3,3'-Diiodothyronine Production, a Major Pathway of Peripheral Iodothyronine Metabolism in Man

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ABSTRACT 3,3'-Diiodothyronine (3,3'-T₂) has been detected in human serum and in thyroglobulin. However, no quantitative assessment of its clearance rate (CR), production rate (PR), or of the importance of extrathyroidal sources of 3,3'-T₂ relative to direct thyroidal secretion is yet available. This study examines these parameters in seven euthyroid subjects, and in eight athyreotic subjects (H) eumetabolic due to thyroxine therapy (HT₄) ($n = 5$) or triiodothyronine replacement (HT₃) ($n = 3$). A highly specific radioimmunoassay for the measurement of 3,3'-T₂ in whole serum was developed. Serum 3,3'-T₂ concentrations were (mean $\pm$ SD) 6.0 $\pm$ 1.0 ng/100 ml in 13 normal subjects, 9.0 $\pm$ 4.6 ng/100 ml in 25 hyperthyroid patients, and 2.7 $\pm$ 1.1 ng/100 ml in 17 hypothyroid patients. The values in each of the latter two groups were significantly different from normal. 3,3'-T₂ was detected regularly in normal concentrations in 11 hypothyroid patients eumetabolic by treatment with synthetic T₄, in 10 eumetabolic patients suffering from nonthyroidal systemic illness, and in 2 subjects with elevated serum T₄-binding globulin. The 3,3'-T₂ CR was assessed from data acquired from the ¹²⁵I-3,3'-T₂ constant infusion technique. The 3,3'-T₂ PR was calculated from CR and serum concentration of 3,3'-T₂ determined by radioimmunoassay. In the HT₄ subjects the 3,3'-T₂ CR averaged 840 $\pm$ 377 liters/day and 3,3'-T₂ PR 33.9 $\pm$ 12.5 μ g/day. These results were not significantly different from those in the control group: 3,3'-T₂ CR 628 $\pm$ 218 liters/day and 3,3'-T₂ PR 39.8 $\pm$ 19.8 μ g/day (all corrected to 70 kg body wt). In addition to 3,3'-T₂ PR, T₃, and reverse triiodothyronine (rT₃) PR were determined in three of the HT₄ subjects. In each case studied, the 3,3'-T₂ PR was close to the combined tri-

iodothyronine (T₃ + rT₃) PR. The mean molar ratio of T₂ PR/(T₃ + rT₃) PR was 1.08 $\pm$ 0.10. The results obtained in the HT₄ subjects indicate that the production of 3,3'-T₂ is a major route of T₄ metabolism. The combined studies of 3,3'-T₂, T₃ and rT₃ PR in the HT₄ subjects indicate that both T₃ and rT₃ are major precursors of 3,3'-T₂. In the HT₃ subjects, the conversion of T₃ to 3,3'-T₂, determined as the molar ratio of 3,3'-T₂ PR to T₃ PR, ranged from 0.36 to 0.92, providing further evidence that T₃ is a precursor of 3,3'-T₂. From the close agreement between the mean values for 3,3'-T₂ PR in the euthyroid and HT₄ group it is concluded that most, if not all of the 3,3'-T₂ produced in normal humans is derived by extrathyroidal conversion from T₃ and rT₃.

INTRODUCTION

The presence of 3,3'-diiodothyronine (3,3'-T₂)¹ in the thyroid gland and the plasma of the rat was demonstrated originally by Roche et al. (1). Subsequently, the same group showed that radioiodine-labeled 3,5,3'-triiodothyronine (T₃) and 3,3',5'-triiodothyronine (reverse T₃[rT₃]) were converted to 3,3'-T₂ in the rat (2). Flock et al. (3, 4) reported similar findings in the intact dog. Chopra et al. (5) have recently presented evidence in rat liver homogenates that the conversion of rT₃ and of T₃ to 3,3'-T₂ is enzymatic in nature.

Three groups have described radioimmunoassays for 3,3'-T₂ in normal human serum, although the reported levels do not agree (6-8). No quantitative estimates have been made, however, of the turnover of 3,3'-T₂ in humans. In an early study, Stanbury and Morris (9) using ¹³¹I-3,3'-D,L-T₂ administered to nor-

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¹ Abbreviations used in this paper: ANS, 8-anilino-L-naphthalene sulfonic acid; C, control group; CR, clearance rate; CV, coefficient of variance; H, hypothyroid group; PR, production rate; 3,3'-T₂, 3,3'-L-diiodothyronine; T₃, triiodothyronine; rT₃, reverse T₃; T₄, thyroxine; TBG, thyroxine-binding globulin.

mal subjects in a single bolus injection, found extremely rapid disappearance of tracer from plasma and accumulation of radioiodide in the urine, indicating a high rate of deiodination of this compound.

The present study was initiated to assess the absolute production rate of 3,3'-T₂ in man, using the technique of constant tracer infusion to estimate plasma clearance and a specific radioimmunoassay to measure plasma concentration of this substance. In addition to studies of subjects with intact thyroid glands, measurements of 3,3'-T₂ kinetics were done in athyreotic patients who were maintained eumetabolic on L-thyroxine (T₄) to determine the importance of extrathyroidal sources of 3,3'-T₂ relative to direct thyroidal secretion. In some of the athyreotic subjects on T₄ therapy, turnover studies of T₃ and rT₃ were performed as well. In three additional cases receiving T₃ as maintenance therapy, T₃ and 3,3'-T₂ kinetics were determined. These studies allowed an assessment of the relative contributions of T₃ and of rT₃ as immediate precursors of 3,3'-T₂ in humans.

METHODS

The subjects participating in this investigation were from the outpatient service of the San Francisco Veterans Administration Hospital. All subjects gave written consent, having been informed of the purpose and nature of the study. Seven male euthyroid control subjects included four normal volunteers and three patients with mild nonthyroidal, nonsystemic illnesses. The eight athyreotic adult males were eumetabolic by clinical and laboratory criteria at the time of study. Five subjects were receiving full replacement doses of L-T₄ (Synthroid, Flint Laboratories, Deerfield, Ill.) and three were maintained on L-T₃ (Cytomel, Smith Kline & French Laboratories, Philadelphia, Pa.). None of the patients was taking any drug known to interfere with normal thyroid function or to affect the metabolism of thyroid hormones. The mean T₄ replacement dose in the athyreotics was 145 µg/day.

Radioimmunoassay of 3,3'-T₂. Unlabeled 3,3'-L-T₂ and the various other thyroid hormone analogues and derivatives were obtained through the courtesy of Dr. Eugene Jorgensen, University of California, San Francisco, Calif. The ¹²⁵I-3,3'-T₂ (labeled presumably in the 3'-position) was prepared from 3,3'-L-T₂ by Abbott Laboratories, North Chicago, Ill. Specific activity ranged from 250 to 350 µCi/µg. At the time of use, each lot of labeled tracer was analyzed for radiochemical purity by Sephadex G-25 column chromatography (by the method described below) and was found to contain <5% of labeled iodide and <1% of labeled rT₃. A conjugate of 3,3'-T₂ and 3,3'-T₂—bovine serum was prepared by the method of Gharib et al. (10). The carbodiimide (1-cyclohexyl-3-carbodiimide metho-*p*-toluene-sulfonate) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and dimethylformamide from Fisher Scientific Co., Pittsburgh, Pa. Antiserum was harvested from rabbits immunized with the 3,3'-T₂—bovine serum albumin conjugate emulsified in complete Freund's adjuvant (Miles Laboratories, Inc., Elkhart, Ind.) and pertussis vaccine (Eli Lilly and Company, Indianapolis, Ind.). The antiserum selected for the present study bound 30–35% of tracer amounts of radioactive 3,3'-T₂ in a final dilution of 1:10,000. The binding of ligand to serum proteins was inhibited by 8-anilino-L-naphthalene sulfonic acid (ANS), supplied by Eastman Kodak Co., Rochester, N. Y.

To determine the need for ANS in our assay we compared the percent (% B/T) of ¹²⁵I-3,3'-T₂ bound by a fixed concentration of antiserum in barbital buffer (0.07 M barbital, pH 8.6) with that bound in iodothyronine-free serum (11). In the absence of ANS, antibody bound only 8–10% of ¹²⁵I-3,3'-T₂ in serum compared to 50–60% in barbital. From a series of experiments utilizing from 75 to 500 µg ANS per tube, we determined that 450 µg per tube allowed optimum binding (30%) of ¹²⁵I-3,3'-T₂ by our antiserum in iodothyronine-free serum.

Serum 3,3'-T₂ was determined in unextracted serum samples. The various reagents were added in the following order: (a) 200 µl of test sample; (b) 300 µl of 2% normal rabbit serum in barbital buffer (0.07 M barbital, pH 8.6); (c) 100 µl (450 µg) of ANS; (d) 100 µl (5 pg) of ¹²⁵I-3,3'-T₂; (e) 100 µl of 1/1,250 diluted T₂-binding antiserum. The samples were incubated for 24 h at 4°C. A sufficient quantity (50 µl) of previously titered goat anti-rabbit gamma globulin (Antibodies Inc., Davis, Calif.) was then added and the mixture further incubated for 24 h at 4°C. The bound precipitate was separated by centrifugation and aspiration of the supernate. Antibody-bound radioactivity was determined in an automatic well counter. Standards containing stable 3,3'-T₂ (0.5–100 ng/dl) were prepared in iodothyronine-free serum (11) and were processed by the same method. A standard curve was constructed as percent bound ¹²⁵I-3,3'-T₂ vs. concentration of 3,3'-T₂. The 3,3'-T₂ concentration in 0.2 ml of test serum was read from the standard curve and the results were expressed in nanograms of 3,3'-T₂/100 ml. Total serum T₃ and rT₃ were measured by radioimmunoassay as previously described (12). Total serum T₄ and free T₄ index were determined by a competitive binding assay (13).

Preparation of 3,3'-T₂ conjugates. ¹²⁵I-3,3'-T₂ was injected into the inferior vena cava of an adult male Sprague-Dawley rat. Bile was collected via a biliary cannula for 4 h postinjection. The radioactive components of the bile were separated by thin-layer chromatography of 20 × 20-cm glass-fiber sheets (Gelman Instrument Co., Ann Arbor, Mich.). Chromatographs were developed in butanol-dioxane-2 N ammonia, 4:1:5 (vol/vol/vol), at 25°C for 1½ h, and dried in a stream of nitrogen. The radioactive bands were located by autoradiography of the dried chromatogram and the various components eluted from the chromatogram in methanol-ammonia (99:1). The 3,3'-T₂ glucuronide and sulfo-conjugate zones were identified by enzymatic hydrolysis using glucuronidase (Worthington Biochemical Corp., Freehold, N. J.) and Mylase-P (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio), respectively (14–16).

Constant-infusion 3,3'-T₂ studies. Subjects were hospitalized on a metabolic ward for the duration of the study. Thyroidal radioiodine uptake was minimized in all euthyroid subjects by giving a saturated solution of potassium iodide orally, 10 drops every 8 h before and during the study. ¹²⁵I-3,3'-T₂, diluted in 1% human serum albumin in normal saline to a concentration of 8 µCi/ml, was sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.) before administration to the subjects. A total dose of 200 µCi (25 ml) was administered by intravenous infusion over 8 h at a constant rate (3.1 ml/h) in each subject. The constant infusion was given with a Harvard Infusion Pump (Harvard Apparatus Co., Inc., Millis, Mass.). The accuracy of the rate of infusion was checked on several occasions in control runs by measuring H₂O infusion into a graduated cylinder over 8 h and also during the actual infusions by recording the volume infused per hour from the graduated infusion syringe. We always prepared 30 ml of tracer solution, as extra volume was needed to fill the tubing connecting the pump (syringe) with the patient. Aliquots for standards were

taken from the solution remaining in the tubing at the completion of the infusion. Samples of blood were obtained at time 0 and every 2 h during the infusion.

T_3 and rT_3 kinetics studies. $^{131}\text{I}-T_3$ was obtained from Amersham Corp. (Arlington Heights, Ill.) at a specific radioactivity of $\approx 40 \mu\text{Ci}/\mu\text{g}$. $^{125}\text{I}-rT_3$ was supplied by Abbott Laboratories at a specific radioactivity of $500 \mu\text{Ci}/\mu\text{g}$. Before administration to human subjects, a mixture of $^{131}\text{I}-T_3$ and $^{125}\text{I}-rT_3$, diluted in 1% human serum albumin-normal saline, was sterilized by Millipore filtration. Tracer doses of $^{131}\text{I}-T_3$ (100 μCi) and $^{125}\text{I}-rT_3$ (200 μCi), in an accurately measured volume, were injected intravenously. Samples of blood were obtained at 15, 30, 60 min and 2, 4, 6, 12 h, and every 12 h thereafter until 72 h after the doses were administered.

In all tracer studies, dose standards were prepared by adding aliquots of tracer doses to undiluted pooled normal serum. These standards were processed in an identical manner to the plasma samples.

Analysis of sera from kinetics studies. Multiple serum samples, collected during the $^{125}\text{I}-3,3'\text{-}T_2$ infusion studies, were analyzed by column chromatography employing Sephadex G-25F, by a modification of the method described by Green (17). A column of Sephadex G-25F, bed volume $2.05 \times 25 \text{ cm}$, was equilibrated with 0.5 M NaCl–0.054 N NaOH (pH 11.3). Samples and standards (3 ml) were diluted with an equal volume of diluent (1.0 M NaCl in 0.2 M phosphate buffer, pH 6.5) before application to the column. Iodoprotein, iodide, and $^{125}\text{I}-3,3'\text{-}T_2$ were eluted in succession by 0.005 M NaCl + 0.1 N NaOH, pH 11.8. 20 mM sodium metabisulfite was added to the equilibrating and eluting solution to minimize deiodination. From 140 to 160 fractions (3 ml each) were collected. Each fraction was counted (10 min) in an automatic well-type gamma counter (mean background = 40 cpm). Those fractions included in the $^{125}\text{I}-3,3'\text{-}T_2$ region (fractions 75–120, approximately) were counted again for longer periods, up to 100 min each, to obtain a relative counting error of 3% or less.

An average of $98.2 \pm 4.7\%$ (SD) of the total ^{125}I in samples and standards applied (40 runs) to the column was recovered in the eluate. Of the total ^{125}I in the standards prepared from the infusate, $86.6 \pm 7.5\%$ was eluted as $^{125}\text{I}-3,3'\text{-}T_2$. The remainder of the ^{125}I was in the form of iodide and iodoprotein in approximately equal proportions. The mean $^{125}\text{I}-3,3'\text{-}T_2$ concentration in two or more samples taken during the steady-state was used in the determination of the clearance rate (CR).

$^{131}\text{I}-T_3$ and $^{125}\text{I}-rT_3$ were separated from other labeled components of serum by means of anion-exchange resin columns (Curtis Nuclear Corp., Los Angeles, Calif.) according to the method described by Nicoloff et al. (18). The experience of our laboratory with this method of analysis for $^{131}\text{I}-T_3$ and $^{125}\text{I}-rT_3$ has been described (12). All samples and dose standards from a kinetics study were processed at the same time. For each tracer, the results are expressed as the percent of the injected dose per liter of serum.

Calculations. In the constant infusion $3,3'\text{-}T_2$ study the CR was calculated from the infusion rate divided by the steady-state serum concentration of $^{125}\text{I}-3,3'\text{-}T_2$ achieved during the infusion (12). The production rate (PR) was calculated from CR multiplied by the serum concentration of stable $3,3'\text{-}T_2$ (mean of three to five samples taken during infusion). In each single injection (T_3 and rT_3) study, the plasma disappearance curve for each tracer was fit to a three-exponential function by the method of least squares. From the Y-intercepts and slopes of the exponential components the CR was computed by the method of Tait (19). The production rate was calculated as described for $3,3'\text{-}T_2$.

Statistical methods. All results have been expressed as mean $\pm$ SD. Statistical evaluations were performed by the unpaired Student's *t* test (20).

RESULTS

Radioimmunoassay for $L-3,3'\text{-}T_2$

Specificity. The reactivities of various compounds relative to $3,3'\text{-}T_2$ were calculated on the basis of the molar amount that caused 50% inhibition of the binding of $^{125}\text{I}-3,3'\text{-}T_2$ to antibody (Table I). The monoiodothyronines, $3\text{-}T_1$ and $3'\text{-}T_1$, demonstrated the greatest degree of cross-reactivity. T_4 and T_3 demonstrated negligible reactivity, whereas rT_3 showed a cross-reactivity of 0.6%. The calculated total contribution of T_4 , T_3 , and rT_3 was $<5\%$ of the observed $3,3'\text{-}T_2$ concentration, based on the cross-reactivity values and the known concentrations of these iodothyronines in normal serum.

Analysis of the autoradiographic bands eluted from the thin-layer chromatograph of rat bile demonstrated the presence of two labeled conjugates. The eluted bands containing the conjugates represented 52% of the total biliary counts applied to the chromatograph. Enzymatic hydrolysis of these bands with β -glucuronidase and Mylase-P in separate experiments released $^{125}\text{I}-3,3'\text{-}T_2$. Before enzymatic hydrolysis there was no detectable binding by specific $3,3'\text{-}T_2$ -binding antibody of the radioactive conjugates of $3,3'\text{-}T_2$. In control experiments it was determined that the enzymes did not affect the antibody binding of tracer.

Standard curve. Fig. 1 demonstrates a typical standard curve of $3,3'\text{-}T_2$ radioimmunoassay. Significant inhibition of the binding of $^{125}\text{I}-3,3'\text{-}T_2$ to anti-serum was evident with as little as 20 pg of stable $3,3'\text{-}T_2/\text{ml}$ serum (4 pg/tube). The sensitivity of the assay was determined from *t*-test analyses of data from multiple standard curves performed in triplicate. The data were expressed as percent bound (mean $\pm$ SD) and the first point on each standard curve demonstrating a significant difference ($P < 0.05$) at >2 SD from the

TABLE I
Related Cross-Reactivity of Various Iodothyronines
with $3,3'\text{-}T_2$ Antiserum

Compound	Relative cross-reactivity
	%
$L-3,3'\text{-}T_2$	100.0
$L-3\text{-}T_1$	10.3
$D,L-3'\text{-}T_1$	7.3
$L-rT_3$	0.6
$D,L-3',5'\text{-}T_2$	0.3
$L-T_3$	0.07
Triac*	0.046
$L-3,5\text{-}T_2$	0.014
$L-T_4$	<0.001
Tetrac*	<0.001

* Triac, 3,5,3'-L-triiodothyroacetic acid; Tetrac, 3,5,3',5'-L-tetraiodothyroacetic acid.

FIGURE 1 Standard curve for 3,3'-T₂ radioimmunoassay and the effects of varying dilutions of serum from a hyperthyroid patient on the binding of ¹²⁵I-3,3'-T₂ by antibody.

basal point was taken as the assay sensitivity. Fig. 1 also demonstrates that the binding curve of dilutions (with iodothyronine-free serum) of serum from a hyperthyroid patient was parallel to the standard curve.

Recovery. Treatment of sera with activated charcoal (11) demonstrated that 96–99% of the added ¹²⁵I-3,3'-T₂ was removed by this procedure. To determine the recovery of 3,3'-T₂ in this assay, various amounts

of stable 3,3'-T₂ were added to sera from normal, hyperthyroid, hypothyroid subjects, and from euthyroid individuals with idiopathic elevation in T₄-binding globulin (TBG). In 12 separate assays 3,3'-T₂ concentration was determined before and after enrichment of samples with stable 3,3'-T₂. A normal (5.9 ng/100 ml) and a hypothyroid serum (3.0 ng/100 ml) sample were enriched with 3.0 ng/100 ml. The percent recoveries were 112 and 100, respectively. A hyperthyroid (10.0 ng/100 ml) and a high TBG serum (5.0 ng/100 ml) sample were enriched with 9.0 ng/100 ml. The percent recoveries were 107 and 93, respectively. The mean 3,3'-T₂ recovery from the series of experiments was 103%.

Reproducibility. The intra- and interassay reproducibility was examined in samples with variable 3,3'-T₂ concentrations. The intra-assay coefficient of variation (SD/mean × 100) was 6.2%. The mean coefficient of variation of 10 specimens, varying in 3,3'-T₂ concentration from 2.0 to 9.0 ng/100 ml and assayed in duplicate four times in different assays, was 11.6%.

Serum 3,3'-T₂ concentration in health and disease. Table II presents data on serum 3,3'-T₂ concentration in normal subjects and patients with diverse levels of thyroid function as determined by clinical assessment and standard laboratory studies. The mean serum 3,3'-T₂ concentration in 13 healthy euthyroid subjects was

TABLE II
Serum 3,3'-T₂, T₄, T₃, and rT₃ Concentrations in Various Thyroid States

Group		Mean	SD	Range
Control subjects (n = 13)	3,3'-T ₂ , ng/100 ml	6.0	1.0	4.5–7.8
	T ₄ , μg/100 ml	7.2	1.1	5.5–8.9
	T ₃ , ng/100 ml	103	15.1	82–125
	rT ₃ , ng/100 ml	26	4	21–37
Hyperthyroidism (n = 25)	3,3'-T ₂ , ng/100 ml	9.0*	4.6	3.0–21.0
	T ₄ , μg/100 ml	14.8†	2.4	12.0–18.5
	T ₃ , ng/100 ml	239†	98	129–500
	rT ₃ , ng/100 ml	77†	33	41–160
Hypothyroidism (n = 17)	3,3'-T ₂ , ng/100 ml	2.7‡	1.1	<2.0–6.0
	T ₄ , μg/100 ml	2.1‡	0.7	<1.8–4.1
	T ₃ , ng/100 ml	62‡	27	24–105
	rT ₃ , ng/100 ml	8‡	4	<3–16
Hypothyroidism treated with T ₄ (n = 11)	3,3'-T ₂ , ng/100 ml	5.3	1.5	3.3–8.5
	T ₄ , μg/100 ml	7.9	2.1	5.4–12.2
	T ₃ , ng/100 ml	88	15	64–119
	rT ₃ , ng/100 ml	33	13	21–60
Patients with acute nonthyroidal systemic illness (n = 10)	3,3'-T ₂ , ng/100 ml	4.8	2.7	2.0–9.4
	T ₄ , μg/100 ml	7.4	2.0	5.6–11.0
	T ₃ , ng/100 ml	49‡	22	<10–87
	rT ₃ , ng/100 ml	43§	25	16–94

* Cf. normal *P* < 0.01.

† Cf. normal *P* < 0.001.

§ Cf. normal *P* < 0.05.

6.0 ± 1.0 ng/100 ml with a range from 4.5 to 7.8 ng/100 ml. The mean serum 3,3'-T₂ concentration in 25 untreated hyperthyroid patients was significantly higher than in normal subjects ($P < 0.01$) but extended over a wide range from 3.0 to 21.0 ng/100 ml. In the 17 untreated hypothyroid patients the mean serum 3,3'-T₂ concentration was significantly lower than in normal subjects ($P < 0.001$). The mean serum 3,3'-T₂ in 11 hypothyroid patients maintained eumetabolic on L-T₄ (100–200 µg/day) was not significantly different from the normal subjects. In 10 patients suffering from acute nonthyroidal systemic illness with decreased total serum T₃ (49 ± 22 ng/100 ml) and elevated rT₃ (43 ± 25 ng/100 ml), the mean 3,3'-T₂ level was within the normal range. These patients had a variety of disorders: three acute hepatitis; three unstable angina; two infectious disorders; one postdiabetic ketoacidosis and one postgastrectomy for peptic ulcer disease. In two subjects with high serum TBG levels the 3,3'-T₂ concentrations were within the normal range. There was no correlation between 3,3'-T₂ concentration and serum T₄, T₃, or rT₃ in any of the groups.

Synthroid and Cytomel tablets ground into powder, dissolved in 0.05 M NaOH, and diluted in iodothyronine-free serum, demonstrated 3,3'-T₂ levels consistent with those anticipated from their respective assay cross-reactivities.

Analyses of stable 3,3'-T₂ concentrations by radioimmunoassay in serum sample collected from controls and athyreotic subjects receiving T₄ (HT₄) or T₃ (HT₃) during the 8-h infusion period of the kinetics study

revealed minimal variation in its concentration. All of the samples were stored frozen for an adequate period of time after the tracer infusion period to allow for radioactive decay. The coefficients of variation (CV) of samples collected during the 8-h period for each group studied were: controls ($n = 7$) 11.9% (mean); HT₄ ($n = 5$) 18.5%, and HT₃ ($n = 3$) 15.9%. There was no evidence for a diurnal (24 h) variation in controls ($n = 2$), HT₄ ($n = 2$), or HT₃ ($n = 2$) subjects. Their respective CV on data analyzed from frequent samples collected over 24 h were 14.9, 22.6, and 21.3%.

Fig. 2 demonstrates that it is possible to maintain in an athyreotic subject the serum T₃ concentrations relatively stable over a 24-h period with frequent small doses of L-T₃. This was necessary to obtain steady-state T₃ kinetics studies in these subjects. The CV of T₃ concentration in these samples was 11.9%. The serum 3,3'-T₂ concentration did vary somewhat more but, as reported above, the CV for the two HT₃ cases studied over 24 h was 21.3%, indicating reasonable stability. In the HT₃ subjects (3) the serum T₃ CV was 12.6% over 8 h.

Kinetics studies. The results of Sephadex column chromatographic analyses of one of the serum samples collected during the ¹²⁵I-3,3'-T₂ infusion in one case are depicted in Fig. 3. Analysis of an aliquot of the infused dose is also shown. In the serum sample and in the infusate, ¹²⁵I-3,3'-T₂ and iodide were the major labeled constituents. The zone between the ¹²⁵I⁻ and ¹²⁵I-3,3'-T₂ peaks contained a number of areas of low radioactivity. These peaks were considered to be metab-

FIGURE 2 Serum concentrations during a 24-h period of T₃ (interrupted line) and 3,3'-T₂ (solid line) in a hypothyroid subject maintained eumetabolic on frequent oral doses (10 µg every 3 h) of L-T₃. The arrows (abscissa) indicate the dose times. On the left hand ordinate is the T₃ concentration, and on the right hand ordinate, the 3,3'-T₂ concentration. In each case, the hatched box indicates the mean $\pm$ 2 SD.

olites of ^{125}I -3,3'-T₂. The elution position of some of these metabolites, 3,3'-T₂ conjugates and 3'-T₁, was determined in separate column analyses. Fig. 3 also demonstrates that the elution profile of stable 3,3'-T₂, added to the 6-h sample before analysis on the Sephadex G-25 column, is superimposable on the ^{125}I -3,3'-T₂ peak. This confirms the position of ^{125}I -3,3'-T₂ in the eluted fractions from the column. In each subject studied at least two samples collected during the infusion period were analyzed to determine the ^{125}I -3,3'-T₂ concentration.

The clearance of ^{125}I -3,3'-T₂ was determined by the constant infusion method. In initial studies three separate serum samples collected during the interval from 2 h to the end of the infusion period were analyzed for ^{125}I -3,3'-T₂. Fig. 4 shows that the concentration of ^{125}I -3,3'-T₂ was relatively constant during this interval in all three cases, indicating that a steady state with respect to this tracer was achieved by the 2nd h after infusion. (No priming dose was given.) The CV of the ^{125}I -3,3'-T₂ concentration in these serum samples averaged 8.5%. In the subsequent studies two serum samples collected during each infusion, usually at 4 and at 8 h, were analyzed on the column. The ratio of the

FIGURE 4 Constant infusion of ^{125}I -3,3'-T₂ in three subjects. The plasma concentration of ^{125}I -3,3'-T₂, in counts per minute per milliliter, is plotted on a linear scale (ordinate) against the duration of infusion in hours (abscissa). The figure indicates that the ^{125}I -3,3'-T₂ concentration achieved a constant level within 2 h. (No priming dose was given).

^{125}I -3,3'-T₂ concentration in the final sample to that in the initial sample averaged 0.95 ± 0.08 in the entire group, again confirming that an isotopic steady state had been reached. In every case calculation of the clearance was based on the average of the ^{125}I -3,3'-T₂ concentrations in all serum samples collected beyond the 2-h point of the infusion.

The plasma concentration of 3,3'-T₂ and the kinetic parameters obtained in each of the subjects studied are shown in Table III. The mean total serum 3,3'-T₂ concentration in the control (C) group ($n = 7$) was not significantly different from the T₄-treated hypothyroid (H) group ($n = 5$). The mean total serum T₄ concentrations and free T₄ indices (normal 0.5–1.5) were almost identical for the two groups, at $7.7 \pm 1.0 \mu\text{g}/100 \text{ ml}$; 0.9 ± 0.2 in C, and $7.9 \pm 1.2 \mu\text{g}/100 \text{ ml}$; 0.9 ± 0.3 in H, respectively. The 3,3'-T₂ CR averaged 628 ± 218 liters/day in C and 840 ± 377 liters/day in H. 3,3'-T₂ PR were not significantly different ($39.8 \pm 19.8 \mu\text{g}/\text{day}$ in C vs. $33.9 \pm 12.5 \mu\text{g}/\text{day}$ in H).

To determine the quantitative significance of 3,3'-T₂ as a product of the metabolism of T₃ and rT₃, the absolute turnover rates of T₃, rT₃, and 3,3'-T₂ were determined in three athyreotic subjects on T₄ replacement. Table IV gives the kinetics studies results of each of these iodothyronines in the three subjects. The 3,3'-T₂ PR in each case was close to the combined PR of T₃ and rT₃. The average ratio of 3,3'-T₂ PR (nanomoles per day) to the combined triiodothyronine PR (nanomoles per day) was 1.08 ± 0.10 .

To estimate the magnitude of conversion of T₃ to 3,3'-T₂, kinetics studies were conducted on three athyreotic subjects receiving L-T₃ replacement at three or four hourly intervals. A single pulse injection of ^{131}I -T₃ and a constant 8-h infusion of ^{125}I -3,3'-T₂ were given. The PR of each was determined as described above. Table V presents the 3,3'-T₂ and T₃ kinetics results

FIGURE 3 Column chromatographic analysis of radioactivity in an aliquot of the administered dose (infusate) of ^{125}I -3,3'-T₂ (A) and in the 6-h plasma sample of a typical subject (B). (See text for details of the chromatographic method.) Arrows indicate position of 3,3'-T₂ and other iodothyronines, 3'-monoiodothyronine (3'-T₁), 3,3'-T₂ conjugates (T₂S), T₃, rT₃, and T₄ determined on identical columns. The insert in the bottom panel (dotted line) indicates the elution profile of stable 3,3'-T₂ added to the sample before column analysis and assayed by radioimmunoassay (RIA). Its position is superimposable on that of ^{125}I -3,3'-T₂.

TABLE III
Serum T_4 , 3,3'- T_2 Concentrations and Results of Kinetics Studies in Controls and T_4 -Treated Hypothyroid Patients

Subject	Body wt.	Age	Serum T ₄	3,3'-T ₂			
				Concn.*	CR†	PR	
	kg	yr	μg/dl	ng/liter	liters/day	μg/day	
Controls							
1	68	57	8.1	49	734	35.9	
2	85	59	7.8	42	688	28.9	
3	95	53	8.9	82	749	61.4	
4	82	30	6.0	45	360	16.2	
5	77	47	7.5	64	418	26.7	
6	66	31	7.0	74	970	71.8	
7	67	30	8.9	78	482	37.6	
Mean			7.7	62	628	39.8	
±SD			±1.0	±17	±218	±19.8	
Treated hypo- thyroids		T ₄ dose					
		mg/day					
8	88	62	0.150	8.3	46	912	41.9
9	71	64	0.150	8.1	47	928	44.1
10	76	79	0.125	6.5	31	1,374	42.6
11	77	60	0.200	7.0	40	623	24.9
12	79	64	0.100	9.5	45	366	16.5
Mean			7.9	42	840	33.9	
±SD			±1.2	±7.0	±377	±12.5	

Concn., serum concentrations.

* Each value is the mean of six or more serum determinations of samples collected.

† Corrected to 70 kg body wt.

in each subject. The mean total serum 3,3'- T_2 concentration (3.4 ± 1.2 ng/100 ml) was significantly lower than in the C group (6.0 ± 1.0 ng/100 ml) ($P < 0.001$). The 3,3'- T_2 CR averaged 653 ± 144 liters/day and the 3,3'- T_2 PR, 22.6 ± 10.7 $\mu\text{g/day}$. Although the latter value was

not significantly less than the C group (39.8 ± 19.8 $\mu\text{g/day}$), a study of a large number of T_3 -treated hypothyroid subjects could very well reveal a difference in 3,3'- T_2 PR compared to controls. The mean total serum T_3 concentration was high (1.53 ± 0.28 ng/100 ml) in

TABLE IV
Serum T_3 , rT_3 , 3,3'- T_2 Concentration and Results of Kinetics Studies in T_4 -Treated Hypothyroid Patients

Treated hypo-	thyroids	Body wt.	Age	T_4 dose	T_3			rT_3			3,3'- T_2			(3,3'- T_2 PR§)/ (T_3 PR + rT_3 PR)
					Concn.*	CR†	PR	Concn.*	CR†	PR	Concn.*	CR†	PR	
		kg	yr	mg/day	$\mu\text{g/liter}$	liters/day	$\mu\text{g/day}$	$\mu\text{g/liter}$	liters/day	$\mu\text{g/day}$	ng/liter	liters/day	$\mu\text{g/day}$	
8		88	62	0.150	0.52	35.0	18.2	0.28	93.5	26.2	46	912	41.9	1.17
9		71	64	0.150	0.78	29.0	22.6	0.28	117.0	32.8	47	928	44.1	0.98
10		76	79	0.125	0.62	22.9	14.2	0.36	95.6	34.4	31	1,374	42.6	1.08
Mean					0.64	28.9	18.3	0.31	102.0	31.1	41.5 [¶]	1,071 ^{**}	42.9	1.08
±SD					±0.13	±6.1	±4.3	±0.05	±13.0	±4.4	±9.1	±262	±1.1	±0.10

Concn., serum concentration.

* Each value is the mean of six or more serum determinations of samples collected during the kinetics studies.

† Corrected to 70 kg body wt.

§ Nanomolar ratio of PR.

Significantly different from the control mean: ^{||} ($P < 0.01$), [¶] ($P < 0.02$), ^{**} ($P < 0.02$).

comparison to the control subjects (1.03 ± 0.15 ng/100 ml) ($P < 0.001$). Although the mean T_3 CR (33.0 ± 4.3 liters/day) was normal, the mean T_3 PR (50.9 ± 13.8 μ g/day) was significantly higher ($P < 0.005$) than the previously reported normal value of 24.2 ± 4.1 μ g/day (12). Table V also shows that the PR T_3 in each subject was close to the daily dose of L- T_3 . It thus appears that almost 100% of the oral T_3 was absorbed. This finding agrees with previous studies (21).

The molar ratio of 3,3'- T_2 to T_3 PR varied among the three subjects from 0.36 to 0.92, indicating that 3,3'- T_2 is an important product of T_3 deiodination.

DISCUSSION

The present study confirms the presence of 3,3'- T_2 in normal human serum. The mean normal serum 3,3'- T_2 concentration obtained in our study (6.0 ± 1.0 ng/100 ml) is intermediate between the value of 7.6 ± 2.4 ng/100 ml reported by Wu et al. (6), who employed ethanol extraction of serum and the recently reported value, 4.3 ± 2.0 ng/100 ml, from Burger and Sakoloff (8), who analyzed unextracted serum. The presence of 3,3'- T_2 in normal whole serum at a slightly higher concentration (17.0 ± 1.0 ng/100 ml) measured by radioimmunoassay has also been reported by Burman et al. (7).

In hyperthyroidism, serum 3,3'- T_2 , like T_4 , T_3 , and rT_3 , was elevated on the average. There was, however, a wide range of values from moderate elevation to markedly high serum 3,3'- T_2 levels. The absence of a positive correlation between the serum total T_4 and the 3,3'- T_2 levels has been noted previously (22). It may be due to an increased 3,3'- T_2 CR, as preliminary studies in our laboratory demonstrate increased 3,3'- T_2 CR in hyperthyroidism (23). The normal serum 3,3'- T_2 levels in patients with elevated TBG concentration is consistent with the reported low binding affinity of 3,3'- T_2 for TBG, compared with T_4 (24), and normal levels in pregnancy (6).

The finding of normal serum 3,3'- T_2 levels in athyreotic subjects maintained eumetabolic on T_4 replacement confirms a previous report (22) and suggests that the major source of circulating 3,3'- T_2 is extrathyroidal production.

The original analysis of rat thyroglobulin by Roche et al. (1) suggested that 3,3'- T_2 was a major component of the iodothyronines present. This finding in the rat was not confirmed by the recent studies of Taurog et al. (25), who demonstrated that 3,3'- T_2 comprised $<2\%$ of the total 131 I in the thyroids of severely iodine-deficient rats and even less in the iodine-replete animal. In a recent report Burman et al. (7) found a 3,3'- T_2 concentration of 0.4 ± 0.03 μ g/g in normal thyroid tissue and a lack of a serum 3,3'- T_2 response secondary to thyrotropin-releasing hormone stimulation, findings indicative of a minimal rate of thyroidal secretion. That the thyroid gland is not a major source of circulating 3,3'- T_2 is confirmed by the similar 3,3'- T_2 PR in the euthyroid control group and in the athyreotic subjects in whom the only source of 3,3'- T_2 was exogenous T_4 . There are no other data available at present to compare with the PR of 3,3'- T_2 obtained in this study.

The rate of disappearance from the blood of tracer 3,3'- T_2 (as the racemic mixture) in man has been examined in a previous study and found to be extremely rapid (9). The achievement of a constant plasma level of 125 I-3,3'- T_2 during the first few hours of a constant infusion is consistent with this finding. However, quantitative estimates of 3,3'- T_2 CR have not been reported previously.

The metabolism of 3,3'- T_2 would be expected to yield moniodothyronines, thyronine itself, deaminated derivatives, and conjugated forms. The glucuronic acid conjugate of 3,3'- T_2 has been identified in the bile of the rat after the injection of labeled 3,3'- T_2 (26) and the sulfate conjugate of 3,3'- T_2 in the bile and urine of the dog after the administration of labeled T_3 (16). Stanbury and Morris (9) demonstrated

TABLE V
Serum T_3 , 3,3'- T_2 Concentration and Results of Kinetic Studies in T_3 -Treated Hypothyroid Subjects

Treated hypothyroid	Body wt.	Age	T ₃ dose	T ₃			3,3'-T ₂			(T ₃ PR)§/ (T ₃ dose)	(3,3'-T ₂ PR)¶/ (T ₃ PR)
				Concn.*	CR†	PR‡	Concn.*	CR†	PR‡		
	kg	yr		μg/liter	liters/day	μg/day	μg/liter	liters/day	μg/day		
9	69	64	5 μg every 3 h	1.24	33.4	41.4	20	599	12.0	1.03	0.36
11	71	60	10 μg every 4 h	1.57	28.6	44.8	41	816	33.5	0.75	0.92
13	85	65	10 μg every 3 h	1.79	37.1	66.4	41	544	22.3	1.01	0.42

Concn., serum concentration.

* Each value is the mean of six or more serum determinations on samples collected during the kinetics studies.

† Corrected to 70 kg body wt.

‡ Uncorrected for body wt.

§ Nanomolar ratio of PR.

the presence of two unidentified compounds in some serum samples chromatographed after the administration of ^{131}I -3,3'-T₂. The Sephadex column employed in the present study separates 3,3'-T₂ conjugates and 3'-T₁ from 3,3'-T₂. Even if the 3,3'-T₂-containing fractions from our columns did include labeled metabolites, however, the calculated CR would have underestimated the true CR in our subjects.

The magnitude of 3,3'-T₂ PR suggests that it is a major metabolite of the deiodination pathways. Tracer studies in both the rat (2, 27, 28) and the dog (4, 16) demonstrated that both T₃ and rT₃ are precursors of 3,3'-T₂.

Recent in vitro studies by Chopra et al. (5) in rat liver homogenates have demonstrated that both T₃ and rT₃ are converted to 3,3'-T₂. The finding of 5'-deiodination (phenolic ring) of rT₃ to 3,3'-T₂ by liver homogenates confirms the previous reports by Flock and Owen (29) who employed perfused rat liver and a recent study of the metabolism of thyroid hormones by cultured monkey hepatocarcinoma cells (30).

There has been no information, however, on the quantitative significance of 3,3'-T₂ as a metabolite of T₃ or rT₃ until the present study in humans. The magnitude of 3,3'-T₂ PR indicates that it is a major metabolite of both triiodothyronines. In all cases studied, the 3,3'-T₂ PR was close to the combined (T₃ + rT₃) PR, accounting for 90–110% of total turnover. This finding is supported by the demonstration that the conversion of T₃ to 3,3'-T₂ (in T₃-replaced subjects) was a major pathway of T₃ metabolism.

The finding that both T₃ and rT₃ are major precursors of 3,3'-T₂ and that the magnitude of their contribution may be variable could explain the observation of a normal serum 3,3'-T₂ concentration in acute nonthyroidal systemic illness with low serum T₃ but high serum rT₃.

Previous studies in normal humans have demonstrated that 5-deiodination of T₄ to rT₃ is at least as active as 5'-deiodination to T₃, the total activity of both pathways accounting for most of the T₄ metabolized (12, 31). The present finding that 3,3'-T₂ is the principal product of the metabolism of both rT₃ and T₃ indicates the importance of 3,3'-T₂ as an intermediate in T₄ metabolism. Furthermore, the finding implies that the other diiodothyronines, 3,5-T₂ and 3',5'-T₂, are of only minor quantitative significance.

Early studies by Roche et al. (1) demonstrated that 3,3'-T₂ was calorigenically active but this has not been confirmed by subsequent in vivo studies (25, 32–34). Recent studies have shown the ability of 3,3'-T₂ to enhance the uptake of radiolucine by thymocytes in culture, but the effect was seen only at high concentrations and so its biological significance is not clear (35). Even if it is not biologically active, 3,3'-T₂ must be regarded as a major intermediate in the deiodination

of iodothyronines. A recent report by Chopra (36) shows that 3,3'-T₂ is a weak inhibitor of the conversion of T₄ to T₃ in rat liver homogenates. This raises the possibility that 3,3'-T₂ may regulate the monodeiodination of T₄. Studies are presently in progress in our laboratory to elucidate further the significance of this intermediate in the peripheral metabolism of iodothyronines.

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