A Radioimmunoassay for Measurement of 3,3',5'-Triiodothyronine (Reverse T₃)

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ABSTRACT A highly specific antiserum to 3,3',5'-triiodothyronine (reverse T3, rT3) was prepared by immunization of rabbits with D,L-rT3-human serum albumin conjugate. Of the various thyroid hormone derivatives tested, only 3,3'-diiodothyronine $(3,3'-T_2)$ crossreacted significantly (10%) with rT3-binding sites on the antiserum, while thyroxine (T₄) and triiodothyronine (T_3) cross-reacted by less than 0.1%. The antiserum was used in a simple, sensitive, precise, and reproducible radioimmunoassay (RIA) for measurement of rT3 in ethanolic extracts of serum. The dose-response curves of inhibition of the binding of [125]rT3 to antibody obtained by serial dilutions of serum extracts were essentially parallel to the standard assay curve. Recovery of nonradioactive rT₃ added to serum before extraction averaged 93%.

Serum rT₃ concentrations were found to be (mean \pm SD) 41 \pm 10 ng/100 ml in 27 normal subjects, 103 \pm 49 ng/100 ml in 22 hyperthyroid patients, 19±9 ng/100 ml in 12 hypothyroid patients, and 54 ± 7 ng/100 ml in five subjects with elevated serum thyroxine-binding globulin; the values in each of the latter three groups of individuals were significantly different from normal. Reverse Ts was detected regularly in normal or supranormal concentrations in serum of 12 hypothyroid patients rendered euthyroid or mildly hyperthyroid by treatment with synthetic T₄. It is suggested that serum rT₃ values noted here should be taken to reflect the relative changes in serum rT₃ rather than its absolute values in health and thyroid disease. True serum rT₃ may be somewhat different because: (a) D,L-rT₃ employed in the standard curve and L-rT3 present in human serum may react differently with anti-D,L-rT₃. (b) Even though 3,3'-T₂, which cross-reacted 10% in rTs RIA, has been considered unlikely to be present in human serum, it may circulate in low levels. (c) Cross-reaction of T₄ in rT₃ RIA of 0.06% although small, could contribute to RIA estimates of rT₃; the effect of T₄ would be particularly important in case of serum of hyperthyroid patients. Thus, serum rT₃ concentration in hyperthyroid patients averaged $89\pm48 \ \mu g/100$ ml after correction for cross-reaction effects of T₄; this value was about 14% lower than that before correction (see above).

Serum rT₃ concentartion in cord sera of seven newborns averaged 136±19 ng/100 ml; it was clearly elevated and within the range of values seen in hyperthyroid patients. This was the case when the mean T₄ concentration in the newborn cord sera was moderately higher than normal and about one-half that in hyperthyroid patients, whereas serum T₃ was markedly below the normal adult level. A Pronase hydrolysate of thyroglobulin prepared from pooled normal thyroid glands contained 0.042, 3.0, and 0.16 $\mu g/mg$ protein of rT3, T4, and T3, respectively. The various data suggest that: (a) rT_3 is a normal component of human serum and thyroglobulin: (b) peripheral metabolism of T₄ is an important source of the rT_3 present in serum; (c) peripheral conversion of T₄ to T₃ and rT₃ may not necessarily be a random process.

INTRODUCTION

On the basis of recent studies of the metabolism of radioactive thyroxine (T₄), it has been proposed that iodine atoms from both the phenolic and tyrosyl rings of this molecule are removed randomly at a similar rate, resulting in the formation of 3, 5, 3'-triiodothyronine (T₃),¹

Dr. Chopra is the recipient of U. S. Public Health Service NIH Research Career Development Award 1K04AM-70225.

Received for publication 19 February 1974 and in revised form 29 April 1974.

¹ Abbreviations used in this paper: HSA, human serum albumin; morpho-CDI, 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-*p*-toluene-sulfonate; PBS, phosphate buffered saline; RIA, radioimmunoassay; $p_{,L-r}T_{s}$, 3,3'5'- $p_{,-}$ L-triiodothyronine (reverse T_{s}); L-r T_{s} , 3,3',5'-L-triiodothyronine; T_{1} , L-monoiodothyronine; T_{2} , L-diiodothyronine; T_{s} , 3,5,3'-L-triiodothyronine; T_{4} , L-thyronine; TBG, thyroxine-binding globulin; Tg: thyroglobulin; TSH, thyrotropin.

when monodeiodination occurs first in the phenolic ring, or of 3, 3',5'-triiodothyronine (reverse T₃, rT₃), when monodeiodination occurs first in the tyrosyl ring (1). While several studies have provided data which have clearly established that T₄ is indeed an important source of the body pool of T₃ both in man and in experimental animals, (2-8), there is a paucity of data on rT_3 . Thus, we do not know if it actually exists in normal man and, if so, what its sources may be. rT3 has been identified both in serum and thyroglobulin (Tg) of the rat (9, 10). However, there are apparently some important differences in the metabolism of rT₃ in the rat and in man. Thus, whereas the urinary and fecal excretion of injected radioactive rT₃ in the rat are very similar to that of radioactive T_4 (11), rT_3 is metabolized in man much faster than either T_4 or T_3 (12). Markedly rapid metabolism of rT₃ has led to the consideration that rT₃ may not exist naturally in man (12). Because of the apparent importance of these issues to an understanding of the metabolism of T₄ in man (1, 8, 13, 14), I undertook to develop a radioimmunoassay (RIA) method for its measurement in serum, which is described in this report. The data obtained with this method indicate not only that rT³ is normally present in the circulation of man but also that significant alterations do occur in its serum level in various thyroid diseases. These studies also suggest that T₄ is an important source of rT₃ in serum.

METHODS

Reagents. D, L-rT₃ was obtained through the courtesy of Dr. Robert I. Meltzer of Warner-Lambert Research Institute, Morris Plains, N. J. Various thyroid analogues were either purchased from Sigma Chemical Co., St. Louis, Mo., or provided by courtesy of Warner-Lambert Research Institute. 3,3'-L-diiodothyronine $(3,3'-T_2)$ was a gift from Drs. Hossein Gharib of the Mayo Clinic, Rochester, Minn. and Hans Cahnmann of National Institute of Health, Bethesda, Md. The radioactive [125] T₃ was kindly prepared from $3,3'-T_2$ by Drs. J. F. Jeffries and W. J. Green of Abbott Laboratories, North Chicago, Ill. It had a specific activity of 400-500 mCi/mg. It allowed approximately 10,000 cpm/30 pg of tracer added per assay tube. Human serum albumin (HSA) was purchased from Sigma Chemical Co., and 1-cyclohexyl-3 (2-morpholinoethyl) carbodiimide metho-p-toluene-sulfonate (morpho-CDI) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Preparation of rT_s -HSA conjugates. rT_s was conjugated to HSA by a modification of the method of Oliver, Parker, Brasfield, and Parker (15). To 25 mg of HSA in 12.5 ml of phosphate-buffered saline (0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.5) (PBS) I added, while stirring, 9 mg of p.L-rTs dissolved in a mixture of 2 ml of dimethylformamide and two drops of 0.4 M sodium hydroxide, 1 ng of [¹²⁵I]Ts, and 15 mg of morpho-CDI. The pH of the solution was adjusted to 7.5 with dilute HCI. After 10 min, an additional 5 mg of morpho-CDI was added. The reaction mixture was stirred continually at room temperature in the dark for 18 h. It was then dialyzed once against distilled water and subsequently against

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three changes of PBS, each time with 4 liters for 24 h at 4°C. The conjugate was stored frozen at -10° C. 50% of [¹²⁵I]T₃ added to the reaction mixture had been incorporated into the conjugate. Radioactive T₃ rather than radioactive rT₃ was employed to monitor the conjugation because the latter was not available at the time. The entire experiment was repeated at a later date with radioactive rT₃. 37% of the tracer [¹²⁵I]rT₃ was incorporated into the conjugate at this time.

Preparation of rT_s -binding antiserum. Five New Zealand rabbits were immunized with rT_s -HSA conjugate described above. 1 ml of solution of the conjugate containing 1 mg of HSA was emulsified in 1 ml of complete Freund's adjuvant (Perrin's modification, Calbiochem, San Diego, Calif.), and the emulsion was injected subcutaneously in the back at four sites. The injections of the emulsion were repeated at 2-wk intervals for four times and at monthly intervals thereafter. Blood for antiserum was drawn at 10– 15-day intervals after the third and subsequent injections.

Antibody to rT₃ was detected by incubation of 40 pg of ¹²⁵IlrT₃ with immunized rabbit serum, diluted 1:100-1: 1000 in 0.075 M barbital buffer, for 24 h and then separation of antibody-bound radioactivity by precipitation of the antibody with goat anti-rabbit gamma globulin serum ("second antibody", 16). When sera obtained from immunized rabbits after four injections of the conjugate were tested in a dilution of 1:100, all bound some (10-65%) of the added ¹²⁵I]rT₈. There was only a modest increase in the antibody titer after subsequent injections of the immunogen. The antiserum selected for use in the studies to be described was obtained from a rabbit after four injections of the conjugate. It was selected on the basis of the maximal specificity of its binding rT₃, the lowest threshold of detection of unlabeled rT₃ in a displacement assay (see below) as well as minimal or no interference in its binding of [125I]rT3 by addition of 300 µl of 63% ethanol in a final volume of incubation mixture (final ethanol concentration, 19%). The first two criteria were the same as used in selection of an antibody reagent for most RIAs. The last criterion was required because it was planned to measure rT_3 in 300-µl volumes of the extracts of the test sera where ethanol was present in a concentration of 63%.

The antiserum employed in the present studies bound 27-35% (mean 32, n=9) of a tracer (40 pg) of [¹²⁵I]rT₃ in dilution of 1:250. The bound to free ratio so obtained has been noted to be optimal for the sensitivity of RIAs (17).

RIA procedure. In 10×75 -mm disposable glass culture tubes, the various reagents were added in the following order: (a) 0.075 M barbital buffer containing 1% normal rabbit serum and 0.01% sodium azide (barbital buffer), adjusted to a final volume of 1 ml. Subsequent experiments indicated that PBS could be substituted for barbital buffer without an appreciable difference in the results. (b) 300 µl of ethanol (95% ethanol: water, 2:1 vol/vol) in standard curve tubes and an equal volume of an ethanol extract (see below) of test sera, representing 100 µl of original serum. (c) Nonradioactive rTs for standard curve. 100 μ l of various solutions of rT₃ was added to place 5 pg-3 ng rT₃ in tubes for a 10-12 point standard curve. rT₃ had been dissolved and diluted to a concentration of 10 μ g/ml in 0.01 M sodium hydroxide. Subsequent dilutions and those used in the standard curve were made in barbital buffer. (d)100 μ l of 1:25 diluted rT₃-binding antiserum. (e) approximately 10,000-15,000 cpm of [1251]rT3 (30-40 pg rT3) in 100 μ l of barbital buffer. After being mixed, tubes were incubated for 24 h at 4°C. Selection of 24 h as the time of incubation was based on pilot experiments that indicated that essentially maximal binding of $[1^{25}T]rT_8$ to antibody in this system had occurred by this time. (f) sufficient quantity (60-100 µl) of previously titered goat anti-gamma globulin to precipitate all of rabbit gamma globulin, including antibody bound radioactive rT_8. Subsequent steps of separation of bound from free radioactivity, correction for nonspecific binding or trapping of $[1^{25}T]rT_8$ in the precipitate, and plotting of the standard curve were undertaken as described previously for RIA of T₈ (18). The rT₈ content in 100 µl of test serum was read off the standard curve and results were expressed in nanograms of p_L -rT₈ per 100 ml.

Preparation of extracts of test sera. 0.5 ml of serum was mixed with 1 ml of 95% ethanol on a vortex mixer. The mixture was centrifuged at 1,000 g for 10 min to sediment precipitated proteins. A $300-\mu l$ aliquot of the supernate was used directly for rT_s assay.

Sources of sera. Serum was obtained from 27 normal subjects, who were healthy laboratory workers or relatives of patients or hospital staff, 22 untreated hyperthyroid patients with Graves' disease, and 12 patients with primary hypothyroidism. The clinical diagnosis of Graves' disease was based on the finding in each case of a diffuse goiter, high 24-h thyroid ¹³¹I uptake, and elevated serum T₄ and T_s concentrations as determined by RIAs (19, 20). Hypothyroidism was diagnosed on the basis of clinical examination, low serum T₄, and elevated serum thyrotropin (TSH) (21). Sera were also obtained from 12 hypothyroid patients when they received treatment with synthetic T₄ (Synthroid, Flint, Eaton & Co., Div. of Baxter Laboratories, Inc., Morton Grove, Ill.). Serum T₄ concentration in these patients varied between 7.5 and 25 μ g/100 ml. Sera of five patients with elevated serum concentration of thyroxinebinding globulin (TBG) were tested; two of them were in the third trimester of pregnancy, and three were receiving exogenous estrogen for contraception. Serum TBG concentration in these subjects, as determined by a competitive ligand-binding assay (22), varied from 4.2 to 5.4 mg/100 ml (normal range 1.6-4.2). Sera were also collected from cord bloods of seven full-term fetuses at the time of vaginal delivery. Alterations in serum rT₃ levels were tested in four Graves' disease patients receiving treatment with propylthiouracil. Additionally, serum rTs levels were measured throughout a day in hypothyroid patients receiving a daily dose of thyroid hormones; two of these patients received Synthroid, 0.2 and 0.3 mg, and two others Ts (Cytomel, Smith, Kline & French Laboratories, Philadelphia,

Pa.), 75 and 100 μ g. Blood was obtained from another patient who had allegedly ingested 900 tablets of Synthroid, 0.2 mg each, 3 days before this study. Sera were also obtained before and at periodic intervals after i.v. administration of 2 mg of rT₃ dissolved in sterile salt-poor HSA (Parke, Davis & Co., Detroit, Mich.) to two normal subjects. After separation by centrifugation, sera were stored frozen at -10° C.

Thyroglobulin. Thyroglobulin (Tg) was prepared from a pool of 10 apparently normal human thyroid glands obtained at autopsy; the methods of preparation have been described previously (23, 24). Tg was hydrolyzed with Pronase (Calbiochem) under the conditions described by Inoue and Taurog (25) and used by us previously (26). The hydrolysate was extracted with 2 vol of 95% ethanol, and rT₃ content of the extract was determined by the RIA described above. T₄ and T₃ content of this Tg was determined by RIA (26, 27).

RESULTS

Specificity. Results of studies showing the ability of nonradioactive rT₃, T₄, T₃, 3,3'-T₂, and monoiodothyronine (3-T₁) to inhibit the binding of $[125]rT_3$ to antibody are shown in Fig. 1. Addition of all of these agents resulted in progressive reduction in the proportion of [125] rT₃ bound to antibody with roughly similar dose-response curves. Significant inhibition of the binding of [125]T3 to antibody occurred with as little as 10 pg of rT₃, and the dose-response curve was essentially linear to 1 ng. The data on relative potency by weight of all of the various compounds compared to rT₃ are presented in Table I. Each of the compounds was tested in four or more doses, which ranged up to 500 ng/assay tube in the case of the iodothyronines and iodotyrosines, and up to 1 mg per assay tube in the case of Tg and potassium iodide. The relative reactivity of the various compounds was calculated on the basis of the amount that caused 50% or close to 50% inhibition of the binding of [125] rT3 to antibody. 3,3'-T2 demonstrated the most cross-reactivity with rT₃-binding sites on the antiserum. T₄, T₃, and the various other thyroid hormone derivatives, as well as iodide, demonstrated little or no effect



FIGURE 1 Dose-response curves. Inhibition of the binding $L^{[125]}I^{T_3}$ to $D,L-rT_3$ antibody by increasing quantities of $D,L-rT_3$, $3,3'-L-T_2$, $3-L-T_1$, $L-T_4$, and $L-T_3$ is shown on a semilogarithmic plot.

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TABLE IRelative Reactivity of Various Thyroid Hormone Derivatives
with rT3-Binding Antibody

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	Compound	Relative reactivity (arbitrary value if D,L-rT ₃ = 100)
	L-T ₃	0.02
	D-T ₃	0.006
	TRIAC	0.003
	3,5,3'-L-Triiodothyropropionic acid	0.005
	L-T4	0.06
	D-T4	0.045
	TETRAC	0.034
	Desaminothyroxine	0.045
	3,5-L-Diiodothyronine	< 0.002
	3,3'-L-Diiodothyronine	10.0
	3,5-L-Diiodothyropropionic acid	0.005
	3-L-Monoidothyronine	0.24
	L-Thyronine	< 0.002
	Diiodotyrosine	0.030
	Monoidotyrosine	< 0.02
	Potassium iodide	< 0.0003
	Tg (unhydrolyzed)	< 0.0003

TETRAC, 3,5,3',5',-L-Tetraiodothyroacetic acid; TRIAC, 3,5,3'-L-triiodothyroacetic acid.

on the binding of $[1^{25}I]rT_3$ to antibody. The cross-reaction data obtained in the presence of ethanol in a final concentration of 19% were no different from those in its absence. Since T₄ is present in very large quantities in serum and its cross-reaction with rT_3 -binding sites on the antiserum could result in a significant error in the measurement of rT_3 , the effect of T₄ on the binding of [¹²⁵I]rT₈ to antibody was checked especially carefully. Thus, the two batches of reagent T₄ and three lots of proprietary T₄ (Synthorid), tested in four assays, showed a cross-reaction varying between 0.06 and 0.09%; variation in cross-reactivity of different batches of T₄ suggests that at least part of the cross-reaction must be a result of contamination of commercial T₄ with rT₈. The smallest dose of T₄ which appreciably reduced the binding of [¹²⁵I] rT₈ to antibody varied between 15 and 20 ng/assay tube (Fig. 1). This amount of T₄ would be expected in 100 µl of serum, tested routinely, only when the serum T₄ concentration is 15 µg/100 ml or higher.

Comparison of samples with standards. The doseresponse curves of inhibition of the binding of [125]rT₃ to antibody produced by the ethanol extracts of serum of one newborn and a hyperthyroid patient, diluted in 63% ethanol, have been compared with the regular standard curve of rT₃ assay in Fig. 2; the final ethanol concentration was constant at 19% at each point. The various dose-response curves were essentially parallel. Similarly parallel dose-response curves were observed with extracts of the sera of another newborn another hyperthyroid patient, and two normal subjects. Also shown in Fig. 2 is a standard curve prepared in barbital buffer in the absence of ethanol. This curve was different from the standard curve used in RIA of serum extracts. The difference was noted mainly in the region of the curve representing low quantities, less than 50 pg/assay tube, of rT₃. It was probably related to the observation that the binding of $[125I]rT_3$ to antibody in zero-rT₃ tubes was about 10% less in the absence of ethanol than in its presence. The reason for the higher binding of ¹²⁵I]rT₃ to antibody in presence of ethanol than in its absence is not clear. It was not attributable to higher



FIGURE 2 Comparison of the standard assay curve and the pattern of inhibition of $L[^{125}I]rT_3$ binding to $p,L-rT_8$ antibody produced by different doses of ethanol extracts of newborn cord serum and serum of a hyperthyroid patient. A dose response curve, obtained with unlabeled $p,L-rT_8$ when ethanol was not added to the reaction mixture, is also shown.

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nonspecific binding (or trapping) of $[1^{25}I]rT_3$ in the second antibody precipitate of the tubes containing ethanol; the nonspecifically bound radioactivity (18) varied between 1.5 and 3% of total radioactivity both in the presence or absence of ethanol.

Effect of charcoal treatment on serum rT_s concentration. To evaluate the effect on the RIA of ethanol extracts of serum with reduced rT_s content, rT_s was measured in extracts of sera before and after treatment of the sera with activated charcoal (28). Pilot experiments with four sera, using [¹²⁵I]rT_s, had shown that 82–88% of added radioactivity was removed by this procedure. The data shown in Table II demonstrate that little or no immunoassayable rT_s remains in sera after charcoal treatment.

Effect of addition of T_* on serum rT_* concentration. Although cross-reaction studies had shown that T_* would have little interference in the rT_* assay, the effect of addition of T_* to serum on immunoassayable rT_* was studied. A T_* preparation, which had shown 0.06% cross-reactivity in the rT_* assay, was added in concentrations varying between 15 and 100 μ g/100 ml to sera with variable rT_* concentration. The data on serum rT_* levels measured in these sera are shown in Table III. It is apparent that addition of this T_* to serum does not increase the rT_* estimated by the RIA by any more than the 0.06% expected from studies of specificity of RIA.

Recovery experiments. The mean recovery of radioactive rT_s added to 10 sera was $89\pm1.5\%^2$ (range 84– 94). Recovery did not vary systematically in sera from different classes of patients. In separate experiments, mean recovery of nonradioactive rT_s added to ethanol extracts of a pooled serum from hospitalized patients in amounts ranging from 50 to 500 ng/100 ml was $96\pm0.5\%$ for 50 ng (n = 4), $101\pm1.5\%$ for 100 ng (n = 4), $99\pm$ 6% for 300 ng (n = 4), and $106\pm3.2\%$ for 500 ng (n =3). The mean recovery of all 15 experiments was $101\pm$

² The data here and subsequently are presented as mean \pm SE unless specified otherwise.

TABLE II Effect of Charcoal Treatment on Serum rT₃ Concentration

Experiment	Before treatment	After charcoal treatment	
	ng/100 ml		
1	$69 \pm 1.2 \ (3)^*$	$<10\pm0$ (3)	
2	62 ± 4.1 (3)	$<10\pm0$ (3)	
3	39 ± 3.1 (3)	11 ± 1.7 (3)	
4	54 ± 0 (2)	$14 \pm 3.9 (7) +$	
5	121 ± 6 (4)	$<10\pm0$ (4)	
6	179 ± 5 (4)	12 ± 4 (4)	
7	95 ± 4 (4)	$<10\pm0$ (4)	
8	153 ± 7 (4)	$<10\pm0$ (4)	

* Serum rT₃ concentration, mean \pm SD (*n*).

2%. The rT₃ concentration in the serum extract was determined to be 54 ng/100 ml. The recovery of nonradioactive rT₃ added to the same pooled serum before ethanol extraction was $96\pm2\%$ for 50 ng (n=4), $91\pm4\%$ for 100 ng (n=4), $91\pm4\%$ for 300 ng (n=4), and $93\pm3\%$ for 500 ng (n=4). The mean recovery for all 16 experiments was $93\pm1\%$.

Reproducibility. Both intra- and interassay reproducibility was examined in samples with variable rT_3 concentration. The mean coefficient of variation (SD/mean \times 100) for 28 samples varying in rT_8 concentration from 28 to 240 ng/100 ml, each of which was tested in duplicate or quadruplicate in the same assay, was $5.6\pm0.8\%$. The mean coefficient of variation of four specimens varying in rT_8 concentration from 50 to 100 ng/100 ml and assayed in duplicate two to six times in different assays was $10.3\pm0.8\%$. No systematic differences in reproducibility were noted in serum rT_8 values read off the various portions of the standard curve.

Serum rT_{3} concentration in health and disease. Table IV presents data on serum rT_{3} , T₄, and T₃ concentrations in normal subjects and patients with various levels of thyroid function. The mean serum rT_{3} concentration in 27 healthy euthyroid subjects was 40.5 ng/100 ml. Nor-

Effect of Addition of T_4 on Serum rT_3 Concentration								
	T_{4}^{*} added, ($\mu g/100 \ ml$)							
Experiment	0	15	25	50	100			
			ng/100 ml					
1	$<10\pm0$ (3)‡		11 ± 1.4 (2)					
2	14 ± 3.9 (7)	21 ± 3.0 (5)	24 ± 4.2 (5)	35 ± 3.0 (6)	49 ± 4.7 (4)			
3	50 ± 3.0 (4)		61 ± 2.3 (4)	74 ± 4.0 (4)	98 ± 5.2 (4)			
4	67 ± 6.9 (3)	68 ± 2.0 (3)	75 ± 2.7 (3)	100 ± 12 (3)	120 ± 5.7 (3)			

TABLE III Effect of Addition of T_4 on Serum rT_3 Concentration

* Tested separately, this T_4 cross-reacted 0.06% in rT_3 assay.

 \ddagger Serum rT₃ concentration, mean \pm SD (*n*).

Group		n	Mean	SD	Range
Normal subjects	rT ₃ ,*ng/100 ml	27	40.5	10.4	27-62
	T ₄ , µg/100 ml	26	8.6	1.6	5.6-13
	T ₃ , ng/100 ml	16	118	33.8	72–195
Hyperthyroidism due to	rT ₃	22	103¶	48.6	54-230
Graves' disease	T4	22	23.3¶	5.7	17-38
	T ₃	22	$744\P$	245	420-1225
Hypothyroidism	rT ₃	12	18.6¶	9.2	<10-35
	T_4	12	5.7¶	1.0	<0.3-2.8
	T_3	11	32.5¶	18.5	<17-85
	TSH, $\mu U/ml$	11	137	60	16->200
Hypothyroidism treated	rT ₃	12	54.8	18.6	20-90
with T ₄ **	T4	12	14.4¶	6.3	7.5-25
	T_3	12	174	74	80-336
Patients with elevated	rT ₃	5	54.2§	6.5	40-64
serum TBG	T_4	5	15.0¶	6.4	8.4-22
	T_3	5	195‡	126	72-328
	TBG, <i>mg/100 ml</i>	5	4.6¶	0.5	4.1-5.4
Newborn	rT ₃	7	136¶	29	100-180
(term fetus cord serum)	T_4	7	11.9¶	2.4	6-15.5
	T_3	7	24¶	9.7	<15-40

TABLE IV Serum rT_3 , T_4 , and T_3 Concentrations in Various Thyroidal States

* Results expressed in terms of weight equivalent of D,L-rT₃.

 \ddagger Cf. normal P < 0.02.

§ Cf. normal P < 0.01.

|| Cf. normal P < 0.005.

¶ Cf. normal P < 0.001.

** Mean (\pm SD) dose of synthetic T₄ was 0.25 \pm 0.07 mg/day.

mal men and women had comparable levels of serum rT₃, and for this reason values in both sexes have been combined. The serum rT₃ concentration of 103 ng/100 ml in 22 hyperthyroid patients was significantly higher than in normal subjects (P < 0.001). When the serum rT₃ values in individual hyperthyroid patients were corrected for the cross-reaction effects of the T4 in their serum, the mean ±SD range value of serum rT₃ in hyperthyroid patients decreased to 89±48 (42-212 ng/100 ml; this value was also significantly higher than that in normal subjects (P < 0.001). The mean serum rT₃ concentration of 18.6 ng/100 ml in 12 hypothyroid patients was significantly lower than the corresponding value in euthyroid subjects (P < 0.001). The mean serum rT₃ in 12 hypothyroid patients treated with synthetic T₄ in a dose of 0.15-0.4 mg/day (mean, 0.25±0.02), was 54.8 ng/100 ml, significantly greater than the normal mean value (P < 0.005). The mean serum T₄ and T₃ concentrations in these patients were also significantly higher than those in normal subjects. The five subjects whose serum TBG concentration was high-normal or high had a mean serum rT₃ concentration of 54.2 ng/100 ml,

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which was significantly higher than the corresponding normal value (P < 0.01). The mean serum rT₃ concentration of 136 ng/100 ml in seven newborn cord sera was also significantly higher than that in normal adults. Serum T₄ concentration in the newborn was modestly higher than that in the adult. On the other hand, the mean newborn serum T₃ of 24 ng/100 ml was significantly lower than the corresponding adult value.

The alterations in serum rT_3 and T_4 concentrations were studied in four Graves' disease patients receiving treatment with propylthiouracil. Serum rT_3 concentration of 65 and 58 ng/100 ml was high or high-normal, respectively, in two patients who were still hyperthyroid during the early phase of treatment; it fell to or below normal levels with continuation of therapy. Serum rT_3 concentration, maintained in the normal range during antithyroid drug treatment in another two patients, increased to supranormal levels (90 and 100 ng/ 100 ml) when they became hyperthyroid four weeks after the termination of a 1-yr course of antithyroid therapy.

Fig. 3 presents the curve describing the disappearance of rT₃ from serum of two healthy laboratory workers who received a bolus of 2 mg of D,L-rT₃ intravenously. The baseline serum rT3 concentration in these subjects was 44 and 33 ng/100 ml. It increased to 41.4 and 43.2 μ g/100 ml, respectively, 10 min after the injection and steadily fell thereafter. The rate of disappearance of nonradioactive rT₃ from serum of these patients was similar to that reported previously for the similar racemic mixture of radioactive rT3 (12). rT3 (% dose/liter) remaining in serum 1 h after injection of rT3 was 2.4 and 2.5% in two cases in the present study, whereas it varied between 1.0 and 9.8% (mean, 4.5) in seven patients reported previously (12). The rapid rate of disappearance of rT3 from serum in the first few hours makes it difficult to estimate the half-life of rTs in the circulation or its volume of distribution with any degree of accuracy.

Serum rT₃ concentration was measured for a day in hypothyroid patients receiving commercially available preparations of T₄ and T₃ for 2 wk or longer. Serum rT₃ levels were normal (36-50 ng/100 ml) throughout the day in two patients taking replacement doses of 0.2 and 0.3 ng of T₄. Serum rT₃ levels were 12 ng/100 ml or less during a day in two patients taking 75 and 100 μ g of T₃/ day; serum T₃ concentration in these patients was 495 and 1.050 ng/100 ml, respectively, 4 h after administration of T₃, and gradually decreased thereafter. Serum levels rT₃, T₄, and T₃, of 250 ng/100 ml, 83 µg/100 ml, and 2,600 ng/100 ml, respectively, in the patient (P.M.) who had allegedly ingested 180 mg of T₄ three days before study were markedly elevated; the values decreased modestly to 196 ng/100 ml, 72 μ g/100 ml, and 2,200 ng/ 100 ml, respectively, 24 h after the initial study.

Reverse T₅, T₄, and T₅ in normal Tg. Reverse T₅, T₄, and T₅ content of Tg prepared from pooled normal thyroid glands of 10 subjects was 0.042, 3.0, and 0.16 μ g/ mg protein, respectively. The T₄/rT₃ ratio in this Tg was 71.4, whereas the T₄/T₃ ratio was 18.7.

DISCUSSION

The present studies have demonstrated that it is feasible to prepare a highly specific rT_{a} -binding antiserum with an affinity suitable for detection of picogram quantities of nonradioactive rT_{a} . These studies highlight again the important influence that presence of a single iodine atom in the thyronine nucleus may have on the nature of the antibodies generated by the rabbit; for example, T_{a} showed little cross-reaction with the rT_{a} antibody. In this respect, the experience with rT_{a} is very similar to the previous experience with T_{a} -binding antibody (18, 19, 28–30). In addition, however, the present studies have brought out another interesting aspect of iodothyronine antibodies by indicating the marked difference made by a shift in the location of one iodine atom from



FIGURE 3 Disappearance of immunoassayable rT_3 from serum of two normal subjects, each of whom received 2 mg of p,L-rT₃ i.v. Serum rT₃ concentration is plotted on the y-axis on a logarithmic scale and time in hours on x-axis on an arithmetic scale. Zero h represents the time of injection of p,L-rT₃.

the 5' position, in case of rT_{s} , to the 5 position, in case of T_{s} .

Of various thyroid hormone derivatives studied, only 3. 3'-T₂ cross-reacted significantly (10%) with rT₃ antibody. This ought to be considered, however, the maximal estimate of the cross-reaction. The possibility that 3, 3'-T2 was contaminated with rT3 cannot be excluded. Significant interference of 3,3'-T2 in rT3 assay may also be related to the possible presence of some 3,3'-T2 antibody in the serum of rabbits immunized with rT₃-HSA conjugate; this could happen if the 5' iodine of rT₃ were lost during the process of conjugation or if the rT₃ used to prepare rT₃-HSA conjugate were contaminated with 3,3'-T2. In any case, the practical significance of cross-reaction with $3,3'-T_2$ is difficult to assess because little information is available on its presence or concentration in human serum. It has, however, been detected in both serum and thyroid gland of the rat (9, 31). Its turnover in man is so rapid that its presence in human serum has been considered highly unlikely (32). In contrast with 3,3'-T2, the effect of the cross-reaction with T4 is clearly impotrant in the rT₃ assay because of large amounts present in serum compared to rT3. However, the remarkably low cross-reaction of T₄ with rT₃ antibody held the effect of T₄ on the rT₃ measurement to an acceptably low level. Thus, in the case of normal subjects, whose serum T₄ concentration averaged 8.6 μ g/100 ml (Table III), the cross-reaction of T₄ with rT₃ antibody of 0.06% would be expected to increase average normal serum rT₃ artifactually by no more than 5.2 ng/ 100 ml.

Distinct from our usual practice of using unmodified serum in RIAs of iodothyronines (19, 20), the present study employed ethanol extracts of serum for RIA of rT_3 . This was done (a) because little information is currently available regarding the nature of serum protein binding of rT_3 and (b) on the basis of a previous report which indicated rapid metabolism of rT₃ in man, we expected to find, if any, only small quantities of rT₃ in serum; therefore it was felt important to devise a RIA where potential interference due to serum proteins would be minimal. The present studies have indicated that ethanol extraction is a simple and reliable procedure for extraction of rT₃ from serum and that antisera can be generated that allow an accurate and precise measurement of rT₃ in ethanol extracts of serum. The experience with rT₃ was similar in these respects to that previously reported for RIA of T₃ in serum (33, 34).

As might have been expected from previous data in the rat and in man (1, 9, 10), the present studies indicate that rT₃ is indeed a normal constituent of human serum. The observed mean serum rT₃ concentration of 41 ng/100 ml could not be attributed to a cross-reaction of T₄ in the rT₃ assay; this value was about seven times greater than what could be expected merely from cross-reaction of T₄ (see above). However, since 3,3'-T₂ cross-reacted 10% in the rT₃ RIA, one could consider the estimated normal mean serum rT₃ concentration of 41 ng/100 ml to be a result of the 410 ng/100 ml of 3,3'-T₂ in serum. However, this appears highly unlikely. The half-time of disappearance of 3,3'-T₂ has been estimated to be 1 h or less (32). Even if 3,3'-T2 is considered to be distributed only in the plasma volume of approximately 3 liters, a serum concentration of 410 ng/100 ml would correspond to a calculated production rate of 3,3'-T2 of about 205 µg/day, which is over twice that of T4. This could occur if 3,3'-T2 were much more abundant in the human thyroid than T₄. There is, however, no evidence in man or in experimental animals to favor such a consideration, nor do the studies of Tg noted here support this possibility at all (see above).

Since the $D_{r}L^{-}T_{s}$ used in the standards is different from the L-rT_s assumed to be present in man, it is possible that the true rT_s concentration in human serum may be somewhat different from that noted in the present study. However, a major difference is unlikely since in several studies, D-isomers of T_s and T₄ have been noted to react with T_s and T₄ antibodies with a potency similar to and varying between 82 and 140% that of the corresponding L-isomers (18, 19, 27, 29). However, D-T_s reacted with one anti-T_s with a potency only 39% that L-T_s (35). In any case, even if the D- and L-isomers of

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rT₃ were to react differently with rT₃ antibody, this would only alter the true serum concentration of L-rT₃ but would not limit the interpretation of relative changes in serum rT₃ in health and disease presented in this report. Serum rT₃ values presented here should be interpreted to reflect relative changes in various situations rather than as absolute (true) serum rT₃ values also because of (*a*) some (0.06%) cross-reaction of T₄ in rT₃ RIA and (*b*) 3,3'-T₂, which cross-reacted 10% in rT₃ RIA. Although the latter has been considered unlikely to be present in human serum (32), it may circulate in low levels.

To assess the sources of rT_3 in human serum, we examined serum rT_3 concentration in hypothyroid or athyreotic patients receiving synthetic T₄. Reverse T₃ was detected in the serum of each of these patients, and this could not be attributed to contamination of proprietary T₄ with rT_3 antibody was found to be no more than that observed with reagent T₄, and this was insufficient to explain the rT_3 measured in serum of these patients. On the other hand, our findings were consistent with the thesis, suggested on the basis of a study of the metabolism of radioactive T₄ (1), that just as in the case of T₃, peripheral metabolism of man.

Study of Pronase digest of human Tg revealed the presence in it of rT_s -like immunoreactive material in an amount approximately 1.4% that of T₄. If 3,3'-T₂ were present in human Tg in quantities approximately 14% that of T₄, it could explain the finding of the observed amount of rT_s in human Tg. However, until later studies can prove this to be the case, it would appear that in addition to peripheral metabolism of T₄ (see above), thyroidal secretion may also be a source of rT_s in human serum.

The changes in serum rT₃ values noted in hyper- or hypothyroid patients appeared similar to those observed in the case of T₄ and/or T₃ levels in serum of these patients, i.e., they were high-normal or clearly high in hyperthyroid patients, and low-normal or low in hypothyroid patients. The changes in serum rT₃ in these patients may signify the influence of the availability of serum T₄ for conversion to rT₃, a change in thyroidal activity, or a combination of these factors. The serum rT₃ values in patients with elevated serum TBG levels also appeared to vary in the same direction as serum T_{4} and T_{3} levels, i.e., they were high-normal or high. These findings suggested that, like T4 and T3, rT8 may also be bound to TBG. The situation in which changes in serum rTs deviated strikingly from those expected from serum T4 and T3 values was in the newborn. Thus, serum rT3 values in the term fetus cord serum were higher than or in the range of values seen in hyperthyroid adults. The mean serum T₄ in newborns, however, was only about one-half that in hyperthyroid patients (Table IV). As against serum rT₃, serum T₃ concentration in the newborn was comparable to that in hypothyroid patients. The serum T₃ and T₄ values in the cord sera reported here are comparable to those previously reported by others and by us (14, 28, 36). The data on serum rT₃ levels in the newborn are intriguing. They could signify that the peripheral metabolism of T₄ may be different in the newborn than in the adult. It has already been suggested that peripheral conversion of T₄ to T₃ may be less in the newborn than in the adult (36). The serum rT₃ and T₃ values in the present study would suggest that conversion of T₄ to rT₃ or to T₃ may not occur randomly in the fetus, as has been suggested to be the case in the adult (1) and that conversion of T₄ to rT₈ may be a major pathway of degradation of T₄ at the time of birth. This in turn raises the question whether nonrandom conversion of T4 to rT3 and T3 might also occur under some conditions in adults. Obviously, studies in the newborn of the peripheral degradation and the thyroidal content of rT₃ are necessary to validate these considerations. However, if the characteristics of the peripheral metabolism of T4 or T3, as well as the thyroidal content of T4 and T3, in the newborn are any indication of the changes in these parameters in the case of rT₃, one could predict that the elevated cord serum rT₃ is due mainly to a difference in metabolism of T₄ in the newborn compared to the adult. Thus, the thyroidal content of T₄ and T₃ in the human term fetus is very similar to that in the adults (36), and the peripheral degradation rate of T₄ and T₃ in the fetal sheep is not lower but much greater than that in the adult (37).

Although the physiological significance of the conversion of T₄ to T₃ (2-8), a biologically more potent iodothyronine (38), can be readily appreciated, the physiological function, if any, of the conversion of T4 to rT_3 , a relatively inert iodothyronine (39, 40), remains unclear. rT₃ has been shown to be capable of inhibiting the calorigenic effects of T₄ as well as slowing its disappearance from the circulation (41-43). These considerations lead one to suspect that rT₃ may not be just an inactivation product of T₄, but that it may be involved in physiological regulation of metabolism and biological action of T₄. Additionally, it is tempting to consider that conversion of T_4 to T_3 and/or to rT_3 may be physiologically regulated phenomena, similar to those proposed recently by DeLuca, Omdahl, and Tanaka for vitamin D₈ (44-46). These workers have made a fascinating discovery of two parallel enzyme systems in the kidney which lead to the conversion of 25-hydroxyvitamin D₃ (25-OH-D₃) to either biologically more active 1,25(OH)2-D3 or to a relatively biologically inert 24,25-(OH)2-D3. Generally, when 1,25-(OH)2-D3 production is diminished, $24,25-(OH)_2$ -D₃ production is increased (44-46). In this respect, the findings of the present study, i.e., that serum rT₃ is markedly elevated in the newborn whose serum T₃ is markedly low, are similar. However, further study is needed to ascertain whether there is indeed an analogy between the metabolism of T₄ and 25-OH-D₃.

ACKNOWLEDGMENTS

I am indebted to Dr. David H. Solomon for helpful advice and critical review of the manuscript. I am very grateful to Dr. R. I. Meltzer, Warner Lambert Research Institute, Morris Plains, N. J., for the generous supply of rT_3 ; to Dr. Hossein Gharib, Mayo Clinic, Rochester, Minn., and Dr. Hans Cahnman, National Institute of Health, Bethesda, Md., for the supply of 3,3'-diiodothyronine; and to Drs. J. F. Jeffries, and William J. Green, Abbott Laboratories, North Chicago, Ill., for preparation of radioactive rTs. I appreciate the help of Dr. Jacques Orgiazzi, the careful technical assistance of Ms. Guadalupe N. Chua Teco, and expert secretarial assistance of Ms. Darling Mata, Ms. Irene Mestad, and Ms. Hollace Sokol.

These studies were supported by the National Institute of Health Grant No. AM-16155 and NIH Research Career Development Award K04 AM-70225.

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