Serum Triiodothyronine: Measurements in Human Serum by Radioimmunoassay with Corroboration by Gas-Liquid Chromatography

TERUNORI MITSUMA, NORIYUKI NIHEI, MARVIN C. GERSHENGORN, and CHARLES S. HOLLANDER

From the Endocrine Division, Department of Medicine, New York University School of Medicine, New York 10016 and the Endocrine Unit, Department of Medicine, University of Rochester, School of Medicine and Dentistry at Rochester General Hospital and Strong Memorial Hospital, Rochester, New York 14620

ABSTRACT Serum triiodothyronine (T_3) has been measured by radioimmunoassay and corroborated by analysis of the identical samples with a previously described gas-liquid chromatographic technique. Special features of the radioimmunoassay procedure which permit determinations in unextracted serum include the use of a T_3-free serum preparation for the construction of the standard curve and of tetrachlorothyronine to inhibit binding of T_3 to thyroxine-binding globulin.

T_3 values by radioimmunoassay were 138 ±23 ng/100 ml (mean ±SD) in 82 normal subjects, 62 ±9 ng/100 ml in 45 hypothyroid patients, and 494 ±265 ng/100 ml in 60 patients with toxic diffuse goiter. In the hypothyroid group, the range was similar in patients with both primary and secondary hypothyroidism. There was no overlap between the three thyroidal states. Elevated T_3 levels were seen in 40 cases that appeared clinically hyperthyroid but had normal serum thyroxine (T_4) determinations, a syndrome we have called T_3 toxicosis. Values obtained with radioimmunoassay agreed closely with those we had previously found by gas-liquid chromatography which were 68 ±2 ng/100 ml in hypothyroidism, 137 ±23 ng/100 ml in normal subjects, and 510 ±131 ng/100 ml in untreated toxic diffuse goiter.

Since T_3 is very potent and its level varies in different clinical states, accurate T_3 measurements are required to assess a patient's thyroid status properly. The radioimmunoassay for T_3 appears to be sufficiently sensitive, precise, and simple to permit its routine clinical application for this purpose.

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INTRODUCTION

L-3,5,3′-Triiodothyronine (T_3)[1] discovered in 1952 (1), has been shown to have a higher metabolic potency, a more rapid turnover, and a larger volume of distribution in the body than thyroxine (T_4) (2). Therefore, despite its low concentration in serum relative to T_4, T_3 apparently contributes a substantial proportion of the metabolic activity of the thyroid hormones (2). It was reported shortly after the discovery of T_3 that T_4 might exert its effect at the tissue level predominantly via conversion to T_3 (3). The suggestion that there is a peripheral conversion of T_4 to T_3 has recently been restated (4–6). Hollander called attention to a hyperthyroid state in which an elevated T_3 concentration appears to be the major pathogenic factor (7, 8), a syndrome he named T_3 toxicosis. Subsequently another group has reported on a similar group of patients (9).

These findings emphasize the physiologic and clinical significance of the hormone and the desirability for an accurate method for its measurement in serum suitable for clinical use. At present, there is little consensus with regard to the actual concentrations of T_3 in serum. Recent estimates of serum T_3 levels in normal subjects by a number of workers using a variety of techniques (7, 10–22) have varied over a ninefold range. The excellent agreement found between the measurements by the radioimmunoassay technique reported here and those

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; LATS, long-acting thyroid stimulator; PBI, protein-bound iodine; T_3, serum triiodothyronine; T_4, thyroxine; TBG, thyroxine-binding globulin; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.

of gas chromatography (10) serve to corroborate the validity of these two entirely independent methods. The inherent simplicity and rapidity of the radioimmunoassay procedure renders it most suitable for routine clinical use for the measurement of serum T_3.

METHODS

The radioimmunoassay method for the measurement of T_3 has two unique features which permit measurements in unextracted serum: (*a*) the use of tetrachlorothyronine, a known inhibitor of T_4 binding to thyroxine-binding globulin (TBG) (23) to inhibit the binding of T_3-^{125}I to TBG and displace endogenous T_3 from TBG; (*b*) the standard curves have been constructed utilizing a T_3-free serum as diluent.

Detailed procedure

Production of antiserum to T_3. T_3 immunogen was prepared by two methods from a purified preparation. T_3 was obtained as the free acid (Sigma Chemical Co, St. Louis, Mo.) and purified by five consecutive paper chromatographic separations in a t-amyl alcohol: hexane: ammonia system (18) to a constant specific activity as previously described (10).

The first procedure, a modification of the method of Brown, Ekins, Ellis, and Reith (24), involved three steps and resulted in the formation of a T_3-poly-L-lysine conjugate. (*a*) Preparation of methyl T_3: chromographically purified T_3 was methylated as previously described (10). (*b*) Preparation of succinylated poly-L-lysine: poly-L-lysine (Sigma Chemical Co.) was reacted with succinic anhydride (Eastman Organic Chemical Corp., Rochester, N. Y.) according to the method of Haber, Page, and Jacoby (25). (*c*) Coupling of the methyl-ester of T_3 to succinylated poly-L-lysine: using the method of Brown et al. (24), the succinylated product was condensed with the methyl-ester of T_3 by coupling with carbodiimide (Sigma Chemical Co.).

The second method, a modification of the procedures of Oliver, Parker, Brasfield, and Parker (26) and Chopra, Nelson, Solomon, and Beall (27), consisted of the direct coupling of T_3 to bovine serum albumin to produce a T_3-bovine serum albumin conjugate. 50 mg of chromatographically purified T_3 was dissolved in 2.5 ml of 0.15 N NaOH and added with stirring into a solution of 100 mg bovine serum albumin (BSA; Sigma Chemical Co.) dissolved in 5 ml of distilled, deionized water. 300 mg of carbodiimide was then added with continued stirring and the pH of the reaction adjusted to 9.0 with hydrochloric acid. The mixture was incubated at 4°C overnight and dialyzed for 5 days against 6 liters of distilled, deionized water which was changed daily.

The resulting immunogens, emulsified in complete Freund's adjuvant (Difco Labs, Detroit, Mich.) (0.75 mg/0.2 ml) were injected into the toe-pads of the rabbits. This amount of immunogen was administered at the same site at 4, 6, 8, and 9 wk after the initial injection and blood for antiserum was drawn 1 wk after the last dose and every 2 wk thereafter. 50 adult 2700 g female rabbits of the New Zealand white strain (Zartman Farms, Douglasville, Pa.) were immunized with the T_3-poly-L-lysine conjugate, and five with the T_3-BSA conjugate.

Detection of antibody. T_3-^{125}I (Abbott Laboratories) was chromatographically purified (see above) before use. Barbital buffer, 0.08 M, containing 1 g/liter of bovine serum albumin to prevent adsorption to glassware (barbital diluent) was used for dilution of the antisera, radioactive T_3 (T_3-^{125}I), and subsequently for test samples (see below).

Rabbit serum in dilutions of 1:10, 1:50, 1:100, and 1:500 was screened for the presence of antibody to T_3 in the following manner. 30 pg of T_3-^{125}I (SA 70–90 μCi/μg; Abbott Laboratories, North Chicago, Ill.) was added to 0.1 ml of the diluted test antiserum. The mixture was incubated at 4°C for 24 hr. After incubation, 0.2 ml of a slurry of activated charcoal and methyl cellulose in barbital diluent prepared as previously described (24, 28) was added, the mixture reincubated for 5 min, centrifuged at approximately 675 *g* for 15 min, the supernatant decanted, and the bound-to-free ratio determined as described below in the section on preparation of standard curves. A B/F ratio in excess of 2 at the lower dilutions (1:10, 1:50), which indicated the presence of potentially usable antibody, was found in 10 of 50 animals immunized with the poly-L-lysine conjugate. Subsequently, antisera to the T_3-BSA conjugate were analyzed and potentially usable antibody was found in all five animals. Four antisera which had the best antibody titers to T_3 were employed for the T_3 radioimmunoassays described in this report; two were harvested at 12 wk from an animal immunized with the T_3-poly-L-lysine conjugate; two others were collected at 9 wk from a rabbit which had received the T_3-BSA conjugate.

Inhibition of serum protein-binding interference. In order to inhibit the binding of the T_3-^{125}I to TBG and displace endogenous T_3 from TBG 3,5,3′,5′-D,L-tetrachlorothyronine, a known inhibitor of T_4 binding to TBG (23) was added to the reaction mixture. It was used in the preparation of T_3-free serum, in the construction of the standard curve and in the analysis of the unknown serum samples. The precise concentration of tetrachlorothyronine used in each instance was empirically determined to be the smallest amount which uniformly yielded a quantitative recovery of added T_3 (see below). The reagent, a gift of Dr. Robert Meltzer of the Warner Lambert Research Institute, was stored in the dry form at 4°C and a stock solution (1 mg/ml) prepared on the day of use by dissolving tetrachlorothyronine 0.13 N NaOH in 70% ethanol.

Preparation of T_3-free serum. To obviate potential differences between standard curves constructed in buffer and in serum, a serum with a very low T_3 content, prepared in the following manner, was used as the diluent for the standard curve. Triosorb sponges (Abbott Laboratories) added directly to serum are known to absorb approximately 30% of the T_3 present (29). The addition of tetrachlorothyronine was found to increase this uptake to 74–76% per sponging. In practice, 40 μg of tetrachlorothyronine (0.1 ml) and a tracer quantity of T_3-^{131}I (30 pg) were added to each 1 ml of hypothyroid serum. A triosorb sponge was then immersed in this mixture and incubated for 1 hr at 25°C. The sponge was then squeezed in the bottom of the tube with a plastic plunger and discarded. The process was repeated three times. The volume of serum recovered was approximately 0.6 ml for each milliliter of original serum and contained less than 4% of the original T_3 (less than 4 ng/100 ml).

Preparation of standard curves. Nonradioactive T_3 was purified chromatographically (see above) and added to T_3-free serum to yield T_3 concentrations of 12.5, 25, 50, 100, 200, and 400 pg/0.025 ml of serum. $\frac{1}{10}$ ml of these standards was added to an assay mixture which consisted of 0.1 ml of antiserum in a 1:100 dilution, 0.1 ml or 30 pg of T_3-^{125}I diluted in barbital buffer, 0.1 ml or 0.2 μg of tetrachloro-

thyronine diluted in barbital buffer and incubated at 4°C for 24 hr. After the incubation, 0.2 ml of a slurry of activated charcoal and methyl cellulose in barbital diluent prepared as previously described (24, 28) was added, the mixture reincubated for 5 min, centrifuged at 675 *g* for 15 min, and the supernatant decanted. The bound T_3 in the supernatant (B) and the free T_3 adsorbed in the charcoal (F) were each counted in a gamma counting spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.) and the B/F ratio calculated from the number of counts in each fraction. The standard curves were constructed by plotting the B/F ratio vs. the quantity of T_3 in the incubation mixture. Each plotted point represents the mean of quadruplicate determinations.

A similar curve was constructed in barbital buffer, i. e. in the absence of T_3-free serum, for T_3, T_4, and tetrachlorothyronine. T_3 and T_4 were obtained as the free acids (Sigma Chemical Co.), T_3 was purified as described above. T_4 was also further purified. A tracer quantity of T_4-^{131}I (Abbott Laboratories) was added at the outset (10,000 dpm/ng of T_4). The resultant mixture was purified by column chromatography on Sephadex LH 20 (30) followed by five successive paper chromatographic separations in a t-amyl alcohol system (18) until a constant specific activity had been achieved. Gas chromatographic analysis (10) of the purified T_4 preparation indicated that its T_3 content was less than 0.01%.

A series of curves was also determined for L-monoiodothyronine, L-3,5-diiodothyronine, L-monoiodotyrosine, L-diiodotyrosine, triiodothyroacetic acid, and poly-L-lysine. All of these were obtained from the Sigma Chemical Co. and had been purified chromatographically by the manufacturer.

Analysis of unknown sera. Mixtures of unknown samples were prepared in an identical manner and at the same time as were the standard solutions, except that 0.1 ml of serum, which had been diluted 1:4 in barbital buffer, was substituted for the test standard at the outset of the procedure so that 0.025 ml of the original serum was actually assayed. The T_3 concentration in the serum sample was determined by comparing the B/F ratio obtained with it with the standard curve. All measurements were the means of quadruplicate determinations.

Clinical material. Normal subjects were clinically euthyroid, had no palpable enlargement of the thyroid gland, and had normal levels of protein-bound iodine (PBI; Bioscience Labs., Van Nuys, Calif.) or T_4 by competitive binding analysis (Boston Medical Labs., Boston, Mass.) and had a normal free T_4 determination (Bioscience Labs.).

Untreated hyperthyroid patients were clinically toxic, had an elevated PBI or T_4, a high free T_4, and an abnormally increased thyroidal uptake of radioiodine. 60 subjects had toxic diffuse goiter, i.e., a diffusely enlarged thyroid gland with no nodules on palpation and a diffuse uptake of radioiodine on scan. Four subjects had toxic multinodular goiter. In these cases the thyroidal uptake of radioiodine was localized to one or more discrete nodules. Patients considered to have T_3 toxicosis on the basis of their clinical presentation and an elevation in the serum T_3 level (7, 8) will be more fully described in the results section.

Patients with treated hyperthyroidism had received radioiodine, undergone surgery, or were taking antithyroid drugs. They were adjudged to be clinically euthyroid and had normal determinations of the PBI and T_4 by the Murphy-Pattee method, free T_4, and a normal uptake of radioiodine.

Patients with hypothyroidism were clinically hypothyroid and had abnormally low PBI or T_4, free T_4, and radioactive iodine determinations. Patients with primary hypothyroidism had an elevated immunoassayable thyroid-stimulating hormone (TSH) level (31), defined as greater than 10 μU/ml, and had no increase in the thyroidal uptake of radioiodine in response to five doses of 10 U of TSH every 12 hr. Those with hypopituitarism had definite clinical and laboratory evidence of failure of at least one other target organ, no immunoassayable TSH, and doubling of the thyroidal uptake of radioiodine in response to administration of exogenous TSH.

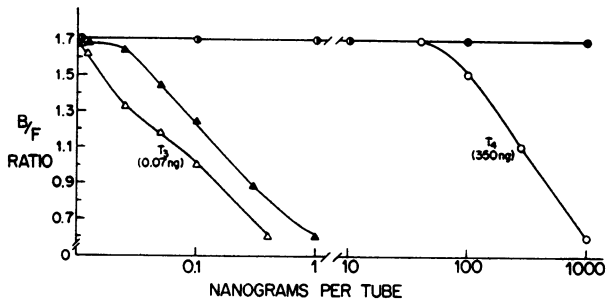

FIGURE 1 Cross-reaction of T_3 antibody with other thyroid analogues. The T_3 standard curve in buffer (open triangles) shows a sensitivity to T_3 in the picogram range. Note the breaks on both the vertical axis which is plotted on an arithmetic scale and in the horizontal axis which is on a logarithmic scale. The extent of cross-reaction between the T_3 antibody and a chromatographically purified T_4 preparation (open circles) was determined by comparing the concentration of nonradioactive T_3 and T_4 at the point at which binding of T_3-^{125}I was reduced by 50% from that found when no unlabeled T_3 was added. Cross-reaction was less than 1:5000 or less than a 1:4185 molar ratio. There was significant cross-reaction of the T_3 antibody to triiodothyroacetic acid (closed triangles) but none with tetrachlorothyronine (solid circles) or a variety of other related compounds (see text).

RESULTS

Methodological results

Cross-reaction with T_4 and other compounds. The extent of cross-reaction between a T_4 preparation, which had been purified chromatographically as described above, and the T_3 antibody was determined by preparing standard curves for each hormone in a barbital diluent and comparing the concentration of nonradioactive T_3 and T_4 at the point at which binding of T_3-^{125}I was reduced by 50% from that found when no unlabeled T_3 was added. Cross-reaction was less than 1:5000, or less than 1.4185 molar ratio (Fig. 1). There was significant cross-reaction of the T_3 antibody to triiodothyroacetic acid, a substance not normally present in the serum but none was found with tetrachlorothyronine (Fig. 1). No measurable cross-reaction was detected with monoiodotyrosine, diiodotyrosine, monoiodothyronine, diiodothyronine, and poly-L-lysine.

Serum standard curves. Standard curves, constructed in T_3-free serum using two different antisera, were vir-

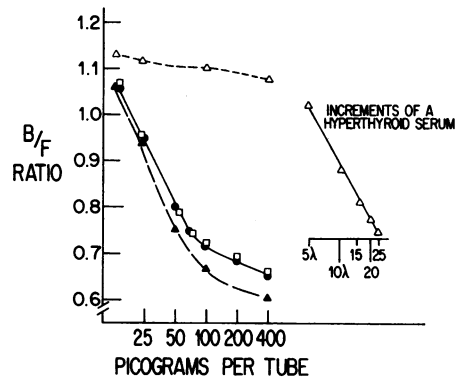

FIGURE 2 T_3 standard curve in T_3-free serum. When increments of T_3 were added to T_3-free serum no change in the B/F ratio was found in the absence of tetrachlorothyronine (dashed line; open triangles). In the presence of tetrachlorothyronine (solid line) identical curves were obtained with antibody A which was formed to the T_3 poly-L-lysine conjugate (closed circles) and antibody B which was produced in response to injection with the T_3 bovine serum albumin conjugate (open squares). Standard curves run with antibodies A and B in T_3-free serum were identical with those depicted when the curves were rerun in the presence of 7.5 ng of purified T_4 per reaction tube. The addition of increments of T_3 to buffer (solid triangles, dashed lines) produced a curve similar but not identical with that obtained in T_3-free serum.

tually identical (Fig. 2). A curve constructed with dilutions of a hyperthyroid serum in place of exogenous T_3 yielded a parallel curve (Fig. 2). Similarly parallel curves were found when dilutions of normal and hypothyroid serum were used. This indicates that alterations in the T_3 level per se are responsible for the changes observed in the B/F ratio. It was also demonstrated that T_4 when present in the concentration of 30 μg/100 ml (7.5 ng/assay tube) did not alter the curve. A standard curve constructed by the addition of exogenous T_3 to barbital diluent was similar but not identical with that obtained by addition of T_3-free serum.

In the absence of tetrachlorothyronine, almost no change was noted in the B/F ratio as the concentration of T_3 increased in the serum assay mixture (Fig. 2). Pilot experiments showed that the addition of tetrachlorothyronine caused a measurable change in the B/F ratio with increments of T_3. A change in the B/F ratio was seen with T_3 additions when these were performed in the presence of 0.05 μg of tetrachlorothyronine per tube, and became more marked at 0.1 μg. No further changes were found in the B/F ratio with additions of tetrachlorothyronine above 0.1 μg/tube. Therefore, it was decided to routinely add $\frac{2}{10}$ of a μg of tetrachlorothyronine per assay tube. This resulted in a change in the B/F ratio sufficient to permit quantitation of 12.5 pg of T_3. This lower limit of detectability (see below) corresponds to a serum concentration of 50 ng/100 ml when the assay is performed with 0.1 ml of serum (0.025 ml of serum per assay tube). Since some hypothyroid sera contain as little as 40–50 ng of T_3/100 ml of serum, samples were requantitated using four times the usual amount of serum (and 0.8 μg of tetrachlorothyronine per tube). Because of the additional total protein and TBG present, a separate serum standard was run for these samples with 0.1 ml of T_3-free serum and 0.8 μg of tetrachlorothyronine per assay tube.

The most sensitive portion of the curve i.e., where the slope is greatest, fell between 12.5 and 100 pg of T_3 per tube, which corresponds to a serum concentration of up to 400 ng/100 ml. The hyperthyroid samples which contain concentrations of T_3 greater than 400 ng/100 ml were remeasured after they had been diluted with 1:4 in T_3-free serum, so that they also fell on this most sensitive portion. In rare samples with concentrations of T_3 above 1600 ng/100 ml, further dilution in T_3-free serum was employed before measurement.

Effect of added T_4. In view of the very minor cross-reaction between the T_3-antibody and T_4, no significant effect on immunoassayable T_3 would be anticipated from clinically observed variations in the T_4 concentration. This was verified by remeasuring the T_3 concentrations of a hypothyroid serum, which initially contained 60 ng/100 ml of T_3 and less than 0.2 μg/100 ml of T_4 after adding 0.4, 2, 10, 20, and 40 μg/100 ml of T_4 in vitro. A similar series of determinations was performed on a T_3-free serum sample, devoid of measurable T_4 (i.e., less than 0.2 μg/100 ml of T_4) to which 200 ng/100 ml of T_3 had been added. In both cases, no change in the measured T_3 concentration was observed with additions of T_4.

T_3 recovery experiments. T_3 determinations were performed on mixtures prepared by the addition of known amounts of T_3 to hypothyroid and T_3-free serum. In seven experiments using T_3-free serum, recoveries were 100 and 108% for 25 pg of T_3, 100% for 50 pg, 95 and 110% for 100 pg, and 96 and 100% for 150 pg. In 14 experiments with normal serum, recoveries were 94 and 104% for 25 pg of T_3, 104 and 111% for 37.5 pg of T_3, 89, 96, 103, and 106% for 50 pg of T_3, 95 and 104% for 75 pg of T_3, 102 and 111% for 100 pg of T_3, and 109 and 109% for 175 pg of T_3. The mean recovery of T_3 in all 21 experiments was 102.2%.

Limits of detectability, precision, and reproducibility of the method. The threshold or lower limit of detectability of the radioimmunoassay was defined as the smallest amount of T_3 which, when added to T_3-free serum, produced a significant change in the observed B/F ratio. To determine this, the standard curve was constructed five times. In each instance, a significant change in the B/F ratio was obtained when 12.5 pg of

TABLE I

Serum T_3 Concentrations in Various Thyroidal States

	Subjects	Mean	Standard deviation	Range	By GLC (10)
	n		*ng/100 ml*		*ng/100 ml*
Normal subjects					
Male	47	142	22	96–168	
Female	35	133	25	100–172	
Combined	82	138	23	96–172	137 ±23
Hypothyroidism					
Primary	45	62	9	44–80	
Secondary	4	57	6	50–69	
Combined	49	59	9	44–80	68 ±12
Hyperthyroidism					
Toxic diffuse Goiter	60	494	265	248–1700	510 ±131
Toxic nodular Goiter	4*	—	—	232–1225	
Euthyroid patients with treated hyperthyroidism:					
after radioiodine	66	135	30	90–200	
after surgery	16	113	20	90–168	
during antithyroid drug administration	19	130	20	100–176	
T_3 toxicosis	40	712	416	228–2000	

* Group is too small and widely spread for meaningful statistical analysis (see text).

T_3 was added/assay tube. The B/F ratio ±SD was 1.15 ±0.02 for the zero point and 1.05 ±0.01 for 12.5 pg of added T_3. This corresponds to a serum T_3 concentration of 50 ng/100 ml which is the lower limit of detectability when 0.025 ml is assayed. For hypothyroid sera, a volume of 0.1 ml is analyzed so that the detectability of the assay is equivalent to a concentration of 12.5 ng/100 ml, a level well below that measured in any hypothyroid sample thus far analyzed. Intraassay precision was repeatedly determined in samples with low, normal, or high T_3 concentrations. The coefficient of variation for 21 samples analyzed in quadruplicate in the same assay was 3.8 ±2.2% (SD). The mean coefficient of variation of 16 samples assayed two to five times in different assays, interassay reproducibility, was 6.1 ±3.0% (SD).

Comparison with measurements by gas chromatography. A gas-liquid chromatographic method (GLC) for the measurement of T_3 in serum has been developed by our group and has been previously described in detail (10). This technique consists of five fundamental steps: (*a*) extraction of T_3 from serum by passage through a cation exchange resin column; (*b*) preparation of a stable, volatile derivative of T_3; (*c*) purification of the derivative with an anion-exchange resin column; (*d*) gas chromatographic separation and quantitation utilizing a nickel-63 electron capture detector, and (*e*) correction for methodological conversion of T_4 and T_3.

28 sera were measured by radioimmunoassay and GLC and the results compared in Fig. 3. The coefficient of the correlation for these measurements equaled 0.99.

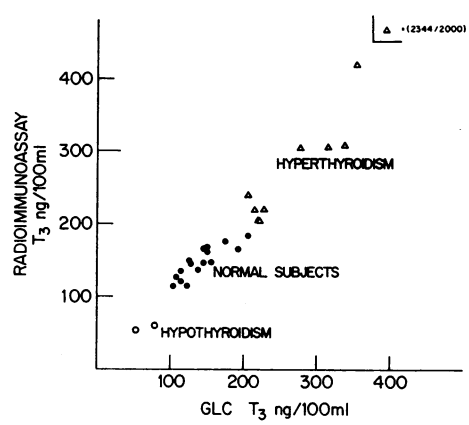

FIGURE 3 Comparison of T_3 measurements by radioimmunoassay and GLC. The coefficient of correlation of the determinations was 0.99. The measurements were performed in hypothyroidism (open circles), hyperthyroidism (open triangles), and in normal subjects (closed circles).

The majority of measurements agreed within 10% and in no instance did the two determinations differ by more than 25%.

Clinical results. In 82 normal subjects, ranging in age from 20 to 60 yr, T_3 ranged from 96 to 172 ng/100 ml with a mean of 138 ng/100 ml and a standard deviation of 23 ng/100 ml. Normal male and female subjects had comparable levels of circulating T_3 and for this reason measurements in both sexes have been combined into a single range (Table I). We have previously obtained a similar normal value, 137 ±23 ng/100 ml by gas-liquid chromatography (10) (Table I).

45 patients with primary hypothyroidism had a mean (±SD) serum T_3 value of 62 ±9 ng/100 ml. This compares with the value of 68 ±12 ng/100 ml found with GLC (10) (Table I). T_3 values in the hypothyroid group were about one-half that found in the normal subjects with no overlap between the two groups. In four cases of hypothyroidism secondary to hypopituitarism, the mean serum T_3 was 57 ng/100 ml, slightly, but not statistically lower than in the primary group.

The mean T_3 value in toxic diffuse goiter was 494 ng/100 ml and again there was no overlap with the normal group. The values had a somewhat wider range varying from 248 to 1700 ng/100 ml with a distribution skewed toward the lower numbers. This compares with a value of 510 ±131 ng/100 ml by GLC. One of a small group of four patients with toxic nodular goiter had a serum T_3 of 1225 ng/100 ml but the other three had only modestly elevated values (232, 296, and 280 ng/100 ml).

The mean serum T_3 was normal in patients with treated toxic diffuse goiter after they became euthyroid. It was 131 ng/100 ml in 60 patients who had been treated with radioiodine, 112 ng/100 ml in 11 patients after surgery, and 132 ng/100 ml in 21 patients after euthyroidism had been achieved during the course of antithyroid drug administration.

Of particular interest were the T_3 values in a third group of 40 patients with a syndrome which we have called T_3 toxicosis (7, 8). These patients, the subjects of a detailed clinical report now in preparation, all had definite clinical evidence of hyperthyroidism but repeatedly normal measurements of total T_4 (PBI and T_4 by column or T_4 by competitive binding analysis) and normal thyroxine-binding globulin capacities. The mean serum T_3 in this group of patients was somewhat higher than that in the conventional form of hyperthyroidism, 712 ng/100 ml vs. 494 ng/100 ml, with a very wide range from 228 to greater than 2000 ng/100 ml (Table I). Six of the patients with this variant of hyperthyroidism had localized accumulation of radioiodine in their glands in addition to a clinically palpable nodule; 34 had diffuse enlargement of their thyroid gland; 6 of 12 tested had measurable long-acting thyroid stimulator (LATS) and all 21 of those tested failed to show a decrease in thyroidal uptake of radioiodine after exogenous T_3.

DISCUSSION

Methodological variables. In order to validly apply the radioimmunoassay method to measurements of small amounts of T_3 present in unextracted serum samples, four potential sources of error were considered: (*a*) T_3 antibody may cross-react with T_4 present in the sample and, therefore, yield a spuriously elevated value for T_3. This was shown to be unimportant in this system since the T_3 antibody-T_4 interaction was less than 1: 5000th that of the antibody's reaction with T_3. (*b*) Competition for T_3 by serum proteins which have a strong affinity for T_3, especially TBG, would interfere with the binding of T_3 to antibody and lead to variations in the B/F ratio depending on their relative binding affinities. Binding of T_3 to TBG was inhibited using 3,5,3′,5′-D,L-tetrachlorothyronine, the chlorinated analogue of T_4, which had been shown by Wolff, Standaert, and Rall to competitively inhibit T_4 binding to TBG (23). (*c*) Serum proteins are known to interfere with antigen-antibody reactions (32) so that even if serum binding of T_3 is inhibited, a standard curve constructed in serum might differ from that obtained in the buffer and such a difference was actually observed in this study (Fig. 2). This problem was solved by using, as a diluent, serum to which increasing amounts of T_3 were added to construct the standard curves. (*d*) Serum from even the most hypothyroid subject contains some endogenous T_3. Measurements by the immunoassay method using a hypothyroid serum to construct a standard curve would be limited to those samples containing more T_3 than in the serum diluent and even in these instances the estimated value would be diminished by the T_3 content of the diluent. Therefore, a serum preparation, which contained less than 4 ng of T_3/100 ml, was prepared with triosorb sponges and used as the serum diluent. By using a T_3-free serum as diluent, it was possible to develop an assay sufficiently sensitive to permit T_3 quantitation and differentiation of normal, hypothyroid, and hyperthyroid subjects.

When performed in this fashion, the radioimmunoassay procedure is an accurate measure of the concentration of T_3 in the serum. This was documented in detail. (*a*) There was virtually quantitative recovery of exogenous T_3 added to normal or T_3-free serum (*b*) Standard curves constructed by the enrichment of serum with exogenous T_3 and by the addition of increments of normal, hypothyroid, and hyperthyroid sera were virtually identical (*c*) The addition of purified T_4 in the physiologic range failed to alter T_3 measurements. The results document the absence of significant cross-reaction

with T_4 in the presence of serum and indicate that no measurable conversion of T_4 to T_3 occurs during the process of incubation or charcoal separation of the bound and free hormone fractions. (*d*) Corroboration of the radioimmunoassayable T_3 with measurements by gas-liquid chromatography was made. In most instances, determinations by the two methods differed by less than 10% and in no case by more than 25%. The coefficient of the correlation between the measurements in the two assays equals 0.99. (*e*) Finally, specificity, satisfactory reproducibility (interassay), and precision (intraassay) were documented.

Published determinations of the level of T_3 in normal human serum have varied widely (7, 8, 10–22) and the actual level has not been agreed upon. Our earlier estimates by gas-liquid chromatography (7) gave a falsely high value, because we failed to correct for methodological conversion of T_4 to T_3, i.e., deiodination of approximately 1.7% of endogenous T_4 that occurs during the GLC procedure. With correction we now obtain values with gas-liquid chromatography which agree well with those reported here with radioimmunoassay (10).

Two groups (17, 18) have published measurements of serum T_3 in which they employed a competitive binding protein analysis method as the final quantitative step in their procedure. The values are significantly higher than those we have obtained. However, we have analyzed the extraction procedures and chromatography system used in these methods by gas chromatography and radioimmunoassay and have demonstrated that there is contamination of the T_3 area on the paper chromatogram with 0.25 and 0.50% of the endogenous T_4 in serum, as well as conversion (deiodination) of 0.20 and 0.25% of the endogenous T_4 to T_3 during the extraction and separation procedures. These in vitro artifacts constitute a negligible proportion of the endogenous T_4 but can cause an error of 50–100% or more in the measured T_3 levels. We find that with one-dimensional paper chromatography, utilizing the t-amyl: alcohol: ammonia, t-amyl alcohol: hexane: ammonia, and butanol-ethanol: ammonia systems, T_4 contamination in the T_3 area can be minimized only by placing the strips in the chromatographic tank after the paper has thoroughly dried, but in confirmation of Taurog, we also find that this results in increased conversion of T_4 to T_3 during the chromatography (33).

Three other groups have also noted these artifacts in the competitive protein-binding method for measuring T_3 serum. Employing different techniques which eliminate T_4 contamination and correct for conversion of T_4 to T_3 these workers have obtained serum T_3 measurements which agree with those reported here (6, 12, 13, 34, 35). Sterling, Bellabarba, Newman, and Brenner (18) and Hagen, Diuquid, Kliman, and Stanbury (20) have obtained values with double isotope derivative assays higher than those reported here but their methods also employ a preliminary extraction and paper chromatographic separation and would give falsely elevated values if either conversion or contamination occurred.

Measurements of T_3 in serum by radioimmunoassay have also proven subject to a variety of artifacts. The authors are aware of four other groups who have developed an immunoassay for T_3. (*a*) Ekins, Brown, Ellis, and Reith initially failed to detect T_3 in serum (36) but later, with a modified technique (19), made determinations by immunoassay after an initial extraction and separation procedure and obtained a mean value of about 120 ng/100 ml for normal subjects. (*b*) Chopra (15) obtained a value slightly lower than we have reported. Conceivably this is due to regional or dietary factors which alter serum T_3 levels. They used hypothyroid sheep serum for construction of the standard curves to compensate for the TBG effects in serum. They were able to measure T_3 in only 20% of the normals and in none of the hypothyroid subjects they studied, probably due to insufficient sensitivity of their antibody. (*c*) Lieblich and Utiger employed Dilantin to block the binding of T_3-^{125}I to TBG and displace endogenous T_3 from TBG (21). Their measurements agree closely with those we have obtained in normal and hyperthyroid subjects but are slightly higher in hypothyroid patients. (*d*) Gharib, Mayberry, Hockert, and Ryan, in a preliminary communication, reported values for serum T_3 in hypothyroidism similar to those we have reported but their measurements of serum T_3 in normal and hyperthyroid subjects appear to be higher than the values we have obtained (14, 37). The reason their results differ from the three other published radioimmunoassays is uncertain. Possibly the discrepancy results from residual binding of T_3 to TBG, which would erroneously elevate the measurements in their double antibody assay.

The physiological role of T_3. There are a number of clinical situations in which available evidence is compatable with a major physiological role for triiodothyronine: (*a*) T_3 toxicosis, i.e., those cases of clinical hyperthyroidism in which thyroxine levels and thyroxine-binding globulin capacities are normal and the patients have an abnormally elevated serum triiodothyronine (7, 8). The precise clinical incidence of this entity remains to be established, but we have found 5 patients on a single clinical service over a period of 1 yr, during which time 91 patients with the more usual form of thyrotoxicosis were seen. This suggests to us that T_3 toxicosis may not be as rare as we initially thought. (*b*) We have recently observed four patients in whom an elevated serum T_3 was an early premonitory laboratory finding preceding a demonstrable rise in the serum T_4 and who 1, 3, 5, and 10 months later developed classic

diffuse goiter (*37a*). (*c*) Iodine deprivation in experimental animals is also known to be associated with high serum T_3 levels (38, 39). More recently, in association with Drs. P. Malvaux and C. Beckers, we have found that normal subjects placed on a diet of less than 50 μg of iodide per day had a high serum T_3 (239 ng/100 ml vs. a normal of 139 ng/100 ml) and a low serum T_4 suggesting that relative hypersecretion of T_3 may represent an important homeostatic mechanism in the face of inadequate iodide substrate in man (40). (*d*) Radechevich and Werner (41) noted that T_3 was elevated in many patients with "hot nodules," i.e., patients having a localized accumulation of radioiodine in one area of their thyroid glands. We have found elevated serum T_3 levels in 40% of patients with toxic adenomata as defined by Ingbar and Woeber (42). Most of our patients with toxic adenomata did not have overt disease but had signs and symptoms compatible with mild thyrotoxicosis. (*e*) The serum T_3 values in toxic diffuse goiter vary over a very wide range and were markedly skewed toward the lower portion of that range. We have previously found using GLC for the measurement of serum T_3 and T_4 that abnormally high T_3/T_4 ratios appear to correlate with the presence of long-acting thyroid stimulator (LATS) in the serum of untreated patients with toxic diffuse goiter. More recently we have observed with the immunoassay technique that T_3 levels in euthyroid patients with treated toxic diffuse goiter are higher in those who have measurable LATS in their blood (43). (*f*) Sterling and coworkers have noted the maintenance of normal clinical status in patients (44) with normal or elevated T_3 but low serum T_4 concentration after ^{131}I therapy for thyrotoxicosis. (*g*) Bowers, Schally, Hawley, Gual, and Parlow (45), Fleischer, Burgus, Vale, Dunn, and Guillemin (46) and many workers have recently demonstrated that intravenous administration of thyrotropin-releasing hormone (TRH) in man is followed by a prompt rise in circulating TSH. Studies in our laboratory with intravenous administration of TRH have revealed elevations not only of TSH, but of T_3 as well (47). This finding would suggest that measurements of T_3 levels after TRH administration may allow for a simple assessment of pituitary and thyroidal reserve.

In normal man, the physiological role of T_3 relative to that of T_4 remains to be established. Woeber and colleagues (48) measured serum T_3 level and the rate of T_3 clearance and found that the absolute T_3 disposal averaged 60 μg/day, a figure comparable with the 80 μg of T_4 which is turned over daily in normal subjects (49). According to preliminary results of tracer studies now underway, we find similar kinetic data, but with our lower values for the serum T_3 concentration, we calculate the T_3 disposal rate to be somewhat lower, averaging 35–40 μg/day. It should not be inferred that these values represent an accurate estimate of the relative rates of secretion of T_3 and T_4 from the thyroid gland, because recent observations (4–6, 50) suggest that a considerable proportion of circulating T_3 derives from T_4. Clearly, as Woeber and colleagues have pointed out (48), available observations do not permit us to draw inferences with regard to relative metabolic contributions of the T_4 and T_3 secreted by the thyroid gland.

Finally, evidence is accumulating that plasma and cellular binding and metabolism of T_3 differ strikingly from that of T_4 (51–54) and that these differences may be of major import in determining the relative metabolic potencies of the two hormones.

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