Radioimmunoassay of Free Thyroxine with Prebound Anti-T_4 Microcapsules

Fuad S. Ashkar, Robert J. Buehler, Teresa Chan, and Mokbel Hourani

University of Miami School of Medicine, Miami, Florida and Damon Diagnostics, Needham Heights, Massachusetts

Free thyroxine (FT_4) may be one of the active thyroid hormones in contact with target end organs. It is unaffected by alterations in serum protein levels. In most cases, measurement of FT_4 reflects an individual's true thyroid function or dysfunction. Previous FT_4 assay techniques have been difficult, tedious, indirect, and inaccurate. A rapid, simple, and accurate radioimmunoassay for FT_4 has been developed using microencapsulated rabbit anti-T_4 antiserum to which I-125 T_4 tracer of high specific activity has been complexed. Addition of FT_4 standards or unknown samples displaces a proportional amount of I-125 T_4 from antibody. Protein-bound T_4 is excluded from the reaction by short incubation time and spatial configurations. Specimens representing known thyroid dysfunction were tested using the above procedure. The normal range of FT_4 was 0.8–2.4 ng/dl. The mean FT_4 for the hyperthyroid group was 6.92 ± 1.38 (range 4.4–9.6) ng/dl. The mean FT_4 for the hypothyroid group was 0.43 ± 0.37 (range 0.1–1.3) ng/dl, and in pregnancy the mean FT_4 was 1.64 ± 0.44 (range of 1.0–2.2) ng/dl (1).

J Nucl Med 20: 956–960, 1979

MATERIALS

The agents used were: rabbit anti-T_4 antiserum*; I-125 thyroxine†; 1,6-hexanediamine, terephthaloyl chloride, cyclohexane, chloroform; Tween-20, and Span-85 were obtained commercially.

METHODS

Microencapsulation. Rabbit anti-T_4 antiserum was microencapsulated within a semipermeable membrane by essentially following the interfacial polymerization procedure described by Chang (*4*). Briefly, antiserum solution containing 1,6-hexanediamine and Span-85 was emulsified in an organic solvent mixture of cyclohexane:chloroform (80:20 v/v). The emulsification was carried out in a glass beaker with a magnetic stirring bar. When emulsion droplets of the appropriate size were achieved (20–80 μ in diameter), a diacid chloride, terephthaloyl chloride, was added to initiate formation of nylon polymer and deposition of membrane around the aqueous droplets of emulsified antibody. Following formation of nylon microcapsules, they were first washed in 50% Tween-20 in 0.15 *M* sodium bicarbonate. (They were centrifuged between washings at 1500 *g* for 10 min, followed by aspiration of the supernatant.) The microcapsules were next washed six times with 0.015 *M* phosphate-buffered saline, pH 7.5 (PBS). An appropriate dilution of this final microcapsule suspension was made to achieve at least 80% binding of I-125 thyroxine after a 15-min incubation at 37°C.

Received Nov. 28, 1978; revision accepted April 5, 1979.

For reprints contact: Robert J. Buehler, Director, R & D, Damon Diagnostics, 111 Fourth Ave., Needham Heights, MA 02194.

Saturation with radiolabeled tracer. Iodine-125 thyroxine of high specific activity (>4 mCi/μg) was used to label previously microencapsulated anti-T_4 antiserum. Twenty picograms of I-125 T_4 were added to each equivalent assay tube. Incubation was carried out at 37°C for 30 min. Following incubation, the radiolabeled microcapsules were centrifuged at 1400 *g* for 5 min and the supernatant aspirated. The microcapsules labeled with I-125 T_4 were then washed four times with 0.015 *M* PBS. After the final wash, they were suspended in 0.8 ml PBS equivalent per assay tube.

Preparation of free T_4 standards. Human serum was obtained from healthy donors. T_4 was removed by essentially following the procedures of adsorption to activated charcoal (*7*) followed by passage through a column of QAE Sephadex‡ (*2*).

Following filtration of the column eluate, the sodium salt of L-thyroxine was analytically added, by weight, resulting in serum FT_4 standards with the following thyroxine values: 0.5, 1.3, 3.0, 5.0, and 7.7 ng/dl. These standards were aliquoted, frozen, and lyophilized. When reconstituted and stored at 2–4°C, they were stable for 2 wk.

Equilibrium dialysis. Equilibrium dialysis on specimens was carried out according to the method of Sterling and Brenner (*11*).

T_3 resin uptake. Specimens were tested using the T_3 resin uptake test.‖

Free T_4 assay. The following protocol was used in the establishment of a standard curve:

1. Pipette 0.8 ml of microcapsule suspension (prelabeled with I-125 T_4) into each 12- × 75-mm polystyrene tube.
2. Pipette 0.025 ml of standard, control, or sample (which may be serum or plasma) into correspondingly labeled tubes. Vortex 3–4 sec each.
3. Incubate all tubes at 37°C for 2 hr. Vortex each tube after 1 hr.
4. Pipette 1.0 ml of 1% bovine serum albumin (BSA) in PBS into each tube. Vortex each tube.
5. Incubate all tubes at room temperature for 20 min.
6. Centrifuge all tubes at 1400 *g* for 10 min. Aspirate supernatant into radioactive waste container.
7. Count all tubes in a gamma counter for 1 min.

RESULTS

Microencapsulation. Rabbit anti-T_4 antiserum was encapsulated within semipermeable nylon microcapsules. The microcapsule size distribution averaged 20–80 μ in diameter. Anti-T_4 antiserum that was encapsulated was not chemically bound to the nylon membrane but was free in solution within the microcapsule (Figs. 1 and 2). This has been shown through physical rupture of the microcapsules in a hand-held glass homogenizer. Microcapsules containing I-125-labeled T_4, previously bound to antibody, were subjected to homogenization. Following centrifugation, over 95% of the radioactivity was found in the supernatant fraction as opposed to the broken-membrane fraction.

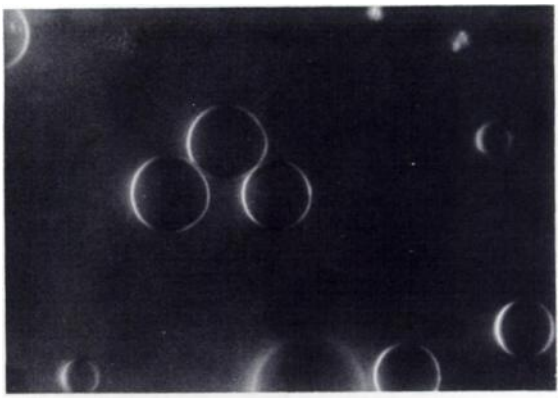

FIG. 1. Phase-interference photograph of microcapsules (×200 before reproduction).

Permeability of the microcapsule membrane was shown by the uptake of bromphenol blue dye solution (1% w/v, mol. wt. 692 daltons). When a few drops of this solution were placed on a glass slide containing microcapsules, uptake of the dye into the microcapsules took place within 2 min. Since the molecular weight of thyroxine is 777 daltons, uptake of bromphenol blue dye offered excellent visualization of the permeability of the microcapsule membrane.

Assay. The FT_4 assay is a single-tube displacement reaction in which the FT_4 value can be read directly from a standard curve. The incubation time should be 120 ± 10 min. The amount of antibody-bound I-125 T_4 that is displaced was found to be proportional to the FT_4 concentration in the sample being assayed. This standard curve may be plotted on semilogarithmic graph paper as counts per minute (or relative percent bound) against FT_4 concentration in ng/dl. (Fig. 3).

Normal range. The normal range of FT_4 values was 0.8–2.4 ng/dl as determined on 217 healthy adult individuals who presented normal routine chemistry and thyroid profiles and were judged to be clinically euthyroid (Fig. 4).

Precision. The coefficients of variation for two control samples, each assayed 20 times within one experiment, are illustrated in Table 1.

The coefficients of variation for two control samples, each assayed five times in five separate experiments, are illustrated in Table 1.

Specificity. The cross-reactivity of various biochemical compounds with the microencapsulated

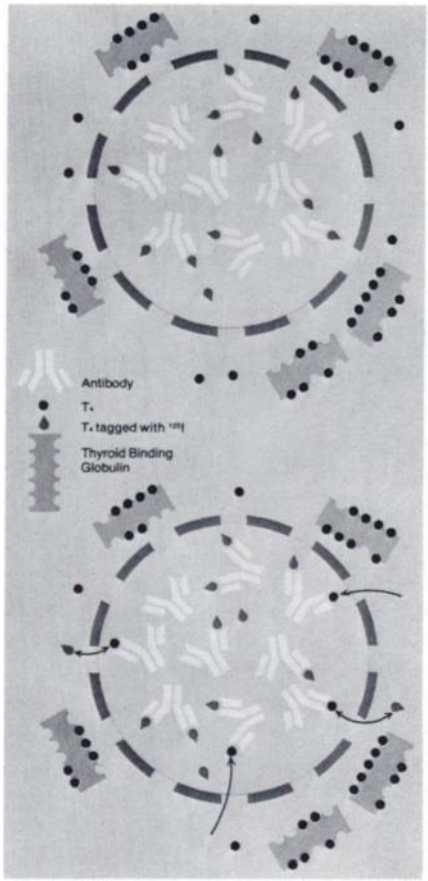

FIG. 2. Schema of free T_4 reaction in microcapsules. Top—conditions before reaction. Bottom—conditions during reaction.

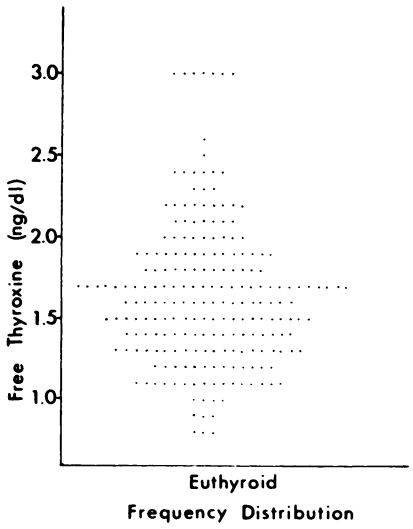

FIG. 4. Euthyroid frequency distribution; normal range 0.8–2.4 ng/dl.

TABLE 1. ASSAY PRECISION

	Mean FT_4 (ng/dl)	CV
Within assay		
Control 1	1.5	8.8%
Control 2	2.6	6.8%
Between assays		
Control 1	1.6	10.5%
Control 2	2.7	8.7%

rabbit anti-T_4 antiserum used in the FT_4 assay are listed in Table 2. The percentage cross-reactivity is defined as the amount of thyroxine required to displace 50% of the labeled thyroxine from antiserum, multiplied by 100 and divided by the amount of cross-reacting substance needed to obtain identical displacement.

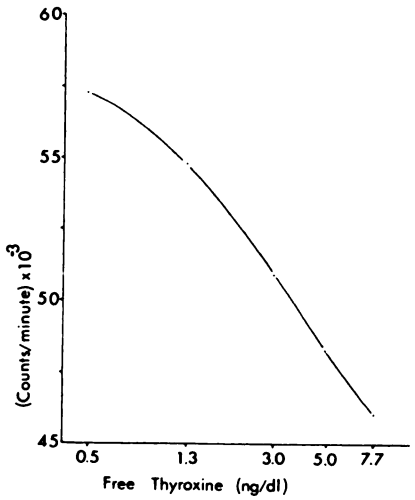

FIG. 3. Free T_4 standard curve.

Intermethod comparison *Correlation with equilibrium dialysis.* Two hundred patient samples were assayed by the present method and by an equilibrium dialysis procedure (see Methods section). The majority of these patients were initially diagnosed as clinically euthyroid. For these values, the coefficient of correlation (r) was 0.88 and the regression equation was: $y = 1.01 \times -0.03$. The mean FT_4 value for samples assayed by equilibrium dialysis was 1.65 ng/dl, while the mean FT_4 value for the present method was 1.60 ng/dl (Fig. 5).

Correlation with free thyroxine index. One hundred fifty patient samples were assayed by the present method and by total T_4 and T_3 resin uptake procedure (see Methods section). The majority of these patients were also initially diagnosed as clinically euthyroid. For these values the coefficient of correlation (r) was 0.91 and the regression equation was: $y = 1.05 \times +0.07$. The mean value of free T_4 index for samples assayed by free thyroxine index was 1.90, while the mean FT_4 value for the present

TABLE 2. CROSS-REACTIVITY

Compound	% Cross-reactivity
L-thyroxine	100.0
D-thyroxine	0.0
L-triiodothyronine	1.9
D-triiodothyronine	0.3
Monoiodotyrosine	0.0
Diiodotyrosine	0.015
Phentoin	0.0
Sodium salicylate	0.0
Aspirin	0.015

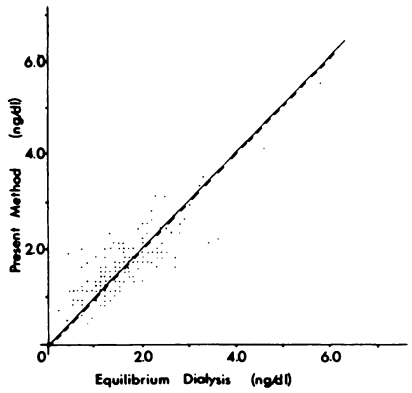

FIG. 5. Correlation between FT_4 by present method and by equilibrium dialysis (solid line = line of identity).

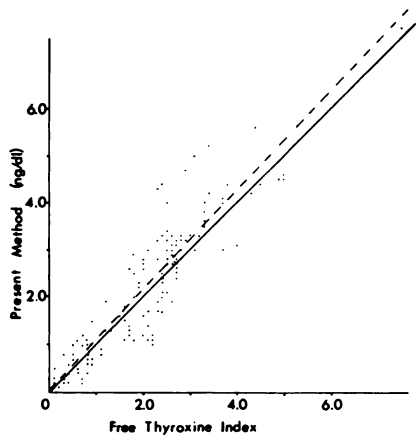

FIG. 6. Correlation between FT_4 by present method and Free Thyroxine Index (solid line = line of identity).

method was 2.05 ng/dl. Free thyroxine was calculated as the numerical product of total T_4 and T_3 resin uptake/100 (Fig. 6).

Clinical correlation. Total T_4 and FT_4 were determined for each of the patients in the four different groups studied. The results are listed in Table 3.

Total thyroxine. The mean TT_4 for the hyperthyroid group was 16.8 ± 3.8 (range 12.0-24.0) μg/dl. The mean TT_4 for the hypothyroid group was 2.53 ± 1.23 (range 1.0-4.8) μg/dl. The mean TT_4 in the pregnant group was elevated to 12.2 ± 3.6 (range 8.2-18.0) μg/dl. The mean TT_4 in the normal group was 7.9 ± 3.0 (range 5.0-11.0) μg/dl.

Free thyroxine. The mean FT_4 for the hyperthyroid group was 6.92 ± 1.38 (range 4.4-9.6) ng/dl. The mean FT_4 for the hypothyroid group was 0.43 ± 0.37 (range 0.1-1.3) ng/dl. The mean FT_4 in the pregnant group was 1.64 ± 0.37 (range 0.5-2.2) ng/dl. This is in agreement with the mean FT_4 value in healthy individuals of 1.64 ± 0.44 ng/dl.

DISCUSSION

Microencapsulation of labile biological material has been carried out for a number of years (*4*). This study has demonstrated our ability to microencapsulate anti-T_4 antiserum and develop a direct radioimmunoassay procedure for FT_4. The antiserum that has been encapsulated is physically trapped within a semipermeable nylon membrane.

The use of microencapsulated antiserum in radioimmunassays offers several advantages over previous techniques. The procedure functions in a liquid, three-dimensional state during the reaction between antigen and antibody. The antibody is not chemically bound to any surface as shown by 95% free radioactivity following rupture of the microcapsule. It is retained within the microcapsule due to the semipermeable nature of the nylon membrane. The separation of free from bound antigen takes place as in most solid-phase assays by simple centrifugation. The semipermeable nature of the

TABLE 3. CLINICAL CORRELATION*

	Euthyroid (N = 217)	Hyperthyroid (N = 16)	Hypothyroid (N = 35)	Pregnant (N = 30)
Total T_4 (μg/dl)	7.9 ± 3.0 (5.0-11.0)	16.8 ± 3.8 (12.0-24.0)	2.53 ± 1.23 (1.0-4.8)	12.2 ± 3.6 (8.2-18.0)
Free T_4 (ng/dl)	1.64 ± 0.44 (0.8-2.4)	6.92 ± 1.38 (4.4-9.6)	0.42 ±0.37 (0.1-1.3)	1.64 ± 0.37 (1.0-2.2)

* Mean ± 1 s.d. (and range) for each parameter measured in each of the four groups of subjects studied.

nylon membrane also serves to reduce interference of serum proteins with the antigen-antibody reaction.

Overall assay reaction time is significantly reduced. Free T_4 measurement by equilibrium dialysis takes between 16 and 24 hr for dialysis alone (*10*). Microencapsulation has reduced the total assay time to approximately 3 hr. The microcapsule, in and of itself, acts as a miniature dialysis system.

Of the available FT_4 assay methods, all others require indirect estimation. Some involve binding to Sephadex (*3*). One involves an estimation of FT_4 through the Free T_4 Index (*6*). The FT_4 Index is nothing more than a mathematical relationship between T_3 resin uptake and total T_4.

All of the previous methods rely on the total thyroxine value to estimate the FT_4 value in some way. The present method is able to measure FT_4 directly using only one test procedure.

Since thyroid hormones are continually being released and bound again to serum proteins such as thyroid-binding globulin (TBG), albumin, and prealbumin, there is a small but consistent portion that can be found at any one time in the free state. It has generally been accepted that circulating thyroid hormone that is not bound to serum protein reflects the biological activity more closely than the total amount of thyroid hormone in the blood (*8*).

Unfortunately, the free T_4 (FT_4) assay procedure has been tedious, complicated, and error-prone as a result of nonstandardization of material and reagents as well as the extreme difficulty of measuring less than 0.1% free hormone in the presence of, and in dynamic equilibrium with, overwhelming amounts of the same hormone loosely bound to protein (*3,6,9,10*). The aforementioned methods use indirect assay procedures and are usually dependent on a total T_4 assay value to obtain a FT_4 patient value.

In the assay described herein, antibody to thyroxine is microencapsulated within microscopic semipermeable nylon microcapsules. Microencapsulated antiserum is complexed with I-125-labeled T_4 of high specific activity. Addition of patient serum, heparinized plasma or FT_4 standards initiate a displacement reaction.

Free T_4 easily enters the microcapsules and displaces a proportional amount of I-125-labeled T_4 from the antiserum. The semipermeability of the microcapsule membrane is similar to that of the dialysis membrance, with two significant differences. The microcapsule membrane is thinner (70 mμ) and the surface area of the microcapsules in the reaction tube is greater than the surface area of the typical dialysis membrane. The effective diffusion rate is therefore about 400 times faster in the microcapsule system (*4*). This highly efficient minidialysis system has created a direct, faster, and technically easier method for measurement of FT_4.

Correlation of FT_4 values, as determined using the present method, with both the equilibrium dialysis procedure and the Free T_4 Index is very good. Most of these specimens were obtained from individuals who were euthyroid. The relatively small number of individuals who were hypothyroid or hyperthyroid did not present binding-protein abnormalities. It is in this area that one begins to see some inconsistencies in the Free Thyroxine Index. (*5*)

Radioimmunoassay procedures using microencapsulated anti-sera can effectively reduce assay time while maintaining the precision of previous procedures that were lengthy, tedious, and expensive.

FOOTNOTES

* Radioassay Systems Laboratories, Inc., Carson, CA.
† Cambridge Nuclear Corp., Billerica, MA.
‡ Pharmacia, Piscataway, NJ.
‖ Nuclear Medical Laboratories, Inc., Dallas, TX.

REFERENCES

1. ASHKAR FS, BUEHLER R, CHAN T: Free thyroxine (FT_4) radioassay with presaturated anti-T_4 microcapsules. *J Nucl Med* 19: 703, 1978 (abst)
2. BURGER A, SAKOLOFF C, STACHELI V, et al: Radioimmunoassays of 3,5,3'-triiodo-L-thyronine with and without a prior extraction step. *Acta Endocrinol* 80: 58-69, 1975
3. CAVALIERI RR, CASTLE JN, SEARLE GL: A simplified method for estimating free-thyroxine fraction in serum. *J Nucl Med* 10: 565-570, 1969
4. CHANG TMS: Encapsulation of enzymes cell contents, cells, vaccines, antigens, antiserum, cofactors, hormones and proteins. In *Biomedical Applications of Immobilized Enzymes and Proteins*, vol 1, Chang TMS, ed, New York, Plenum Press, 1977, pp 69-89
5. CUARON A, DECUARON CH: Tables to estimate total binding capacity of thyroxine-binding globulin from the in vitro thyroid function tests. *J Nucl Med* 20: 67-71, 1979
6. HOWORTH PJN, MACLAGAN NF: Clinical application of serum-total-thyroxine estimation, resin uptake, and free-thyroxine index. *Lancet* 1: 224-228, 1969
7. LARSEN PR, DOCKALOVA J, SIPULA D, et al: Immunoassay of thyroxine in unextracted human serum. *J Clin Endocrinol Metab* 37: 177-182, 1973
8. ROBBINS J, RALL JE: Proteins associated with thyroid hormones. *Physiol Rev* 40: 415-489, 1960
9. SCHUSSLER GC, PLAGER JE: Effect of preliminary purification of ^{131}I-thyroxine on the determination of free thyroxine in serum. *J Clin Endocrinol Metab* 27: 242-250, 1967
10. STERLING K, HEGEDUS A: Measurement of free thyroxine concentration in human serum. *J Clin Invest* 41: 1031-1040, 1962
11. STERLING K, BRENNER MA: Free thyroxine in human serum: Simplified measurement with the aid of magnesium precipitation. *J Clin Invest* 45: 153-163, 1966