of patient serum was diluted to 4.1 ml and dialyzed against phosphate buffer. After dialysis to equilibrium, carrier T_4 was added to the dialysate and T_4 was precipitated with magnesium chloride. The precipitate was washed to remove non-T_4 activity, and counted. FT_4 concentration was estimated by multiplying the percent free dialyzed T_4 by TT_4 concentration determined by radioimmunoassay.[¶]

Free thyroxine index. TT_4 by radioimmunoassay and RT_3U were estimated following the manufacturer's protocols without modification[¶]. Free thyroxine index (FTI) was calculated as the product TT_4 times RT_3U (*14*). Normal values established in this laboratory for these assays are: TT_4 4.5–11.5 μg/dl, RT_3U 35–45%, FTI 1.6–5.2.

Calculations. Activity present in assay tubes was measured in one of several gamma scintillation counters calibrated to count I-125 in a 15- to 75-keV window.

The Corning FT_4 assay (A/B) and (TT_4 × A/TC) standard curves were plotted by hand on linear coordinates. Patient and control dose estimates were obtained using these curves.

In addition, we considered the result of TT_4 × A/TC to be an index since "A" tube counts over total counts is analogous to a T_3 uptake, and examined the clinical applicability of this index without reference to a standard curve.

The Damon and Clinical Assays FT_4 standard curves

TABLE 1. MEASURED CONCENTRATION AND CHARACTERISTICS OF COMMERCIAL FT_4 STANDARDS

	Nominal FT_4 (ng/dl)	vs. Clinical Assays	vs. Corning 03057	vs. Corning 00689	vs. Damon	vs. Equilibrium dialysis	%FT_4	A/TC	Total T_4 (RIA) (μg/dl)	RT_3U (%)	FTI	TBG (μg/ml)
Clinical	0.21		<0.5	<0.3	0.5	0.1	0.14	0.25	1.0	22	0.2	21
Assays	0.88		0.9	0.8	0.9	0.6	0.15	0.24	4.0	24	1.0	21
1751	2.94		2.6	2.8	2.4	2.0	0.16	0.21	12	31	3.7	18
	5.51		4.3	4.6	4.9	3.9	0.19	0.18	20	35	7.0	16
	9.80		<6.0	>6.0	7.5	6.4	0.21	0.16	30	39	12	15
Corn-	0.5	0.3		<0.3	0.7	0.3	0.31	0.26	1.0	43	0.4	17
ing	1.0	0.8		1.0	1.2	1.3	0.33	0.25	4.0	43	1.7	17
03057	2.0	2.1		2.3	2.0	3.3	0.39	0.24	8.3	48	4.0	17
	4.0	5.6		4.4	4.1	7.5	0.46	0.21	16	50	8.2	15
	6.0	8.4		5.6	>7.7	12.5	0.51	0.18	25	52	13	15
Corn-	0.3	0.4	0.6		1.0	0.3	0.22	0.25	1.5	41	0.6	19
ing	1.0	1.0	1.0		1.6	1.0	0.24	0.24	4.3	45	1.9	18
	2.0	3.5	1.6		3.3	4.5	0.60	0.23	7.4	46	3.4	20
	4.0	4.3	3.4		5.6	4.5	0.25	0.18	18	48	8.6	17
	6.0	8.2	6.4		>7.7	8.9	0.42	0.20	21	54	11	23
Damon	0.1	0.2	<0.5	<0.3		0.2	0.20	0.23	0.8	42	0.3	17
	0.6	0.6	0.8	0.6		0.8	0.22	0.22	3.4	43	1.5	19
	1.3	1.8	1.4	1.5		1.4	0.23	0.21	6.1	47	2.9	18
	3.0	4.3	2.5	3.2		3.7	0.32	0.20	12	51	5.9	17
	5.0	6.9	3.8	4.5		6.9	0.41	0.19	17	55	9.3	15
	7.7	>9.8	>6.0	>6.0		10	0.45	0.18	23	57	13	15

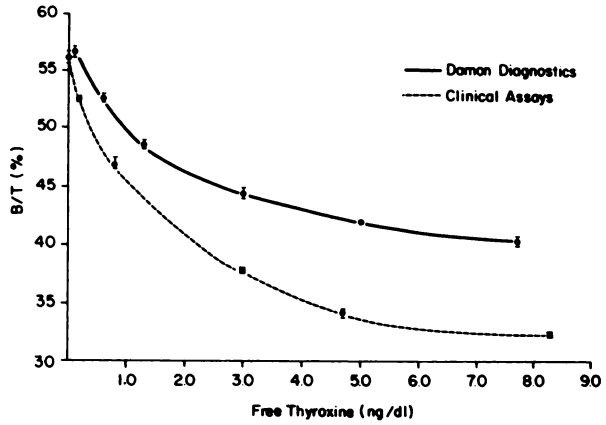

FIG. 1. Representative Damon (—) and Clinical Assays (- - -) standard FT_4 dose/response curves.

and the TBG standard curve, plotted as logit (bound counts/total counts) against log dose, were found to be linear, and patient and control data for these assays were reduced using a logit/log computer program run on a laboratory computer (*15*). The standard curve for TT_4¶, plotted as logit (B/B_O) against log dose, was found to be linear, and patient and control data were reduced using a logit/log computer program (*15*).

RT_3U was calculated as the patient or control counts times standard uptake/standard counts.

RESULTS

Concentrations of FT_4 standards, as measured by equilibrium dialysis and by three kit methods, are shown in Table 1. TT_4 concentrations given by the manufacturers were verified by radioimmunoassay¶. The table shows measured parameters $\%FT_4$ and A/TC, as well as RT_3U, calculated FTI ($RT_3U \times TT_4$), and TBG concentration found in each standard.

Representative FT_4 standard curves for the Damon and Clinical Assays methods are shown in Fig. 1.

For control samples, run-to-run assay precision (expressed as the coefficient of variation) is shown in Table 2.

The dependence of the $\%FT_4$, RT_3U, and A/TC on TBG concentrations is shown in Figs. 2A, B, and C.

Figure 3 shows the effects of FT_4 and TBG on the time course of I-125 thyroxine association with solid-phase antibody in the Corning "A" tube. Solid-phase antibody-bound I-125 thyroxine counts and the initial rates of antibody binding are similar for sera from hyperthyroid, hypothyroid, and euthyroid patients with similar TBG concentrations. Rapid and increased binding is observed in sera from euthyroid patients who have low TBG concentrations. Less antibody-bound I-125 thyroxine at marginally lower rates is observed in sera from euthyroid patients who have elevated TBG concentrations.

Figure 4 shows the effects of FT_4 and TBG on I-125 thyroxine bound to antibody after 30 min of incubation in the Corning "A" tubes.

Figure 5 plots the amounts of T_4 (closed circles) bound to antibody during the Corning "A" tube incubations with sera of various FT_4 and TBG concentrations. The mass bound is relatively independent of TBG concentration and is increased in sera from hyperthyroid patients, decreased in hypothyroid sera.

The time course of Damon microcapsule-T_4 equilibration in sera of various FT_4 and TBG concentrations is shown in Figure 6. Iodine-125 thyroxine is displaced from the microencapsulated antibody more rapidly by sera from hyperthyroid patients, less rapidly by sera from hypothyroids, and appears to be unaffected by TBG concentration. The time dependence of these results is evident.

The effects of varying Damon microcapsule concentration on FT_4 estimates from sera of various TBG concentrations are shown in Fig. 7. Increasing the mi-

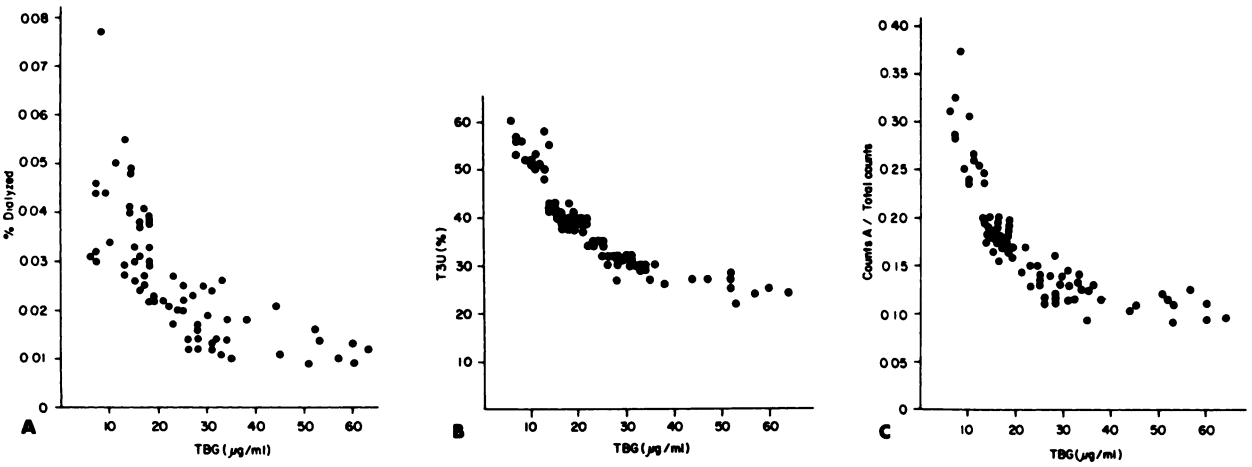

FIG. 2. A. Effect of TBG concentration on % dialyzed fraction (by equilibrium-dialysis assay). B. Effect of TBG concentration on % silicate T_3 uptake.¶ C. Effect of TBG concentration on % "A" tube counts (Corning assay).

TABLE 2. FT_4 CONCENTRATION ESTIMATED IN FIVE CONTROL POOLS (ng/dl ± 1 s.d.)

	Pool	C1	C2	D1	D2
Equilibrium dialysis	2.4 ± 0.5 (N=17)	2.3 ± 0.2 (N=8)	4.3 ± 0.4 (N=7)	2.3 ± 0.4 (N=8)	3.9 ± 0.3 (N=6)
CV*	20%	9%	10%	17%	9%
Corning†	1.4 ± 0.1 (N=10)	1.4 ± 0.1 (N=10)	2.9 ± 0.1 (N=10)	1.6 ± 0.1 (N=5)	2.6 ± 0.2 (N=5)
CV	6%	6%	5%	8%	6%
Damon	1.9 ± 0.3 (N=16)	1.5 ± 0.3 (N=8)	3.3 ± 0.5 (N=10)	1.7 ± 0.2 (N=26)	2.8 ± 0.3 (N=24)
CV	15%	16%	15%	10%	9%
Clinical Assays		1.7 ± 0.3 (N=8)	3.5 ± 0.4 (N=8)	1.4 ± 0.2 (N=16)	2.4 ± 0.3 (N=16)
CV		15%	10%	11%	14%

* CV = coefficient of variation.
† Calculated as Total T_4 × A/TC.

crocapsule concentration increases apparent TBG dependence.

The variability and apparent dependence of Damon assays on TBG are shown in Table 3. Initially we obtained apparently spuriously elevated results in sera from euthyroid patients with elevated TBG concentrations. This apparent dependence on TBG was reduced coincident with changes made by the manufacturer, and currently is not seen.

Figure 5 shows the mass of T_4 (open circles) extracted from sera of various FT_4 and TBG concentrations by the Clinical Assays solid-phase antibody-coated tube. Reequilibration occurs rapidly. Extracted mass is increased in sera from hyperthyroid patients, decreased in hypo-

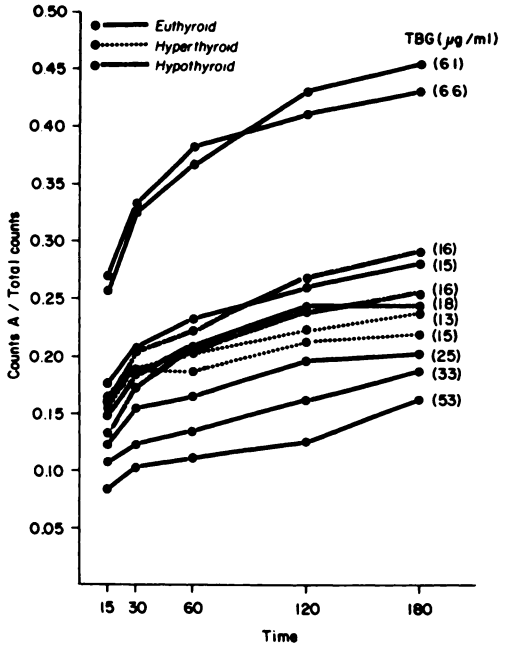

FIG. 3. Effects of TBG concentration and thyroid functional status on time course of antibody binding to radiolabeled T_4 in Corning A tube.

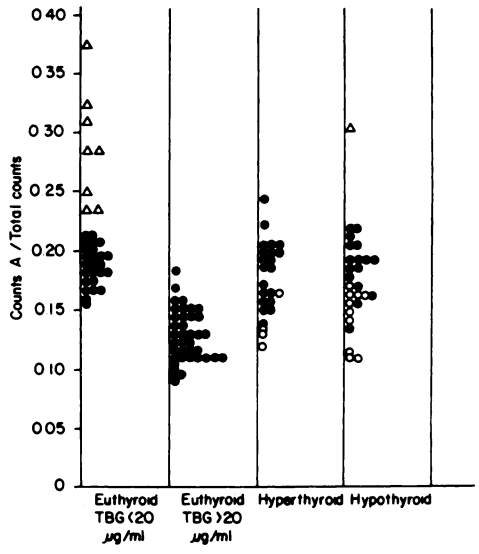

FIG 4. Effects of TBG concentration and functional thyroid status on percentage of added radiolabeled T_4 bound by solid-phase antibody after 30 min in Corning "A" tube. Δ = TBG <10 μg/ml. O = <20 μg/ml. ● = TBG 10–20 μg/ml (normal) in all columns except euthyroid, TBG >20 μg/ml.

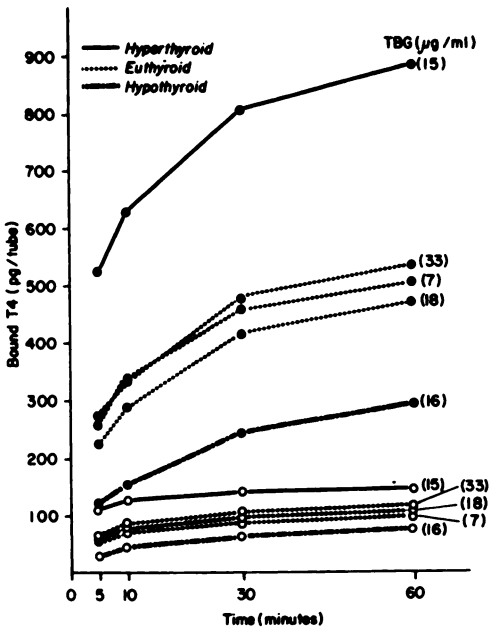

FIG. 5. Effects of TBG concentration and functional thyroid status on mass of T_4 bound by solid-phase antibody in Corning (●) and Clinical Assays (O) systems.

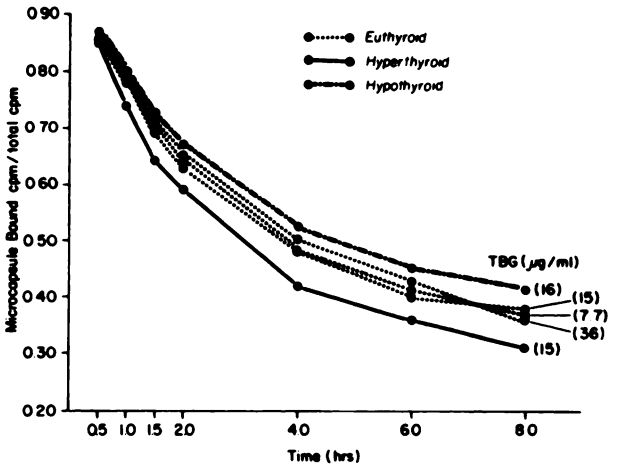

FIG. 6. Effects of TBG concentration and functional thyroid status on release of radiolabeled T_4 from microcapsules in Damon assay.

thyroid sera, and appears to be relatively independent of TBG concentration.

Figures 8–14 plot the FT_4 concentrations in sera of various FT_4 and TBG concentrations as estimated by equilibrium dialysis, by FTI, and by the three commercial kits. Corning results calculated from A/B (Fig. 10) and $TT_4 \times A/TC$ (Fig. 11) are shown. Corning results expressed as an index without reference to a standard curve are shown in Fig. 12.

A summary of results from all methods in patients with various FT_4 and TBG concentrations is presented in Table 4.

DISCUSSION

In most individuals, functional thyroid status can be determined by simply measuring the concentration of TT_4. This would be true invariably if all individuals had similar concentrations of thyroxine-binding proteins but, unfortunately, they do not. TT_4 varies with total binding-protein concentrations, principally TBG; thus the measurement of greatest clinical interest is the estimation of the free or unbound T_4 concentration.

For a number of years, the use of an FTI, obtained by multiplying TT_4 concentration by RT_3U, has been a useful means of obtaining information regarding FT_4 concentration (*3–6*). Although most of the limitations of the FTI arise from the RT_3U test, occasionally an index cannot be calculated because the patient's TBG concentration is very low and the resultant TT_4 concentration is below the sensitivity of the assay. A T_3 uptake test requires the partition of radiolabeled T_3 between available TBG-binding sites and a secondary binder such as resin, red blood cell membranes, or silicate (*16,17*). Depending upon the concentration and affinity of the secondary binder used, this test will function well over a wide range of TBG concentrations but will be limited by very high or very low TBG concentrations (Fig. 2B). The product of an elevated TT_4 and a reduced

TABLE 3. EFFECTS OF TBG CONCENTRATION ON THE FT_4 CONCENTRATIONS MEASURED BY THE DAMON MICROCAPSULE ASSAY IN EUTHYROID PATIENTS

Assay date*	TBG normal (10–20 μg/ml) No.	mean FT_4 (ng/dl)	σ	TBG elevated (>20 μg/ml) No.	mean FT_4 (ng/dl)	σ	Significance of mean FT_4 differences (P)†
12/78	25	1.7	0.4	26	2.7	0.6	<0.001
3/79	25	1.3	0.3	21	2.2	0.3	<0.001
6/79	10	1.5	0.5	20	1.7	0.3	Not significantly different

* The same sets of patients' samples were assayed 12/78 and 3/79. A different set of patients' samples was assayed 6/79.

† Student's t-test.

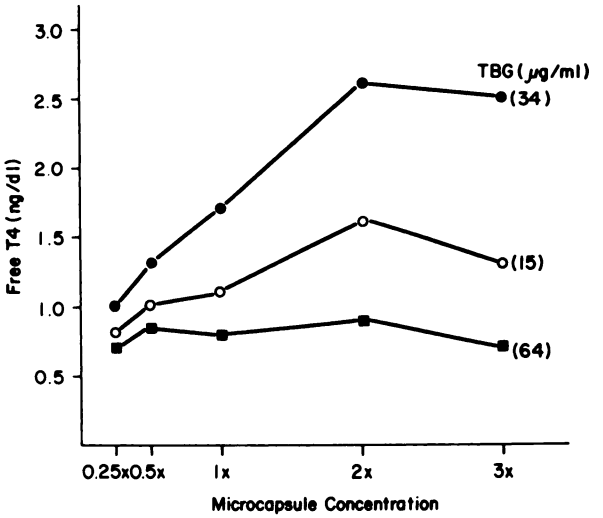

FIG. 7. Effect of microcapsule concentration on measured FT_4 concentration in samples from clinically euthyroid patients with elevated (●), normal (O), and low (■) TBG concentrations (Damon assay).

RT_3U in patients with elevated TBG, and the product of a low TT_4 and an elevated RT_3U in patients with low TBG, should yield a normal FTI. In fact, because of the limitations of the RT_3U test, the index will be slightly elevated with high TBG concentrations and slightly low at very low TBG concentrations (Table 4). Nevertheless, the FTI, when reported with TT_4 and RT_3U, has been—and still is—of considerable clinical use in the assessment of functional thyroid status (Fig. 9).

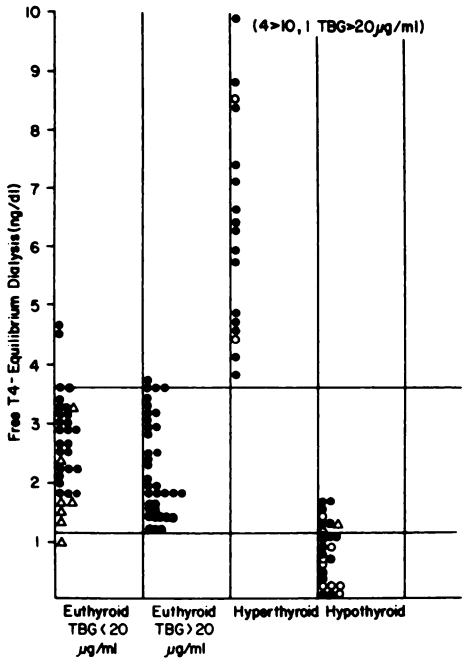

FIG. 8. FT_4 estimated by equilibrium dialysis related to clinical thyroid status. Δ = TBG <10 μg/ml. O = TBG >20 μg/ml. ● = TBG 10–20 μg/ml (normal) in all columns except euthyroid, TBG >20 μg/ml.

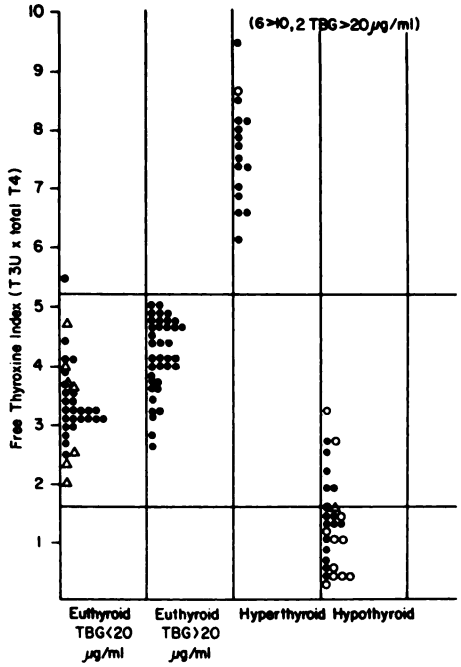

FIG. 9. FTI (total $T_4 \times T_3U$)[1] related to clinical thyroid status. Δ = TBG <10 μg/ml. O = TBG >20 μg/ml. ● = TBG 10–20 μg/ml (normal) in all columns except euthyroid, TBG >20 μg/ml.

Equilibrium dialysis, described first by Sterling (*1*), usually provides clinically accurate assessment of FT_4 concentration, but is technically difficult and is not often done in clinical laboratories. An FT_4 estimate is obtained from equilibrium dialysis as the product of the dialyzable fraction ($\%FT_4$) and TT_4. The $\%FT_4$ is dependent upon TBG concentration, as shown in Fig. 2A. A change in $\%FT_4$ occurs over a wider range of TBG concentrations than is observed for RT_3U; therefore, the calculated FT_4 value remains normal in euthyroid individuals (Fig. 8,

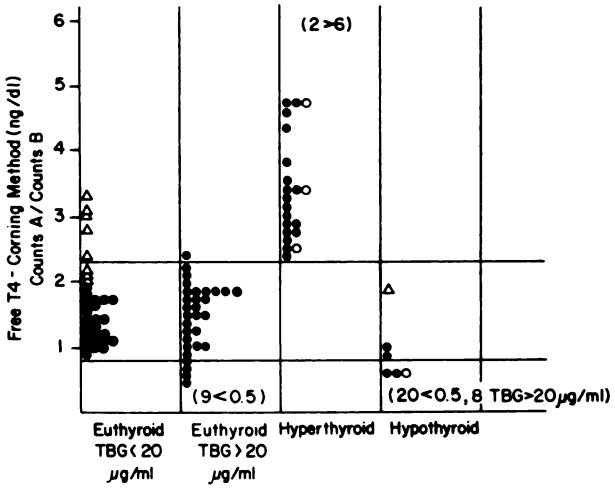

FIG. 10. Corning FT_4 related to clinical thyroid status. Results calculated by Corning's original suggestion (counts A/counts B). Δ = TBG <10 μg/ml. O = TBG >20 μg/ml. ● = TBG 10–20 μg/ml (normal) in all columns except euthyroid, TBG >20 μg/ml.

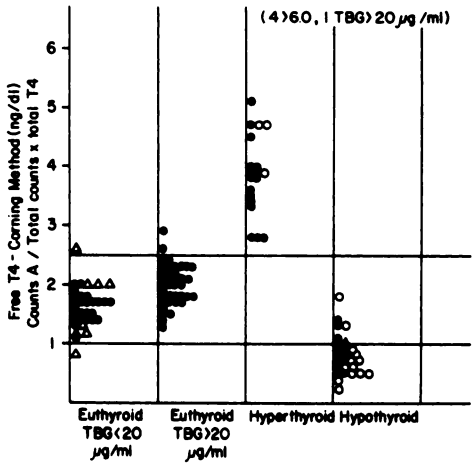

FIG. 11. Corning FT_4 related to clinical thyroid status. Results recalculated from total T_4 × counts A/total counts. Δ = TBG <10 μg/ml. O = TBG >20 μg/ml. ● = TBG 10–20 μg/ml (normal) in all columns except euthyroid, TBG >20 μg/ml.

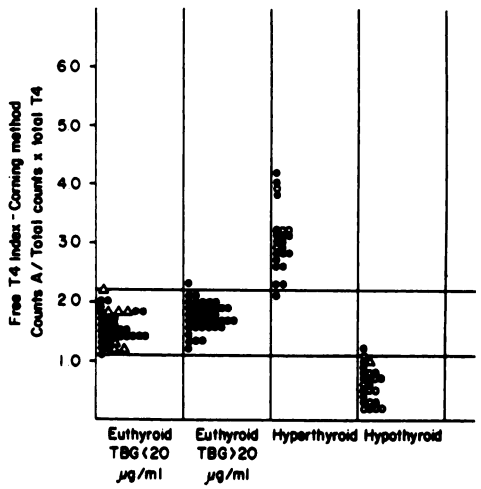

FIG. 12. FTI by Corning method (T_4 × counts A/total counts *without* reference to standard curve), related to clinical thyroid status. Δ = TBG <10 μg/ml. O = TBG >20 μg/ml. ● = TBG 10–20 μg/ml (normal) in all columns except euthyroid, TBG >20 μg/ml.

Table 4). In addition, the $\%FT_4$ tends to be increased in hyperthyroidism and decreased in hypothyroidism (*1*). Therefore, the dialysis-derived FT_4 is high in hyperthyroidism because both the $\%FT_4$ and the TT_4 are high, and it is low in hypothyroidism because both are low.

The ability of the commercial FT_4 assays to reflect the true functional thyroid status of a patient depends upon assay sensitivity, precision, accuracy, and freedom from effects not attributable to FT_4.

Satisfactory displacement of I-125 thyroxine from antibody was observed with increasing concentrations of FT_4 in the Damon and Clinical Assay determinations (Fig. 1). The sensitivity of all assays was adequate to

TABLE 4. SUMMARY OF FT_4 RESULTS OBSERVED IN CLINICAL SAMPLES

						Corning:				Damon			Clinical Assays:	
	Total T_4 (RIA)	RT_3U	FTI	Equilibrium dialysis: % FT_4	Equilibrium dialysis: FT_4	A/TC (30 min)	dA/dT*	FT_4 from (A/B)	FT_4 from (A/TC × TT_4)	Effect of inappropriate increases in microcapsule concentration on measured FT_4	dB/dT† (microcapsule concentration optimized)	FT_4	Mass T_4 extracted (10 min)	FT_4
Euthyroid														
Very low TBG (<5 ng/ml)	↓↓	↑	↓	↑↑	N	↑	↑↑	↑↑	↓	N	N	N	N	N
Low TBG	↓	↑	N	↑	N	↑	↑	↑	N	N	N	N	N	N
Normal TBG (10–20 ng/ml)	N	N	N	N	N	N	N	N	N	N	N	N	N	N
High TBG	↑	↓	N	↓	N	↓	↓	↓	N	↑	N	N	N	N
Very high TBG (>40 ng/ml)	↑↑	↓	↑	↓↓	N	↓	↓↓	↓↓	↑	↑↑	N	N	N	N
Hypothyroid (normal TBG)	↓	↓	↓	↓	↓	N	N	↓	↓	↓	↓	↓	↓	↓
Hyperthyroid (normal TBG)	↑	↑	↑	↑	↑	N	N	↑	↑	↑	↑	↑	↑	↑

* Initial rate of antibody-I-125 thyroxine association.

† Initial rate of microcapsule-thyroxine equilibration.

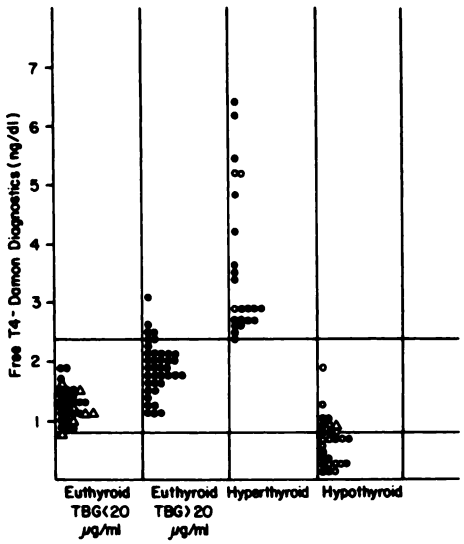

FIG. 13. FT_4 by Damon method (March 1979), related to clinical thyroid status. Δ = TBG <10 µg/ml. O = TBG >20 µg/ml. O = TBG 10–20 µg/ml (normal) in all columns except euthyroid, TBG >20 µg/ml.

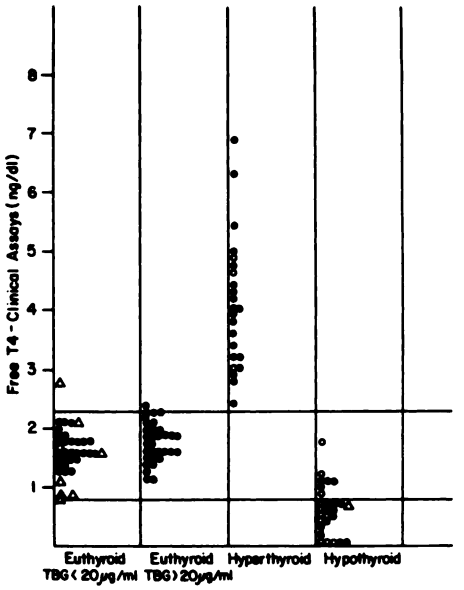

FIG. 14. FT_4 by Clinical Assays method, related to clinical thyroid status. Δ = TBG <10 µg/ml. O = TBG >20 µg/ml. ● = TBG 10–20 µg/ml (normal) in all columns except euthyroid, TBG >20 µg/ml.

allow identification of depleted concentrations of FT_4 in sera from hypothyroid patients (Figs. 11–14). The FT_4 in some hypothyroid patients (as evidence by elevated thyroid-stimulating hormone) was in the normal range. These patients were encountered early in the course of their illnesses, were just beginning replacement thyroxine, or were receiving inadequate doses of replacement thyroxine.

The reproducibility of replicate control serum measurements over time was similar for all assays (Table 2).

Accuracy—the agreement between the true FT_4 concentration and the measured concentration—depends on the accuracy of the FT_4 standards in each commercial assay. Unless the assay is entirely unaffected by endogenous binding proteins, differences between the standard protein matrix and individual patient sera may affect the measured FT_4 concentrations. All four commercial FT_4 standards are prepared in sera and calibrated by equilibrium dialysis. The properties in these standards differ substantially (Table 1). The measured concentrations of individual standards differ with the method of measurement.

The $\%FT_4$ increased with nominally increasing FT_4 concentrations in the Clinical Assays and Damon standards as well as Corning standard 03057. In all of these standards, TBG concentration was lowest in the higher standards. The magnitude of increase in $\%FT_4$ cannot be explained by this factor alone but must be due to increasing FT_4 as well. The $\%FT_4$ changes in Corning standard 00689 were inappropriate, apparently reflecting the greater changes in TBG concentrations in this set of standards.

The standard RT_3U measurements do not approximate those obtained in hypothyroid and hyperthyroid patients with similar TBG and FT_4 concentrations. The RT_3U values increased in each set of standards, as would be expected, but the Clinical Assays RT_3U values were unusually low in the high standards, and the Corning and Damon RT_3U values were unusually high in the low standards. The failure of the RT_3U measurements (and, by inference, the degree of TBG saturation) to approximate pathophysiologic values reflects the differences between the in vitro addition of T_4 to sera to prepare standards and the in vivo development of hypothyroidism or hyperthyroidism.

Changes in A/TC ratio are particularly interesting because, except with Corning standard 00689, A/TC *decreased* as the nominal FT_4 concentration increased. This was not observed in sera obtained from hyperthyroid patients with similar TBG concentrations (Figs. 3 and 4), which suggests that the higher FT_4 concentration standards provide the solid-phase antibody with a very large T_4 pool that has nominally normal TBG concentrations, with resultant low antibody-binding of I-125 thyroxine. This results in an apparently anomalous decrease in (TT_4 × A/TC) as the "free thyroxine" concentration increases, and explains why the Damon, Clinical Assays, and equilibrium dialysis assays "overestimate" the high Corning standards. If this effect were demonstrated in clinical samples, the (TT_4 × A/TC) calculation would be invalid.

Since both the Clinical Assays and Damon assays appear to be relatively free of effects from a sample's endogenous thyroxine-binding protein, the significance of standards with different protein concentrations is unclear. Both equilibrium dialysis and indexing yield a

result related to FT_4 without use of standard curves. The Corning standards behave least like physiologic or pathophysiologic serum samples and the A/TC response appears to be the reverse of that expected. The Corning results ($TT_4 \times A/TC$) without reference to a standard curve apparently provide a clinically valid FT_4 index (Fig. 12) and avoid the issues of standard problems.

The method available from Corning Medical is purported to allow measurement of FT_4 by relating the "A" tube response to functional thyroid status. As suggested by Ekins (*13*), effects due to changes in TBG concentration in this system can be corrected for to some degree by calculating $TT_4 \times A/TC$. In this way, the "A" tube response is in effect like the T_3 uptake response, and the calculated result is an index. This system differs from an FTI in that, unlike T_3, T_4 is bound by all endogenous binding proteins. Rather than reflecting the degree of TBG saturation, radiolabeled T_4 equilibrates with the TT_4 pool.

In contrast with data reported by Odstrchel (*7*) and Hertl (*8*), the A/TC response we obtained was dependent upon TBG concentration and was *not* altered by functional thyroid status, i.e., by changes in FT_4 (Figs. 3 and 4). Changes in "A" tube binding reflect the concentrations and affinities of all binders present in the test system. Consequently, when TBG concentrations are high, antibody binding will be low, and vice versa. This TBG-dependent antibody binding, like T_3 resin uptake, is responsive over a relatively wide range of TBG concentrations but is limited by low and high TBG concentrations (Fig. 2C). When TBG concentrations are greater than ~30 μg/ml, no further decrease in "A" tube binding is observed. As with the FTI, the estimate of FT_4 obtained by calculating $TT_4 \times A/TC$ will increase as further increases in TBG and TT_4 occur. This effect was most evident when ($TT_4 \times A/TC$) was referenced to a standard curve (Fig. 11) rather than when the results were treated as an index (Fig. 12). The original A/B method of calculation did not compensate for the TBG-dependent effects shown in Fig. 3, which resulted in marked overestimation of results in patients with very low TBG concentrations (Fig. 10). Low results were also obtained in sera from patients with very high TBG concentrations (Fig. 10). The $TT_4 \times A/TC$ calculation did produce normal results for euthyroid patients with low TBG concentrations. However, the mean value obtained for euthyroid patients with elevated TBG concentrations was higher than when the TBG concentrations were normal ($P < 0.01$, Figs. 11 and 12). In some euthyroid patients with elevated binding-protein concentrations, values were in the hyperthyroid range. Although these errors occur with a frequency similar to that with the FTI, clinicians are aware of the significance of an elevated TT_4 and low RT_3U value. Diagnostic errors with the Corning method could be avoided if clinicians were educated as to the meaning of A/TC, and if A/TC were reported along with the TT_4 and resultant "free T_4."

The Damon method has been under evaluation since November 1978. Initially, we observed a rather pronounced overestimation of apparent FT_4 concentration in patients with elevated TBG concentrations (Table 3). This effect was not reported by Ashkar et al. (*11*) using materials predating our experience, and apparently was due to excesses in microcapsule concentration (Fig. 7). This situation was resolved to some degree by the manufacturer in March 1979 (Fig. 13), and no longer presents a significant problem (Table 3, Fig. 6). We have examined the rate at which radiolabeled T_4 leaves the microcapsule in samples from hyperthyroid, hypothyroid, and euthyroid patients with either elevated or low TBG concentrations (Fig 6). The microcapsule binding is responsive to changes in functional thyroid status. However, changes due to binding-protein concentrations do not now appear important.

The Clinical Assays system might be expected to demonstrate effects caused by distribution of T_4 among all available binders in the initial extraction reaction. In fact, the solid-phase antibody binding occurs rapidly and TBG effects appear to be quite minimal (Fig. 5, open circles), probably because of the system's geometry as well as because the antibody's affinity for T_4 is less than the affinity of TBG for T_4. This assay performed quite satisfactorily in all patients' samples we studied (Fig. 4).

SUMMARY

Expected results when measurements are made on sera of various TBG and FT_4 concentrations are summarized in Table 4. All FT_4 assays yield appropriate results when TBG concentrations are normal.

The Corning method does not yield a direct measurement of FT_4 but rather an index. The A/TC uptake is currently neither as linear nor as responsive to change in TBG concentration as is the RT_3U test we used. Reference to a standard curve is unnecessary, requires more time and calculation, and compounds errors. Concerns over standard validity should further discourage this practice. If indexing is done without reference to a standard curve, reporting A/TC and TT_4 as well as the resultant FT_4 index would enhance clinical understanding of these results.

Both the Damon and Clinical Assays methods respond to FT_4 and are apparently free of significant effects from endogenous T_4-binding protein. Both manufacturers' standards differ from pathophysiologic sera, however. The results from both assays are a single FT_4 value, and neither TT_4 nor an indication of binding-protein concentration or saturation is available without another measurement.

Although the response of an RT_3U is limited by very

high or very low TBG concentrations, a calculated FTI reported along with the TT_4 and RT_3U values provides maximum information adequate to indicate correctly the functional thyroid status in most patients. In clinical circumstances where the FTI may be misleading (*18*), it is not at all clear that the newer FT_4 methods are better.

None of these methods for the estimation of FT_4 is definitive, and careful clinical assessment and confirmation of functional thyroid status with appropriate testing should be done after any FT_4 screening procedure.

FOOTNOTES

* Corning Medical, Medfield, MA.
† Damon Diagnostics, Needham, MA.
‡ Clinical Assays, Cambridge, MA.
‖ Thypinone, Abbott Laboratories, Chicago, IL.
§ Amersham Corporation, Arlington Heights, IL.
¶ Nuclear Medical Laboratories, Inc., Dallas, TX.

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