JCI The Journal of Clinical Investigation

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J Clin Invest. 1972;51(12):3104-3113. https://doi.org/10.1172/JCI107137.

Research Article

A new procedure for the radioimmunoassay of l-triiodothyronine (T_3) in human plasma is described in which the iodothyronines are separated from the plasma proteins before incubation with a specific antiserum to T_3. The antibody bound and free T_3 are separated with dextran-coated charcoal. In this system, the mean recovery of T_3 added to plasma was 97.9% and both in vitro conversion of l-thyroxine (T_4) to T_3 and cross-reaction between T_4 and the anti-T_3 antibody were undetectable (less than 0.1%). The assay procedure allowed the measurement of T_3 in up to 0.5 ml of plasma resulting in improved assay sensitivity (6 ng/100 ml). The mean plasma T_3 in normal subjects was 146±24 ng/100 ml (sd). Mean T_3 concentration was increased in hyperthyroidism (665±289 ng/100 ml) and decreased in hypothyroidism (44±26 ng/100 ml). In patients with severe hypothyroidism, plasma T_3 was between 7 and 30 ng/100 ml. Plasma T_3 concentration was relatively constant throughout the day in three euthyroid subjects. In contrast, in hypothyroid subjects on replacement therapy with T_3, a T_4: T_3 combination or desiccated thyroid plasma T_3 was markedly elevated for several hours after ingestion of the medication. Plasma T_3 was unchanged throughout the day in patients treated with T_4. Thus, insofar as plasma T_3 levels are concerned, replacement therapy with T_4 appears to mimic the euthyroid state more closely […]

A New Radioimmunoassay for Plasma L-Triiodothyronine: Measurements in Thyroid Disease and in Patients Maintained on Hormonal Replacement

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ABSTRACT A new procedure for the radioimmunoassay of L-triiodothyronine (T_3) in human plasma is described in which the iodothyronines are separated from the plasma proteins before incubation with a specific antiserum to T_3. The antibody bound and free T_3 are separated with dextran-coated charcoal. In this system, the mean recovery of T_3 added to plasma was 97.9% and both in vitro conversion of L-thyroxine (T_4) to T_3 and cross-reaction between T_4 and the anti-T_3 antibody were undetectable (less than 0.1%). The assay procedure allowed the measurement of T_3 in up to 0.5 ml of plasma resulting in improved assay sensitivity (6 ng/100 ml). The mean plasma T_3 in normal subjects was 146±24 ng/100 ml (SD). Mean T_3 concentration was increased in hyperthyroidism (665±289 ng/100 ml) and decreased in hypothyroidism (44±26 ng/100 ml). In patients with severe hypothyroidism, plasma T_3 was between 7 and 30 ng/100 ml. Plasma T_3 concentration was relatively constant throughout the day in three euthyroid subjects. In contrast, in hypothyroid subjects on replacement therapy with T_3, a T_4: T_3 combination or desiccated thyroid plasma T_3 was markedly elevated for several hours after ingestion of the medication. Plasma T_3 was unchanged throughout the day in patients treated with T_4. Thus, insofar as plasma T_3 levels are concerned, replacement therapy with T_4 appears to mimic the euthyroid state more closely than other preparations.

INTRODUCTION

During the last four years considerable attention has been paid to the development of methods for measurement of the plasma concentration of 3,5,3′-triiodo-L-thyronine (T_3).[1] The interest of many investigators has been stimulated by three factors: (*a*) the well-known greater biological activity of T_3 compared with L-thyroxine (T_4) (1); (*b*) the demonstration by Braverman, Ingbar, and Sterling (2) that T_4 is converted to T_3 in vivo; and (*c*), the recent capability to measure T_3 turnover rates in man (3).[2] These data suggest that the major portion of thyroid metabolic activity may be due to T_3 rather than T_4. The possibility that T_4 has no intrinsic biological activity but serves as a prohormone for T_3 has been suggested (4–7).

Several methods for the determination of T_3 have been reported which involve extraction of the iodothyronines from the serum followed by chromatographic separation of T_3 from T_4 and measurement of T_3 by displacement analysis (8, 9). These procedures are technically difficult and have resulted in overestimation of serum T_3 due to conversion of T_4 to T_3 in vitro or from incomplete separation of T_4 from T_3 before T_3 measurement (10, 11). More recently, the production of specific antibodies to T_3 has been achieved (12–15), and several reports describing the determination of T_3 by radioimmunoassay of unextracted plasma have appeared (12, 13, 15–19). The current report describes a new procedure for the radioimmunoassay of T_3 which allows measurement of T_3 in large serum volumes (up to 0.5 ml). The sensitivity of the assay is thereby improved enabling an accurate as-

Dr. Oppenheimer is a Career Scientist of the Health Research Council of the City of New York (I-222).

Received for publication 23 June 1972 and in revised form 15 August 1972.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; RIA, radioimmunoassay; T_3, triiodo-L-thyronine; T_4, L-thyroxine; B-T_3, antibody bound T_3; F-T_3, antibody free T_3.

[2] Surks, M. I., A. R. Schadlow, and J. H. Oppenheimer. 1972. Determination of iodothyronine absorption and conversion of L-thyroxine (T_4) to L-triiodothyronine (T_3) using turnover rate techniques. Submitted for publication in *J. Clin. Invest.*

sessment of the T_3 concentration in hypothyroidism. The concentration of T_3 in euthyroid, hypo- and hyperthyroid subjects as well as in hypothyroid patients under treatment with different thyroid hormone preparations will be presented.

METHODS

The concentration of T_3 in serum or plasma was determined by radioimmunoassay after separation of the hormone from the plasma proteins on small Sephadex columns (20). The barrels of disposable 3.0 ml syringes (Becton-Dickinson & Co., Rutherford, N. J.) with small glass-wool plugs were used for columns (I.D. = 7 mm). Columns were packed with 3.0 ml of a well-mixed 10% suspension of Sephadex, G25 fine (Pharmacia) in 0.1 N NaOH. After packing, they were washed with 2.0 ml 0.1 N NaOH and the bottoms were closed. For the development of standard curves, mixtures of 0.1 ml T_3-^{125}I (75 pg), 0.1 ml gravimetrically prepared standard T_3 solutions (50–1000 pg/0.1 ml 0.3% bovine serum albumin [BSA] in 0.075 M barbital buffer, pH 8.6), and 0.1 ml 0.1 N NaOH were applied to the columns with Pasteur pipettes. When sera were analyzed, 0.02–0.5 ml human serum was substituted for the T_3 standards. All of the T_3-^{125}I in the system was thus added before fractionation on the Sephadex columns. The test tubes in which the samples were prepared were washed with 0.1 N NaOH and the washes were applied to the columns. The columns were then opened. After the samples had percolated through the Sephadex, the columns were washed twice with 1.5 ml 0.075 M barbital, pH 8.6 (barbital buffer), in order to ensure elution of the plasma proteins and to equilibrate the columns at pH 8.6. Studies with different mixtures of serum or albumin enriched with albumin-^{131}I and T_3-^{125}I or T_4-^{125}I showed that more than 99% of the plasma proteins but only 4–8% of the applied iodothyronines were eluted by these procedures. The iodothyronines were then eluted with 0.3% BSA in barbital buffer (BSA buffer). First 0.4 ml BSA was applied and allowed to drain into the column. Four 0.5 ml portions of BSA buffer were then separately applied and the eluates collected as a single pool (approximately 2.0 ml). After gentle mixing, two 0.9 ml portions of the combined BSA eluate from each column were pipetted into disposable 12 × 77 mm plastic test tubes (Falcon Plastics, Los Angeles, Calif.) The tubes were placed in an ice bath and 0.2 ml of a rabbit anti-T_3 antibody solution (see below) was added. The tubes were then mixed (Vortex, Scientific Industries, Inc., Springfield, Mass.) and incubated at 4°C. Trial experiments had shown that equilibration between antibody bound and free T_3 occurred within 16 hr. Therefore, after 18–20 hr incubation, the antibody bound (B-T_3) and free T_3 (F-T_3) were separated by the addition of 1.0 ml of a dextran-coated charcoal suspension at 4°C. The charcoal suspension was prepared by mixing equal volumes of 1% solutions in barbital buffer of activated charcoal (Norit A, Fisher Chemical Co., New York) and dextran, clinical grade, mol wt 70,000–90,000 (Nutritional Biochemicals Corp., Cleveland, Ohio). The charcoal and supernatant were separated by centrifugation 15 min after the charcoal solution was added. The counting rates of both the supernatant containing B-T_3 and the charcoal (F-T_3) were measured in a Packard Autogamma Spectrometer, Model 410A. Samples were counted for a sufficient time to limit counting error to 5%.

Antibodies to T_3 were raised in six rabbits and eight guinea pigs by injection of T_3-BSA conjugates which were prepared by the method of Gharib and Mayberry (13, 15). The T_3: BSA molar ratios of the different preparations of conjugates which were synthesized varied between 7–10: 1 as determined from the recovery of tracer amounts of T_3-^{125}I added before conjugation. ½ mg of conjugate was homogenized in 0.5 ml complete Freund's adjuvant and injected into each animal every 14 days. Blood was collected by cardiac puncture 1 wk after the fourth injection. Sera from all rabbits and from four of the eight guinea pigs contained antibodies to T_3 which were suitable for the radioimmunoassay of T_3. A rabbit serum (R4-T_3-AB-#3) was selected for these studies and used at a final dilution of 1: 8000 in BSA buffer. The binding properties of R4-T_3-AB-#3 and various T_3 and T_4 analogues were not assessed. However, since no significant differences in T_3 concentration were observed in 25 human sera obtained from hypothyroid, euthyroid, and hyperthyroid subjects between assays performed both with R4-T_3-AB-#3 and with a well-characterized rabbit antiserum to T_3 (R9, 10/17/70) (kindly supplied by Dr. Robert E. Mayberry) it is doubtful whether small amounts of T_3 or T_4 analogues which may circulate in the plasma interfere with our T_3 measurements.

T_3 labeled with ^{125}I (Triomet-^{125}I, specific activity 70–90 mCi/mg), T_4-^{125}I (Tetramet-^{125}I, specific activity 70 mCi/mg), and albumin-^{131}I were obtained from Abbott Laboratories, North Chicago, Ill. A single T_3-^{125}I preparation was used for 4-6 wk without purification since paper chromatographic analyses demonstrated that these preparations contained 93–96% T_3 and less than 2% iodide-^{125}I even after this interval of time. Since the recovery of T_3 from the Sephadex columns was not uniform (65–80% in the 2.0 ml BSA eluate) it was necessary to determine precisely the specific activity of the added tracer so that appropriate corrections for the mass of T_3 in the added T_3-^{125}I could be made. The specific activity of each batch of T_3-^{125}I was therefore determined by radioimmunoassay by the method outlined by Berson and Yalow (21). Mixtures of T_3 standards (50-400 pg in 0.1 ml BSA buffer) were added to 0.1 ml T_3-^{125}I (50 pg T_3 as estimated from the specific activity of T_3-^{125}I furnished by the manufacturer). Some tubes contained 0.2 ml and 0.3 ml of the T_3-^{125}I tracer solution without nonradiative T_3. T_3 antibody was added and the volume of all tubes adjusted to 1.1 ml with BSA buffer. After 18–20 hours incubation, B-T_3 and F-T_3 were separated as described above. A standard curve was plotted (B/F ratios vs. added nonradiative T_3) and the mass of T_3 in the T_3-^{125}I solution was determined from the B/F ratios of the tubes containing two- and threefold volumes of tracer solution.

Calculations. Since the BSA eluate from each column was divided (two 0.9 ml samples), two assay tubes were processed for each Sephadex column. The two B/F ratios for each BSA column eluate were plotted against the total T_3 in each assay tube and a standard curve was drawn. Total T_3 was calculated as the product of the recovery of T_3-^{125}I in each assay tube (30–35%) (cpm B-T_3 + cpm F-T_3/cpm added T_3-^{125}I) and the mass of T_3 (standards + T_3-^{125}I) applied to each column. Total T_3 content in assay tubes containing serum was determined from their B/F ratios and the standard curve in the usual fashion. The contribution of T_3 in the T_3-^{125}I added to each sample was calculated as the product: recovery of T_3-^{125}I in each tube and the mass of T_3 in the added tracer. The total T_3 was then corrected for T_3 in the tracer. The resulting figure corrected for recovery (T_3-^{125}I) and sample volume is equivalent to the concentration of T_3 in serum. The average contribution of T_3 in the added T_3-^{125}I to the total T_3 of sera

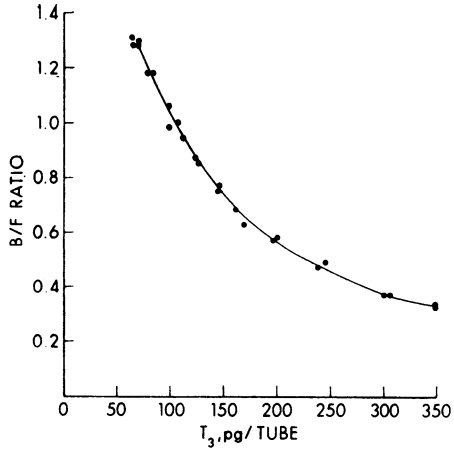

FIGURE 1 Representative standard assay curve. Mixtures of T_3-^{125}I, 0.1 N NaOH, 0.3 ml T_3-free serum, and increasing amounts of nonradioactive T_3 were applied to the Sephadex columns. The two B/F ratios for each column eluate are plotted.

from euthyroid and hyperthyroid subjects was approximately 20%. For sera in the hypothyroid range the T_3 in T_3-^{125}I constituted 30-40% of the total T_3 measured.

T_4 to T_3 conversion in vitro. Since the serum concentration of T_4 greatly exceeds that of T_3, monodeiodination of T_4 in vitro can result in a major overestimation of serum T_3. Special attention was, therefore, paid to assessing T_4 to T_3 conversion during the assay procedure. Our approach was to measure the T_3 concentration of T_3-free serum enriched with purified T_4. In three experiments, different amounts of L-thyroxine (Mann Research Labs, Inc., New York) purified by serial paper chromatography (two cycles) in *t*-amyl alcohol : 2 N NH_4OH : hexane, 5 : 6 : 1 were added to T_3-free serum which was then assayed for T_3. T_3-free serum was prepared by adding 20 ml of a serum pool from euthyroid subjects enriched with T_3-^{125}I (0.1 μCi/ml) to 4 g Dowex 1-X2 anion-exchange resin (BioRad Laboratories, Richmond, Calif.). After mixing for 20 hr at 4°C, the serum was separated from the resin by centrifugation. Fresh resin was added and the procedure was repeated. As assessed from the counting rates of T_3-^{125}I before and after mixing with resin, more than 99% of the T_3 was removed from the serum.

Another approach was to assess the generation of T_3-^{125}I after carrying out the assay procedure in the presence of purified T_4-^{125}I. T_4-^{125}I, purified as described above for nonradioactive T_4, was added to 2.5 ml BSA buffer. ½ ml of this solution was processed by the Sephadex column procedure described above for the assay of T_3. The 2.0 ml BSA buffer eluate containing the eluted iodothyronines was incubated with T_3 antibody for 18 hr at 4°C. After incubation, the iodothyronines were extracted with 95% ethanol (four extractions with 10 ml 95% ethanol). T_3-^{131}I was added to the combined ethanol extracts and the amount of T_3-^{125}I (relative to T_4-^{125}I) was determined by serial chromatography of the T_3 area on paper and thin-layer silica gel sheets to constant isotopic ratio (T_3-^{125}I/T_3-^{131}I) as previously described (4). The T_3-^{125}I content of the added T_4-^{125}I was simultaneously measured by the same procedures in solutions of 2.0 ml BSA buffer which were incubated under the same conditions but had not been subjected to Sephadex column fractionation.

T_4 cross-reaction. The extent of cross-reaction between T_4 and T_3-antibody was assessed in three experiments by determining the T_3 concentration of pooled serum before and after selective removal of T_4. T_3-free serum was diluted 1 : 20 in barbital buffer and enriched with nonradioactive T_4 and T_3, as well as T_3-^{125}I and T_4-^{131}I. The concentration of T_3 was measured in eight samples (0.15 ml) from this pool. Eight additional samples (0.5 ml) were applied to individual columns containing Sephadex G-25 M (Pharmacia) equilibrated with barbital buffer, pH 8.6. The column size and amount of Sephadex were identical to that described above for the T_3 assay. At pH 8.6, the plasma proteins bind T_4 to a greater degree than T_3. Thus, a greater fraction of the added T_3 than the added T_4 is retained by the Sephadex. The columns were then washed with 3.0 ml barbital buffer and the retained iodothyronines were eluted in 2.0 ml BSA buffer. T_3 antibody was added to two 0.9 ml portions of the eluate and the T_3 concentration determined as described above. The recovery of both T_4 and T_3 was calculated from the counting rates of their respective isotopes.

Clinical studies. The concentration of T_3 was determined in the serum or plasma of 37 euthyroid subjects, 29 patients with hypothyroidism, and 22 patients with hyperthyroidism. The euthyroid subjects were hospital personnel with normal thyroid glands by palpation and with no family history of thyroid disease. The hypo- and hyperthyroid patients had typical clinical syndromes. Diagnoses were confirmed by other laboratory tests including serum T_4 concentration (competitive protein binding) (Bioscience Laboratories, Van Nuys, Calif.), 24 hr thyroidal uptake of ^{131}I, and serum TSH concentration (22). The human TSH and anti-TSH antiserum used in the radioimmunoassay of TSH were gifts from the National Pituitary Agency. Research Standard A was kindly supplied by the Medical Research Council, Mill Hill, Great Britain.

Serum T_3 and T_4 concentrations were also measured in three euthyroid subjects at frequent intervals throughout the day and in patients with hypothyroidism receiving hormone replacement therapy. The hormone preparations which were administered to these patients were L-T_3 (Cytomel; Smith, Kline & French Laboratories, Philadelphia, Pa.), L-T_4 (Synthyroid, Flint Laboratories, Morton Grove, Ill.), L-T_4 : L-T_3 combination, 4 : 1 (Euthyroid, Warner-Chilcott Laboratories, Morris Plains, N. J.), and desiccated thyroid (Armour Pharmaceutical Co., Chicago, Ill.). Serum T_3 and T_4 were measured before and at frequent intervals after the various preparations were ingested. After separation by centrifugation, serum or plasma was stored at −20°C.

RESULTS

Methodologic procedures. The capacity of the Sephadex columns to retain T_3 when different amounts of plasma were applied was assessed by addition of mixtures of T_3-^{125}I in 0.1 N NaOH and 0.1 to 0.5 ml normal plasma to the columns followed by elution of the plasma proteins with 3.0 barbital buffer. Comparison of the counting rates of the Sephadex to that of the added T_3-^{125}I showed that when 0.1–0.3 ml plasma was applied, 95–98% of the T_3 was retained by the Sephadex. When the volume of plasma was increased to 0.4–0.5 ml, 83-

92% of the added T_3 was retained. An average of 80% (range: 70–90%) of the Sephadex-bound T_3 was eluted by 2.0 ml 0.3% BSA. Thus, the average recovery of T_3 in the 2.0 ml BSA eluate was approximately 70%. Since two 0.9 ml portions of the BSA eluates from each column were assayed, the net recovery of T_3 in each assay tube averaged 32% (range: 25–35%). Recovery was equally good when sera containing low endogenous T_3 concentrations were processed. Essentially similar recoveries for T_4 were observed in experiments in which T_4-^{125}I was substituted for T_3-^{125}I. The equal recovery of T_3 and T_4 by these procedures can be attributed to the essentially complete disruption of iodothyronine binding by the plasma proteins at the high pH of the Sephadex columns. The B/F ratios for paired tubes from the same column eluate differed by less than 5%.

In order to determine whether the T_3-free serum or the Sephadex contained substances which under the conditions of the assay might displace T_3 from T_3 antibody, 0.1 ml 0.1 N NaOH alone or with 0.3 ml T_3-free serum were applied to separate Sephadex columns followed by 3.0 ml barbital buffer and 2.0 ml BSA buffer. Duplicate 0.9 ml portions from each column BSA eluate were added to tubes containing T_3-^{125}I (50 pg) and T_3-antibody. The B/F ratios in these tubes were identical to those of other tubes which contained fresh BSA buffer, indicating that no interfering substances were present in either the Sephadex or serum. In the same assays, the addition of 10 pg T_3 reproducibly decreased the B/F ratio.

Standard curves were then developed by applying mixtures containing T_3-^{125}I (75 pg), 0.1 N NaOH, 0.3 ml T_3-free serum and increasing amounts of nonradioactive T_3 (50–1000 pg) to the Sephadex columns (Fig. 1). Since, in three experiments, substitution of BSA buffer for T_3-free serum resulted in standard curves which were indistinguishable from those in which T_3-free serum was used, all subsequent curves were set up in BSA buffer. Standard curves developed as described above were also compared with curves derived from incubating comparable mixtures with the exception that fresh BSA was used instead of the column eluates. Although the curves with fresh BSA and BSA from column eluates were generally superimposable, they occasionally diverged in the area of the lowest B/F ratios. Thus, for assay of plasma, standard curves using the Sephadex columns were always employed.

TABLE I
T_3 Recovery

Experiment	Plasma T_3			Recovery
	Initial	Added	Final	
	ng/100 ml			%
1	3(0–6)*	200	198±9.2	97.7
2	100.2±6.1	67	160.8±7.2	90.6
		100	193.4±11.2	93.2
3	157.8±6.8	67	220.6±12.4	93.9
		400	574.2±20.9	104.0
4	200.7±8.1	75	281.4±11.4	107.5
		150	362.6±14.7	101.0
		300	485.4±16.7	95.0

T_3 concentrations are expressed as mean ±SD for four determinations.

* Range indicated by parentheses. Serum T_3 was undetectable in two samples and 6 ng/100 ml in two samples.

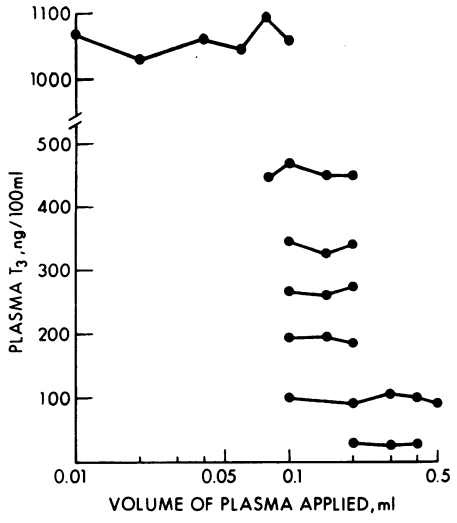

FIGURE 2 The effect of application of different volumes of plasma onto the Sephadex columns on plasma T_3 concentration.

Since the recovery of T_3 in the Sephadex column BSA eluates was not significantly reduced when up to 0.5 ml plasma was applied, the assay procedure allowed flexibility in the selection of appropriate volumes of plasma for assay so that the B/F ratio would fall in the most sensitive portion of the standard curve. Fig. 2 shows that the T_3 concentrations of different volumes of plasma from representative hypothyroid, euthyroid, and hyperthyroid subjects varied less than 10% from the average of three to five determinations. The constancy of the T_3 concentration when different volumes of the same plasma were assayed and the lack of detectable crossreaction with other moieties in plasma indicates that the observed changes in B/F ratio reflect displacement of T_3-^{125}I from antibody by endogenous T_3. In practice, the assay of 0.1 to 0.2 ml plasma from euthyroid subjects, 0.2–0.4 ml from hypothyroid subjects and 0.02–0.08 ml plasma from hyperthyroid subjects resulted in B/F ratios which fell in the steepest

TABLE II

Effect of T_4 Addition to T_3-Free Serum on T_3 Determination

Experiment	Purification	T_4 added	Total T_3	T_3/T_4
		pg × 10	pg	%
1	–	5	470	1.12
	+	2.5	80	0.38
	+	5.0	189	0.45
2	–	5	395	0.94
	+	5	129	0.31
	+	10	286	0.34
3	–	5	458	1.09
	+	2.5	48	0.23
	+	5.0	126	0.30
	+	10.0	226	0.27

The T_3 concentration of the T_3-free serum was undetectable to 5 ng/100 ml. T_4 was purified by two cycles of paper chromatography in *t*-amyl alcohol:2N NH_4OH:hexane, 5:6:1. Nonpurified or purified T_4 was added to 0.1 ml T_3-free serum. The T_3 content of the T_3-free serum (0.1 ml) was less than 5 pg. T_3/T_4 = 100 × moles T_3/moles T_4.

portion of the standard curve (50–150 pg per assay tube).

The recovery of added T_3 was assessed by measuring the T_3 concentration in plasma before and after addition of different amounts of nonradioactive T_3 (Table I). Exact amounts of T_3 were added to T_3-free serum or plasma samples with baseline T_3 concentrations in the low, middle, and high euthyroid range (see below). In four experiments, 90.6–107.5% of added T_3 was recovered. The mean recovery for all T_3 additions was 97.9%.

T_4 to T_3 conversion and T_4 crossreaction. The large T_4 to T_3 concentration ratio in plasma (40–70:1) and the well known property of T_4 to deiodinate in vitro are potentially major problems in the determination of T_3.

TABLE III

Determination of T_3-^{125}I After Addition of T_4-^{125}I to T_3-Free Serum

Experiment	Condition	T_3-$^{125}I/T_4$-^{125}I
		%
1	C	0.162
	E	0.211
2	C	0.227
	E	0.133

T_3-^{125}I was determined by cochromatography to constant isotopic ratio to added T_3-^{131}I as described in Methods. C = control (incubated at 4°C for 18 hr); E = experimental (sample subjected to complete T_3 assay procedure).

As little as 0.5% conversion of T_4 to T_3 in vitro or cross-reaction between T_4 and T_3 antibody will result in a 30–50% overestimation of the T_3 concentration in normal serum. Both of these potential sources of error were therefore carefully examined in the current assay.

T_4 to T_3 conversion was studied by measuring the T_3 content of T_3-free serum which had been enriched with T_4. For three separate lots of T_4 the measured T_3 was equivalent to 1.05% (range: 0.94–1.12%) of the T_4 added (Table II). After chromatographic purification of the T_4 preparations, the mean observed T_3 was substantially reduced to 0.36% (range: 0.23–0.45%). Since in pilot studies, the generation of 0.2–0.4% T_3 during serial paper chromatographic purification of T_4 has repeatedly been observed in this laboratory, it was considered probable that the T_3 measured after addition of chromatographically purified T_4 was a result of residual T_3 in the T_4 preparations and not to T_4 to T_3 conversion. Other experiments were therefore designed in which the percentage of T_3-^{125}I was measured after purified T_4-^{125}I was processed through the Sephadex columns and incubated with T_3 antibody (Table III). In two experiments, the percentage of T_3-^{125}I (100 × T_3-$^{125}I/T_4$-^{125}I) observed after the Sephadex column procedure and that of the added T_4-^{125}I were indistinguishable within the limits of precision of the chromatographic analyses. Thus, it was concluded that no detectable T_4 to T_3 conversion (less than 0.1%) occurred during the assay procedure.

The variable T_3 contamination of chromatographically purified T_4 preparations precluded studies of T_4 cross-reaction in which the effect of added T_4 on the B/F ratio of T_3-^{125}I is examined. A new approach to this problem was therefore taken. The concentration of T_3 was determined in diluted T_3-free serum enriched with T_3 and T_4 before and after the serum T_4:T_3 concentration ratio was substantially reduced by Sephadex chromatography at pH 8.6 (Table IV). If the antibody exhibits significant cross-reaction between T_4 and T_3, measurement of the apparent T_3 concentration in sera with high T_4:T_3 concentration ratios should be greater than measurements after the T_4:T_3 ratios are reduced. In three experiments in which the T_4:T_3 concentration ratios were reduced three- to eightfold, the extent of T_4 cross-reaction varied from undetectable to between 0.1 and 0.2% of the T_3 value. The average T_4 cross-reaction for the three experiments was less than 0.1%. Cross-reaction of 0.1% should result in less than a 7% overestimation of the T_3 level in euthyroid sera.

Assay conditions. After it was shown in eight subjects that the T_3 concentration was not significantly different in serum or heparinized plasma, serum or plasma were used interchangeably in clinical studies. In two experiments no change in T_3 concentration was

observed after repeated freezing and thawing (six times in 24 hr) or after plasma was allowed to stand at room temperature for 24 hr. The T_3 concentration was unaltered after storage for at least two months at −20°C.

Sensitivity. The lowest T_3 concentration detectable depends on the maximum volume of serum which can be introduced into the assay (0.5 ml), the recovery of T_3 in the assay tubes and the sensitivity of the standard curve. Since a significant reduction in B/F ratio is regularly observed with the addition of 10 pg T_3, the lowest limit of detection is 10 pg T_3 ÷ recovery (average 33%) = 30 pg. This is equivalent to a concentration of 6 ng/100 ml when a 0.5 ml serum sample is assayed (30 pg ÷ 0.5 × 100).

Precision. The mean coefficient of variation for 8–10 determinations of the same serum in three different assays was 5.8%. The coefficient of variation for a single serum determined in 23 consecutive assays over a 10 wk interval was 8.2%. Duplicate determinations within an assay using either the same or different serum volumes differed by less than 6%. There was no difference in precision when the T_3 concentration was in the hypothyroid, euthyroid, or hyperthyroid range.

Clinical studies. The concentration of T_3 in the serum or plasma of euthyroid, hypothyroid, and hyperthyroid subjects is shown in Fig. 3. In euthyroid subjects, the range of T_3 concentration was 100–196 ng/100 ml. In this group the mean serum T_3 was 146±24 (SD) ng/100 ml. The mean T_3 concentration in 29 hypothyroid patients was 44±26 ng/100 ml. None of the values in the hypothyroid group fell within 2 SD of the mean of the euthyroid group. The T_3 concentration generally correlated with the severity of hypothyroidism. The lowest value, 7 ng/100 ml, was found in a patient in myxedema coma. In other patients with severe hypothyroidism, serum T_3 concentration was generally between 10 and 30 ng/100 ml. In 22 hyperthyroid patients, mean serum T_3 concentration was 665±289 ng/100 ml. The lowest value in this group, 289 ng/100 ml, occurred in a patient with clinically mild hyperthyroidism due to Graves' disease. No cases of T_3 thyrotoxicosis were encountered in a 6 month interval in which 19 consecutive hyperthyroid patients were studied.

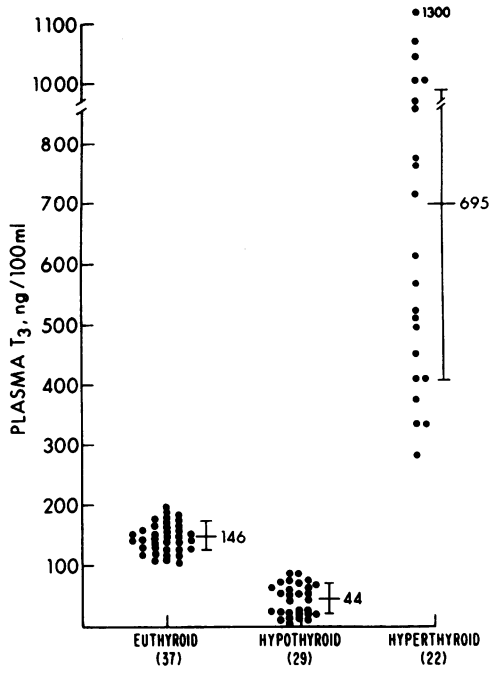

FIGURE 3 Plasma T_3 concentrations in euthyroid, hypothyroid, and hyperthyroid subjects. Individual values are plotted. The mean ±SD for each group is indicated.

Plasma T_3 and T_4 concentrations were measured at frequent intervals during a 24 hr period in three euthyroid subjects. As shown in Fig. 4, the plasma levels of both hormones were relatively constant throughout the day.

TABLE IV
T_4 Cross-reaction

Experiment	Before Sephadex column		After Sephadex column			Cross-reaction
	T_4/T_3	T_3	T_4/T_3	T_3 expected	T_3 observed	
		ng/100 ml		*ng/100 ml*	*ng/100 ml*	%
1	26.2	255.8±11.4	8.8	65.2±2.1	67.5±2.3	0.1
2	49.7	134.8±7.3	16.2	22.8±1.1	20.3±2.7	0.1–0.2
3	43.6	153.5±5.7	5.3	38.6±1.0	42.2±0.8	—

The concentration of T_4 added to the 1/20 diluted T_3-free serum was 8 μg/100 ml. T_3 expected was calculated as the product initial T_3 concentration and recovery T_3-^{125}I from the Sephadex columns. The calculated cross-reaction was based on the precision of the T_3 determinations, recovery of T_3-^{125}I and T_4-^{131}I, and the extent of change in the T_4/T_3 ratio. Cross-reaction was calculated by determining if the difference between the T_3 expected and the T_3 observed could be accounted for by a fraction of the T_4 remaining after Sephadex chromatography. T_3 concentration is expressed as mean ±SD for eight samples.

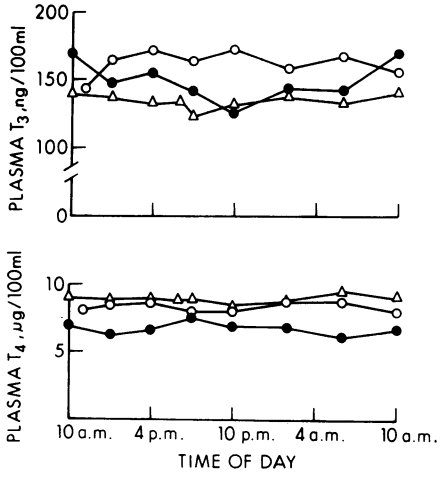

FIGURE 4 Plasma T_3 and T_4 concentrations at different times throughout the day in three euthyroid subjects.

Serum T_3 and T_4 concentrations were also measured in hypothyroid patients who were maintained euthyroid by treatment with different thyroid medications. Sera were obtained at frequent intervals (1–2 hr) after the entire daily dose of medication was ingested. After T_3 ingestion, the plasma T_3 concentration of four subjects, initially in the normal range, increased to peak values of 450–700 ng/100 ml within 1–2 hr (Fig. 5). Thereafter, T_3 concentration decreased toward the normal range. 24 hr after ingestion, just before administration of the next daily dose, plasma T_3 concentration had returned to the initial euthyroid values. An entirely different pattern of hormone concentrations was observed in four patients who were taking L-thyroxine (Fig. 6). Plasma T_3 concentration remained essentially unchanged throughout the 24 hr after ingestion of T_4. Plasma T_4, however, increased transiently 20–40% in three of four patients studied within the first 5 hr after hormone administration. In the patients treated with T_4: T_3, 4:1 mixtures (Fig. 7) or desiccated thyroid (Fig. 8) the serum T_4 concentration was relatively constant throughout the day. The pattern of T_3 concentration was, however, quite variable after ingestion of these medications. After the synthetic T_4: T_3 mixture plasma T_3, initially at or slightly above the upper limit of the normal range, increased transiently to levels which ranged from 320 to 450 ng/100 ml. The increase occurred within the first 1–2 hr in one patient but not until 4–8 hr in two other patients. After desiccated thyroid administration, plasma T_3 was unchanged in one patient but increased significantly (100%) in two others. Serum T_3 in one patient, who was taking 60 mg desiccated thyroid per day, increased from a hypothyroid level (70 ng/100 ml) to an euthyroid value (142 ng/100 ml) after drug ingestion (Fig. 8).

DISCUSSION

This report describes a new procedure for the radioimmunoassay (RIA) of serum T_3 in which the iodothyronines are separated from the plasma proteins by Sephadex column chromatography before incubation with anti-T_3 antibody. The assay of T_3 in the presence of plasma proteins using a standard curve set up in buffer should theoretically result in an overestimation of T_3 concentration since T_3 bound by the plasma proteins is not available for binding by anti-T_3 antibody. The observed percentage of antibody bound T_3 is thereby reduced.

This theoretical consideration is supported by comparison of the mean plasma T_3 concentration of euthyroid individuals using the same antibody but different assay conditions. Gharib, Ryan, Mayberry, and Hockert have reported that the mean plasma T_3 concentration of a euthyroid group was 218±55 ng/100 ml as measured by RIA in the presence of plasma proteins (15). Using an assay procedure which separates the iodothyronine from the plasma proteins before incubation with antibody, there was no significant difference between the T_3 concentration of a large number of sera when assayed with this antiserum or an antiserum produced in our laboratory. Mean plasma T_3 in euthyroid subjects as measured with our antiserum is 146±24 ng/100 ml. Moreover, others have reported that the mean plasma T_3 of normal subjects is between 100–145 ng/100 ml as determined in the presence of plasma proteins but with plasma binding blocked by the addition of *d,l*-tetrachlorothyronine (16), 5,5′-diphenylhydantoin (17), acetylsalicylic acid (19) or

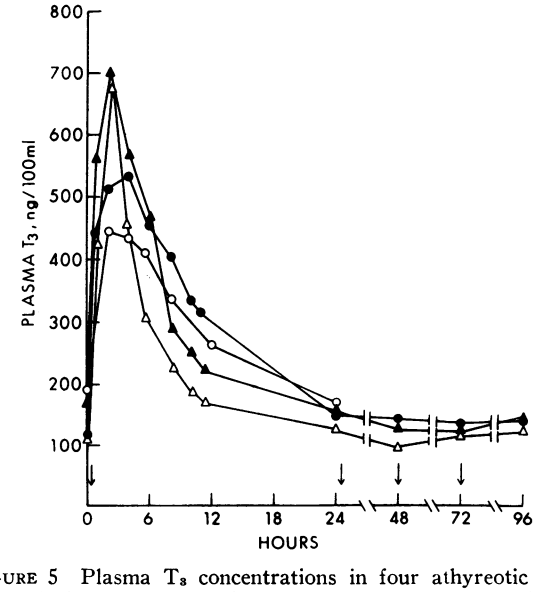

FIGURE 5 Plasma T_3 concentrations in four athyreotic patients during treatment with T_3. The vertical arrows indicate the time at which the entire daily T_3 dose was ingested. The dose of T_3 was 75 µg/day for three subjects and 50 µg/day for one subject (▲—▲).

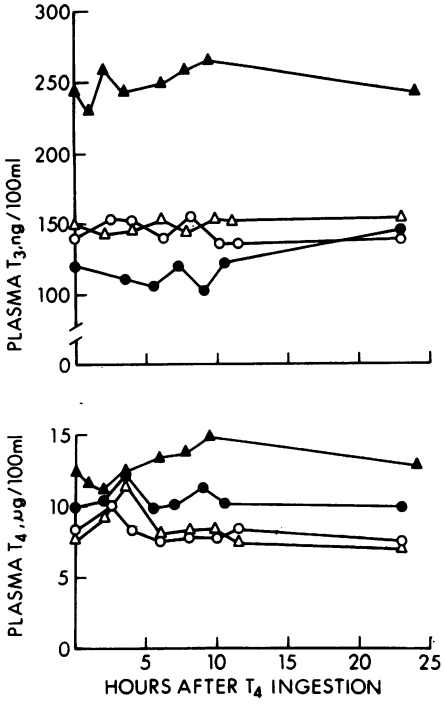

FIGURE 6 Plasma T_3 and T_4 concentrations in four athyreotic patients during treatment with T_4. All patients received 200 μg T_4 per day. The dose of T_4 in the one subject with elevated plasma T_3 and T_4 levels (▲—▲) was subsequently reduced to 150 μg/day. This change resulted in a decrease in plasma T_3 and T_4 to the normal range without an elevation in serum TSH concentration.

8-anilino-*l*-napththalene-sulfonic acid (18). A physical method for the removal of the plasma binding proteins, Sephadex column chromatography, was selected for the present studies since from published reports concerning the agents listed above, we were not certain that the added compounds completely blocked plasma protein binding in plasma with increased iodothyronine binding capacity. Moreover, one of the compounds, *d,l*-tetrachlorothyronine, may not be generally available in a pure and stable form.[3] At the time of this writing, however, there is insufficient experience with any of the reported assay procedures to allow generalizations regarding the superiority of any particular procedure with the exception that it appears important either to remove the plasma proteins or block their binding of T_3.

[3] Evidence suggesting that the latter compound might contain or generate during standing sufficient *d,l*-trichlorothyronine, a T_3 analogue, to displace T_3 from antibody sites was obtained in two experiments performed in our laboratory before the present method was developed. In these, we noted displacement of tracer T_3 from anti-T_3 antibody by addition of *d,l*-tetrachlorothyronine (kindly supplied by Dr. Robert E. Meltzer, Warner-Lambert Research Laboratories, Compound W1633).

The assay procedure described herein satisfies a number of criteria which are of cardinal importance for any T_3 measurement. First, T_3 added to sera of widely different T_3 concentrations was quantitatively recovered. Second, the calculated T_3 concentrations of hypo-, eu-, and hyperthyroid sera were independent of the volume of serum applied to the columns. Third, conversion of T_4 to T_3 during the assay procedure was undetectable ($< 0.1\%$). Fourth, the capacity of the anti-T_3 antibody to cross-react with endogenous T_4 was also undetectable ($< 0.1\%$). Fifth, as demonstrated from the identical standard curves run in different volumes of T_3-free serum or 0.3% BSA, the Sephadex column procedure effectively removed all possible interfering substances from the plasma. Moreover, the Sephadex columns did not introduce any materials which alter the B/F ratio of tracer T_3 and anti-T_3 antibody. Finally, the precision of measurement within an assay, reproducibility between assays, and sensitivity of the assay system were all well within the limits required for both clinical and investigative use.

The sensitivity of T_3 measurement by the Sephadex column procedure is substantially improved over published methods in which the volume of serum which is assayed is generally limited to 0.1 ml (13, 15–17). Without altering assay conditions, as much as 0.5 ml serum can

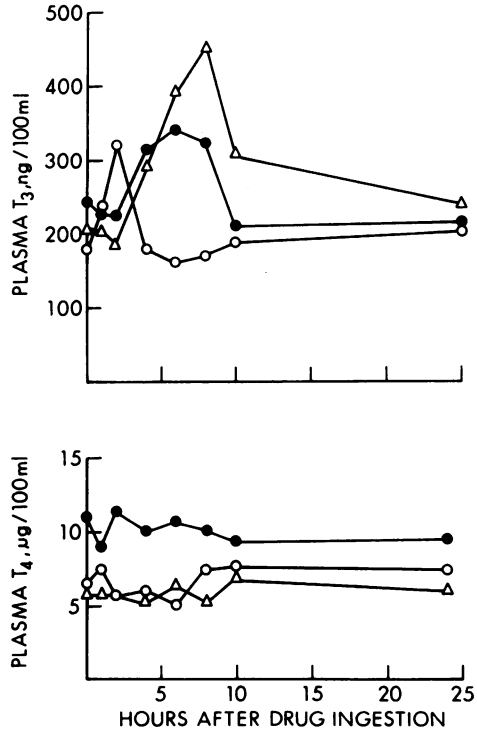

FIGURE 7 Plasma T_3 and T_4 concentrations in three hypothyroid patients during treatment with a T_4:T_3 (4:1) mixture. The dose was 180 μg T_4 and 45 μg T_3 per day.

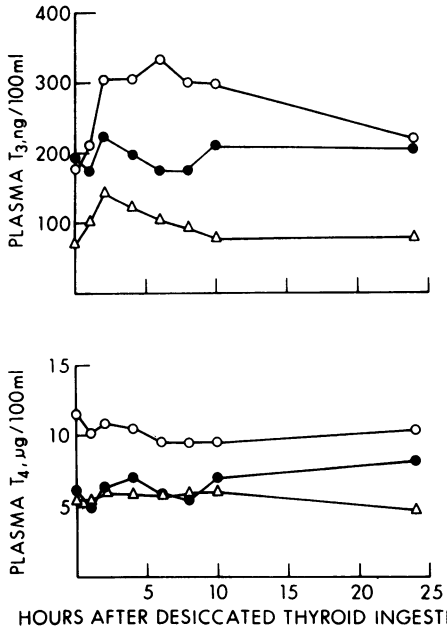

FIGURE 8 Plasma T_3 and T_4 concentrations in three hypothyroid patients during treatment with desiccated thyroid. The dose was 120 mg/day in two subjects and 60 mg/day in a third (△—△). This subject (△—△) was the only patient in this series of experiments who was somewhat hypothyroid (serum TSH = 55 µU/ml).

be processed on the Sephadex columns. The lowest limit of detection is approximately 6 ng/100 ml. Indeed, the T_3 concentration in one patient in myxedema coma was 7 ng/100 ml and in a number of patients with severe hypothyroidism was between 10 and 25 ng/100 ml. The low T_3 concentrations observed in severe hypothyroidism and lack of overlap between the hypothyroid and euthyroid range contrasts with other radioimmunoassays of T_3 in which the reported mean ±SD for hypothyroid subjects was 103±43 (15), 99±24 (17), and 59±9 ng/100 ml (16). In the system developed by Mitsuma, Nihei, Gershengorn, and Hollander (16), there was no overlap between the hypo- and euthyroid range. However, although their assay system, modified for hypothyroid sera by increasing fourfold the volume of sera and amount of added *d,l*-tetrachlorothyronine, can theoretically detect T_3 concentrations as low as 12.5 ng/100 ml, T_3 concentrations less than 44 ng% were not observed in hypothyroid subjects. The disparity between their data and those from the present hypothyroid patients cannot be attributed to geographic factors. The possibility exists, therefore, that hypothyroid sera or the increased amount of added *d,l*-tetrachlorothyronine may introduce a blank which prevents measurement of extremely low T_3 concentrations by the procedure of Mitsuma et al. Thus a major advantage of the Sephadex column procedure appears to be the capacity to measure T_3 concentrations in the hypothyroid range with greater precision than has heretofore been possible.

The application of radioimmunoassay technology with antibodies specific for T_3 has rendered the measurement of serum T_3 concentration relatively simple and precise. Serum T_3 determinations have thus become an important tool for the investigation of numerous aspects of thyroid physiology and pathophysiology and should soon become generally available to aid clinicians in the diagnosis and treatment of thyroid disease. A number of experimental considerations based on different lines of evidence suggests that, in the rat, T_4 is a prohormone without intrinsic biologic activity (4–7). We have recently shown that a similar relationship exists in man [2] and have suggested that under conditions of adequate iodine supply and in the absence of excess TSH stimulation, the normal gland secretes primarily T_4. Under such circumstances almost all T_3 appears to be derived from the extrathyroidal conversion of T_4. Thus, the serum T_3 concentration should be a more direct measure of thyroidal status than serum T_4. Indeed, clinically euthyroid patients with decreased T_4 concentrations but normal concentrations of T_3 have been described by others (9) and have been noted in our clinic too. The use of serum T_3 in the assessment of thyroidal status in hypothyroid patients receiving replacement therapy with different thyroid hormone preparations has not been emphasized previously. The increase in serum T_3 concentration to the hyperthyroid range which was observed after ingestion of T_3 is similar to that reported by Lieblich and Utiger (17) while these studies were in progress. An increase in T_3 concentration was also noted after ingestion of desiccated thyroid and a T_4:T_3 mixture but not after ingestion of T_4. The difference in the magnitude of increase and time course of T_3 concentration during therapy with these medications may be related to the chemical form of hormone administered, the nature of the pharmaceutical preparation, the absorption of hormone by the patient, or a combination of these factors. Of particular interest, is that the mean plasma T_3 concentration calculated by integrating the area under the plasma T_3 curves (Figs. 5–8) was significantly greater (200–290 ng/100 ml) than the upper limit of the normal range for all patients who were clinically euthyroid after treatment with T_3, T_4:T_3 mixtures, or desiccated thyroid. Although no direct data are currently available, possible deleterious effects on different organ systems of a modest increase in plasma T_3 concentration sustained over the course of years should be considered. Until this problem is evaluated it would appear reasonable to use synthetic T_4 for hormone replacement since the constancy of serum T_3 and T_4 after administration of this preparation most closely mimics the plasma hormone concentrations observed in euthyroid subjects throughout the day. It is

clear, however, that if the other medications are employed, careful consideration must be given to the relationship of the time medications are ingested to that of blood sampling in order to obtain T_3 concentrations which reflect the mean plasma hormone concentration and not a transient postabsorptive elevated value. Additional studies to assess the comparative adequacy of various thyroid hormone preparations in replacement therapy are in progress.

ACKNOWLEDGMENTS

The authors thank Mr. Kenneth Gans for expert technical assistance and Mrs. Annette Friedman for secretarial help. We also thank Mr. Barry Shapiro for the illustrations.

This work was supported by U. S. Public Health Service Grant 9 RO1 AM 15421-12 and U. S. Army Contract DA-49-193-MD-2967.

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