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Measurement of Serum Free Thyroxine by RIA in Various Clinical States

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A radioimmunoassay for quantitatively measuring the serum concentration of free thyroxine is described. This method does not require equilibrium dialysis, and is rapid and reproducible. The serum values obtained by this radioimmunoassay and by equilibrium dialysis are similar in normal subjects, hyperthyroid and hypothyroid patients, pregnant women, "sick euthyroid" patients, and euthyroid patients with hereditary TBG abnormalities. The method also provides a total serum thyroxine concentration in the same assay procedure.

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The most commonly used test in the diagnosis of thyroid function is the serum thyroxine (T_4) concentration. Since circulating T_4 is almost totally bound (~99.97%), primarily by the thyronine-binding globulin (TBG) but also by albumin and prealbumin (TBPA), measurement of serum total T_4 concentration will be influenced by both the quantity of T_4 secreted by the thyroid gland and the concentration of TBG. It is generally accepted that the free or unbound T_4 concentration (FT_4) is more readily available to the tissues for metabolic action, and that the measurement of serum FT_4 more accurately reflects thyroid function, since it is not altered by the concentration of the thyronine-binding proteins, especially TBG. Many methods have been described for the calculation of the free T_4 concentration, and all depend on measurement of the serum T_4 concentration and an assessment of the percentage of the total T_4 that is unbound. The triiodothyronine uptake (T_3U) is the most commonly used test to determine the relative saturation of the TBG binding sites by T_4, and it indirectly reflects the proportion of the free T_4 in serum. The indirect measurement of the serum FT_4 is then calculated as the product of the T_4 and T_3U and is reported as a free T_4 index (FTI). The FTI can also be calculated by the quotient of the serum T_4 and TBG concentrations measured by radioimmunoassay (RIA). These indirect methods do have clinical utility but do not give a quantitative FT_4 concentration (*1–4*).

A direct measurement of serum FT_4 can be assessed as the product of the serum T_4 and the fraction of free T_4 measured by equilibrium dialysis (*5–7*). Other methods for measuring FT_4, such as Sephadex filtration (*8*), ultrafiltration (*9*), charcoal absorption (*10*), ion-exchange or gel filtration (*11*), and a combination of dialysis and gas chromatography (*12*) as well as RIA methods (*13,14*), have also been described. Most of these are not suitable for routine laboratory use, since they are often time-consuming and technically more difficult to perform.

The present report describes a rapid, reproducible, and simple RIA method for the quantitative measurement of FT_4 in the sera of patients with thyroid dysfunction, in patients with the estrogen-induced increase in TBG that occurs in pregnancy, in euthyroid patients with hereditary TBG abnormalities, and in sick euthyroid patients.

MATERIALS AND METHODS

FT_4 by RIA. The IMMO PHASE FT_4 ^{125}I Radioimmunoassay Test System* was used. Each kit contains the following reagents, sufficient for 60 FT_4 determinations: The T_4 antibody (rabbit anti-T_4), covalently bound to porous glass particles, suspended in phos-

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phate-buffered saline, pH 7.4, containing bovine serum albumin and sodium azide; I-125-labeled T_4, Tracer A containing red dye and Tracer B containing green dye and thimerosal; five vials of lyophilized human-plasma-based standards containing FT_4 at concentrations of 0.5, 1.0, 2.0, 4.0, and 6.0 ng/dl; and two vials of lyophilized human-plasma-based controls. The FT_4 standards were determined by using the equilibrium dialysis method as described by Sterling and Hegedus (*5*). Before use, the lyophilized reagents are reconstituted with distilled or deionized water. Tracer A and Tracer B are reconstituted with 6 ml water, and standards and controls with 3 ml water. The vials are swirled and then intermittently mixed for 10 min.

The total T_4 can also be measured, since values for T_4 are given for each of the FT_4 standards. A total T_4 standard curve can be obtained by using Tracer B. In a study by Hertl and Odstrchel (*15*), it was demonstrated that the binding of T_4 to its specific immobilized antibody (IMA) obeys second-order reaction kinetics. The rate of the reaction is a function of the FT_4 concentration and the antibody concentration. The FT_4, in turn, is related to total concentrations of T_4 and thyronine-binding protein. The FT_4 method was developed in conjunction with a basic kinetic and thermodynamic study (*15*).

The key concept of the FT_4 (RIA) method is that the concentration of thyroxine bound to the immobilized antibody (IMA) in Tube A after a 30-min incubation is functionally related to the FT_4 concentration in the sample. This functional relationship is described by the following equation:

$$f[IMA \cdot T_4] = [FT_4]_0 k_1 t$$

Where f is a symbol for "function of", $[FT_4]_0$ is the concentration of unbound thyroxine in the serum sample, k_1 is the forward rate constant, and t is the reaction time in minutes. More details on the theory of the assay are described by Ekins (*16*) and Fullarton and Lidgard (*17*). The derivation of the full integrated rate equation is described by Schoemaker (*18*). In the FT_4 (RIA) assay, two reactions, A and B, are measured. In reaction A, T_4 antibody immobilized onto porous glass particles (IMA) binds the T_4 that is not bound to the thyronine-binding proteins throughout the incubation period. The count from T_4 bound in reaction A (cpm_A) divided by the total counts (TC) represents the fraction of T_4 molecules bound to IMA and is thus a function of the percentage of free T_4 in the serum. In reaction B, the bound T_4 is displaced from the proteins by thimerosal, and then binds to the IMA. In this classical RIA, the patient's total T_4 concentration is calculated from the radioactive T_4 bound to IMA. The product of the fraction of T_4 bound to IMA in the A series (cpm_A/TC_A) and the total T_4 concentration (B series) gives a measure of the amount of T_4 bound to IMA in the A series, which is a function of the free T_4 concentration. A standard curve is constructed on linear graph paper by plotting $cpm_A/TC_A \times T_4$ (μg/dl) as abscissa against FT_4 (ng/ml) as ordinate. A smooth curve is drawn through the points and the FT_4 in ng/ml is determined from the standard curve for both the supplied control samples and the unknown serum samples.

All reagents should be brought to room temperature before starting the assay. Before pipetting the immobilized antibody, the vial is thoroughly mixed until the glass particles are suspended. The assay procedure is described in the product insert.

FT_4 by equilibrium dialysis. Serum T_4 concentration was measured by RIA by a solid-phase technique.* The percent free T_4 was measured by equilibrium dialysis as described by Sterling and Hegedus (*5*). FT_4 (ng/dl) was calculated as the product of the total T_4 and fraction of free T_4.

Free thyroxine index. The FTI was calculated by using commercially available reagents for measuring T_4, TBG, and T_3U. Serum T_4 and TBG concentrations were measured by RIA,* and FTI (IMMO PHASE) calculated as: T_4 (μg/dl)/TBG (μg/ml) $\times$ 10.

Serum T_4 (RIA) and T_3U were measured with commercial kits,† and the FTI (NML) calculated as: $T_3U/100 \times T_4$.

Serum T_3. Serum T_3 concentration was measured by a solid-phase RIA method.*

Serum TSH. Serum TSH concentration was measured by a commercial double-antibody method.‡

In this study, FT_4 (RIA) was measured in the sera of 243 subjects: 89 healthy euthyroid subjects, 29 hypothyroid patients, 25 hyperthyroid patients, 31 pregnant women, 47 sick euthyroid patients, and 22 patients with hereditary TBG abnormalities. The "sick" euthyroids suffered from a wide variety of systemic illnesses including metastatic carcinoma, renal failure, severe infection, cirrhosis, and congestive heart failure; none was receiving drugs known to affect thyroid function or the

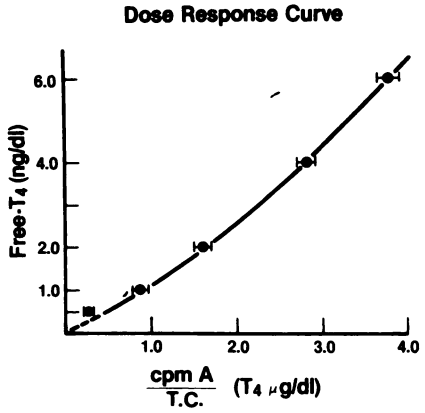

FIG. 1. Representative standard curve. $cpm_A/TC_A \times (T_4$ μg/dl) is plotted against FT_4 concentration. Solid circles represent mean and bars ± s.e.m., computed from six consecutive assays.

TABLE 1. PRECISION OF THE FT_4(RIA) ASSAY AT TWO FT_4 CONCENTRATIONS

	Mean FT_4 (ng/dl)	Coefficient of variation* (%)
Intra-assay†	1.4	1.1
	2.7	1.3
Interassay‡	1.4	6.4
	2.7	7.1

* ± Coefficient of variation = 100 × s.d. of mean.
† Duplicate determinations in six consecutive assays.
‡ Means of duplicates in six consecutive assays.

T_4-binding proteins. In the study comparing the FT_4 (RIA) method with the equilibrium dialysis technique for measuring FT_4, 170 of the 243 subjects were evaluated: 71 euthyroid subjects, 15 hypothyroids, 23 hyperthyroids, 20 patients with hereditary TBG abnormalties, 18 pregnant subjects, and 23 sick euthyroids. The diagnoses of hyperthyroidism or hypothyroidism were confirmed clinically and by serum T_4, T_3, and TSH concentrations. The euthyroid subjects were healthy volunteers participating in this and other studies. Serum FTI was measured in the 47 sick euthyroids. All assays were carried out in duplicate.

RESULTS

Variability of the standard curve, and precision of FT_4 (RIA). The standard curve in six consecutive assays demonstrated satisfactory assay-to-assay reproducibility (Fig. 1). Inter- and intra-assay variability (*19*) was well within the accepted range (Table 1).

Serum FT_4 (RIA) compared with FT_4 (equilibrium dialysis) (Fig. 2). FT_4 (RIA) correlated extremely well with FT_4 (equil. dial.) in the 170 patients evaluated, irrespective of thyroid function, concentration of TBG and nonthyroid illness. The regression line correlating the two methods was highly significant ($r = 0.96$, $P < 0.001$); its equation is FT_4 (RIA) = 0.68 FT_4 (equil. dial.) + 0.47. Both methods confirmed the diagnosis in all patients. As expected, FT_4 by both methods was increased in hyperthyroidism and decreased in hypothyroidism. Serum FT_4 was almost always within the normal range in pregnant and systemically ill euthyroid patients and in patients with hereditary abnormalities in serum TBG concentration.

Serum FT_4 (RIA) in various clinical states (Table 2, Fig. 3). Compared with the 89 euthyroid subjects evaluated in the present study, the serum FT_4 (RIA) was elevated in all hyperthyroid patients and decreased in all hypothyroids. The normal range in a larger group of 160 euthyroid subjects (including these 89) was somewhat broader (1.0–2.5 ng/dl). FT_4 (RIA) in the sera of the

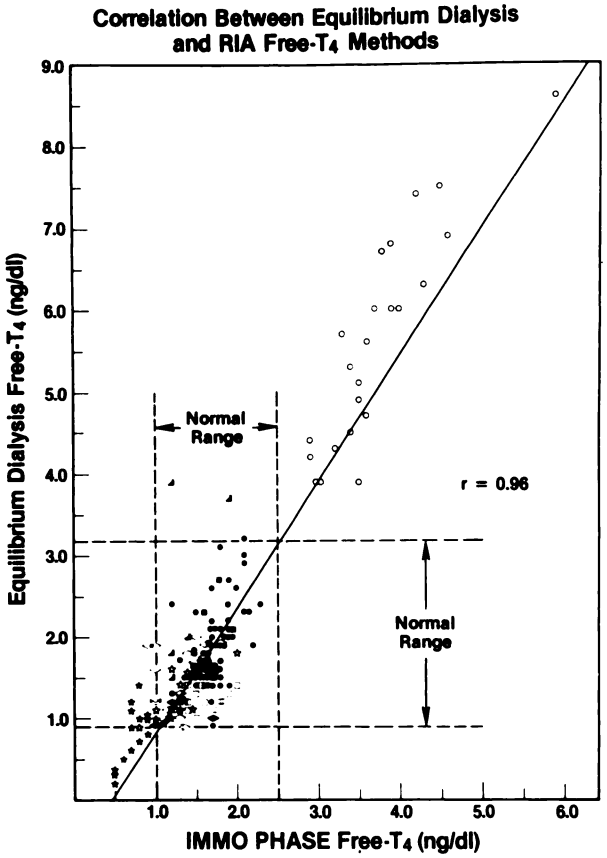

FIG. 2. Comparison of FT_4 (RIA) with FT_4 (equil. dial.). Regression gives following relationship: FT_4 (RIA) = 0.68 FT_4 (equil. dial.) + 0.47, with $r = 0.96$. Symbols indicate various clinical states in 170 patients as follows: 71 euthyroid (●); 15 hypothyroid (*); 23 hyperthyroid (O); 18 pregnant (□); 17 "sick" euthyroids with *low* T_4 (◇); six "sick" euthyroids with *normal* T_4 (■); four hereditary TBG *deficient* (◆); 11 hereditary *low* TBG (☆); and five hereditary high TBG (◪). Normal range for each method is also indicated.

pregnant women was always within the euthyroid range, and the mean value did not differ from that observed in the euthyroid subjects. Others have reported that the mean serum FT_4 (equil. dial.) in pregnancy may be significantly lower than that observed in euthyroid subjects (*2,6,20,21*). Serum TSH concentration was well within the normal range (0–10 ng/ml) in the pregnant subjects.

The serum FT_4 (RIA) was normal in patients with an hereditary absence of serum TBG (n = 5), an hereditary decrease in serum TBG (n = 11), or an hereditary increase in serum TBG (n = 6). The serum T_4 concentration in 30 sick euthyroid patients was within the normal range, and the mean value did not differ from the euthyroid mean. Serum T_4 was below the normal range in 17 sick patients as determined by the IMMO PHASE method. Serum free T_4 (RIA) was within the normal range in all except one sick patient (0.9 ng/dl), irrespective of whether the serum T_4 was normal or low, although the serum FT_4 (RIA) was significantly de-

TABLE 2. SERUM T_4 AND FT_4 (RIA) CONCENTRATIONS IN VARIOUS CLINICAL STATES

Clinical Diagnosis	n	T_4 (μg/dl)*	Range	FT_4 (ng/dl)*	Range
Euthyroid	89	8.0 ± 0.3	6.1–13.1	1.6 ± 0.04	1.3–2.3
Hypothyroid	29	2.8 ± 0.3	0.5–4.9	0.5 ± 0.05	0.3–1.0
Hyperthyroid	25	16.9 ± 0.5	12.7–22.0	3.8 ± 0.14	2.9–5.9
Pregnancy	31	11.8 ± 0.4	7.4–16.8	1.5 ± 0.04	1.1–2.0
"Sick" Euthyroid† (Normal T_4)	30	7.5 ± 0.4	5.9–11.9	1.6 ± 0.06	1.0–2.1
"Sick Euthyroid† (Low T_4)	17	3.7 ± 0.3	1.6–5.3	1.3 ± 0.08	0.9–1.8
Hereditary Absent TBG	5	2.7 ± 0.3	2.0–3.7	1.3 ± 0.12	0.9–1.7
Hereditary Low TBG‡	11	3.9 ± 0.5	1.9–8.3	1.3 ± 0.09	0.8–2.0
Hereditary High TBG‡	6	18.1 ± 1.5‖	13.3–23.2	1.4 ± 0.11	1.2–1.9

* Mean ± s.e.m.

† *Normal* and *low* T_4 concentrations are defined as greater or less than 5.5 μg/dl, respectively, using the IMMO PHASE, T_4 RIA.

‡ *Low* and *high* TBG concentrations are defined as less than 12 μg/ml or more than 60 μg/ml using the IMMO PHASE, TBG RIA.

‖ To determine T_4 concentration more accurately, a 10 μl sample was used in the six samples with *high* TBG and T_4 concentrations.

creased in the patients with a low serum T_4 ($P < 0.001$).

Various thyroid function tests in sick euthyroid patients (Tables 3 and 4). In some euthyroid patients with systemic illness, serum T_4 concentration and FTI may be decreased or increased, FT_4 (equil. dial.) elevated, and serum T_3 concentration decreased, leading to false diagnoses of hypothyroidism or hyperthyroidism. Multiple tests of thyroid function were therefore carried out in the 47 sick euthyroid patients. The percent free T_4 by equilibrium dialysis was significantly increased in the 23 sick patients evaluated by this method ($P < 0.001$) and was above the normal range in 18. The serum T_3U was also increased and was above the normal range in 16 patients. As noted above, serum T_4 was within the normal range in 30 patients, but was decreased in 17 patients as measured by the IMMO PHASE RIA. In these 17 patients FTI (NML) was decreased in nine and FTI (IMMO PHASE) in seven. Serum FT_4 (RIA) was within the normal range in all except one sick patient (0.9 ng/dl), but values in patients with a low serum T_4 were significantly decreased. FT_4 (equil. dial.) was also within

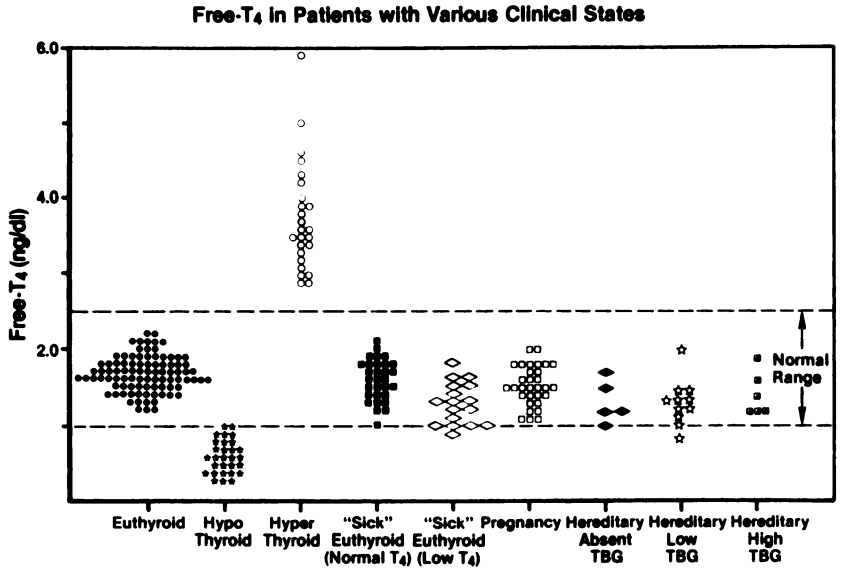

FIG. 3. The serum FT_4 (RIA) frequency distribution for various groups of patients (n = 243): 89 euthyroid; 29 hypothyroid; 25 hyperthyroid; 31 pregnant; 30 and 17 "sick euthyroids" with normal and low serum T_4 concentrations respectively; five, 11, and six euthyroid subjects with hereditary TBG abnormalities.

TABLE 3. THYROID FUNCTION TESTS IN SICK EUTHYROID PATIENTS WITH NORMAL SERUM T_4 CONCENTRATION*

	IMMO PHASE			NML			IMMO PHASE	Equilibrium dialysis†			
	T_4 (μg/dl)	TBG (μg/ml)	FTI	T_4 (μg/dl)	RT_3U (%)	FTI	FT_4 (RIA) (ng/dl)	FT_4 (%)	FT_4 (ng/dl)	T_3 (ng/dl)	TSH (μU/ml)
Mean	7.5	16.9	3.9	7.9	39.7	3.2	1.6	0.030	2.0	69	3.6
s.e.m.	0.4	0.8	0.1	0.2	1.2	0.07	0.06	0.002	0.08	5.8	0.2
Range	5.9–11.9	10.1–32.2	2.7–5.7	5.9–9.8	32.7–53.3	2.2–4.3	0.8–2.1	0.022–0.036	1.6–2.7	12–156	2.4–9.0
Normal range	5.5–12.0	12–30	2.5–6.0	5.5–11.5	35–45	2.2–4.7	1.0–2.5	0.017–0.028	0.9–3.2	80–220	0–10

* Normal serum T_4 concentration is defined as greater than 5.5 μg/dl by IMMO PHASE.

† Due to the limited supply of sera, equilibrium dialysis data were obtained on only six of the 30 patients.

TABLE 4. THYROID FUNCTION TESTS IN SICK EUTHYROID PATIENTS WITH LOW* SERUM T_4 CONCENTRATION

Patient no.	IMMO PHASE			NML			IMMO PHASE FT_4 (RIA) (ng/dl)	Equilibrium dialysis		T_3 (ng/dl)	TSH (μU/ml)
	T_4 (μg/dl)	TBG (μg/ml)	FTI	T_4 (μg/dl)	RT_3U (%)	FTI		FT_4 (%)	FT_4 (ng/dl)		
1	5.1	15.4	3.3	5.9	55.3	3.3	1.5	0.027	1.6	25	3.0
2	3.8	9.4	4.0	5.0	59.5	3.0	1.4	0.041	1.8	18	3.3
3	2.4	14.1	1.7	4.3	44.7	1.9	1.0	0.039	1.2	13	3.5
4	4.9	15.4	3.2	6.4	50.9	3.3	1.6	0.034	1.9	38	2.9
5	3.0	17.5	1.7	3.9	48.6	1.9	1.6	0.045	1.7	194	3.0
6	4.7	18.7	2.5	5.6	38.6	2.2	1.0	0.035	1.9	46	4.3
7	5.0	13.5	3.7	6.2	44.8	2.8	1.3	0.022	1.3	40	2.9
8	5.1	13.3	3.8	5.8	47.3	2.7	1.3	0.017	1.0	18	2.4
9	4.7	10.6	4.4	5.7	49.1	2.8	1.5	0.037	2.0	22	3.4
10	1.6	2.5	6.4	1.6	66.8	1.1	1.0	0.100	1.6	0	6.5
11	2.9	10.8	2.3	3.5	55.5	1.9	0.9	0.076	1.9	35	3.2
12	2.8	16.3	1.7	3.3	45.9	1.5	1.2	0.056	1.1	0	1.8
13	5.3	10.3	5.1	5.1	51.8	2.6	1.8	0.036	1.9	45	7.1
14	5.2	17.2	3.0	4.6	40.2	1.8	1.0	0.023	1.2	49	3.8
15	2.2	13.1	1.7	3.7	48.6	1.8	1.2	0.041	1.1	20	4.9
16	1.6	6.7	2.4	2.7	52.6	1.4	1.1	0.052	1.0	150	2.9
17	2.3	16.9	1.4	3.2	43.4	1.4	1.3	0.040	0.9	27	9.5
Mean	3.7	13.0	3.2	4.5	49.6	2.2	1.3	0.042	1.5	44	4.0
± s.e.m.	0.3	1.9	0.5	0.3	3.0	0.1	0.11	0.009	0.20	9	0.3

* Low serum T_4 concentration is defined as less than 5.5 μg/dl using IMMO PHASE.

the normal range in all except one sick patient. Serum TBG (RIA) was below normal in seven of the 47 sick patients, six of whom had a low serum T_4 concentration. There was a positive correlation between serum TBG and T_4 concentrations in these 47 sick euthyroid patients ($r = 0.713$, $P < 0.001$). Since illness is associated with decreased peripheral outer-ring deiodination of T_4 to T_3, serum T_3 concentration was below the normal range in 33 sick patients. Serum TSH concentration was normal in the 47 euthyroid, sick patients.

DISCUSSION

Measurement of the serum FT_4 concentration is an excellent test of thyroid function, since it is not usually affected by TBG abnormalities or by the decreased outer-ring monodeiodination of T_4 to T_3 associated with a wide variety of acute and chronic illnesses (*22–26*). The FTI is an indirect assessment of the serum FT_4 concentration, but even though the relationship is not quantitative, the FTI correlates well with the FT_4 con-

centration in most patients' sera. The FTI requires two tests, a direct measurement of TBG by RIA or the relative saturation of TBG by T_3 (T_3U), and the serum T_4 concentration. Most FTI or FT_4 methods are compared with the FT_4 as determined by equilibrium dialysis, which quantitatively measures the free T_4 concentration in serum. The present method for measuring FT_4 by RIA also uses two measurements that are carried out in the same assay. This method results in values similar to those obtained by equilibrium dialysis in normal subjects, in patients with hyperthyroidism and hypothyroidism and abnormalities in TBG, and in most sick euthyroid patients. This method is simple, rapid, and reproducible. In addition to an FT_4 value, the two-tube test also provides values for the serum T_4 concentration.

Patients who are severely ill may present a diagnostic problem, since the serum concentrations of T_3, T_4, FTI, and FT_4 may be abnormal, suggesting the possibility of thyroid dysfunction. In the present study, the low serum T_3 concentration in many of the systemically ill patients confirms the findings from many laboratories (*22–26*). It is evident, therefore, that in sick patients the measurement of the serum T_3 concentration may result in a false diagnosis of hypothyroidism. The serum T_4 concentration is usually normal in patients with systemic illness, although in some patients it may be elevated (*26–30*) or decreased (*1,2,6,7,27,31–33*). In the present study, the serum T_4 concentration was not elevated in any of the 47 sick patients, but was below the normal range in 17. The FTI is usually normal in euthyroid sick patients, although increased (*26,29*) or decreased (*31,32*) values have been reported to occur with variable frequency. In the present study, the FTI was decreased in nine of 47 sick patients, and elevated in none. The serum T_4 was low in these nine patients. The FT_4 measured by equilibrium dialysis and RIA was normal in all except one of these patients with a low FTI, although the mean FT_4 (RIA) was significantly decreased. It is evident from the discrepancy between the FTI and FT_4 in these nine patients that the T_3U was not sufficiently elevated to result in a normal FTI. Others have reported a similar discrepancy between the percent free T_4 and the T_3U, with the result that the FT_4 (equil. dial.) may be normal and the FTI decreased in some systemically ill patients (*2,32,33*). Burrows and Chopra and their coworkers (*28,34*) have suggested the possibility of a nondialyzable inhibitor (IgM) of T_4 binding in sick patients, detectable only by equilibrium dialysis, although Schussler and Welski have not found such an inhibitor (*35*). In view of the normal values for FT_4 as determined by RIA and equilibrium dialysis in almost all the sick patients with a low serum T_4, the FT_4 (RIA) can be substituted for the more cumbersome and less reproducible equilibrium dialysis technique.

As reported previously (*2,5–7,21,23,24,27,30,32*), the percent free T_4 measured by equilibrium dialysis was significantly increased in the sick patients, especially in those with a low serum T_4 concentration. The majority of the sick patients in the present study had a normal serum FT_4 (by RIA and equil. dial.) and might not have been as severely ill as the patients in these other studies in whom the serum FT_4 (equil. dial.) was also frequently increased. It is possible that the FT_4 (RIA) would be elevated in some more severely ill patients with a normal serum T_4 concentration, but less so than the FT_4 (equil. dial.), since the former was somewhat lower than the latter in the 17 patients described above, who had a decreased serum T_4 concentration.

Although this method for assessing the free T_4 concentration is reliable, simple, and reproducible, its efficacy in patients with nonthyroid illness must be evaluated in other laboratories and in larger groups of sick patients. On the basis of our study, it would be premature to recommend the FT_4 (RIA) as "the" screening test for thyroid function. However, it warrants further study by other investigators.

FOOTNOTES

* Corning Medical, Medfield, MA.
† Nuclear Medical Laboratories, Inc.,
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