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Research Article

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A Radioimmunoassay for Measurement of 3,3′,5′-Triiodothyronine (Reverse T_3)

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ABSTRACT A highly specific antiserum to 3,3′,5′-triiodothyronine (reverse T_3, rT_3) was prepared by immunization of rabbits with D,L-rT_3-human serum albumin conjugate. Of the various thyroid hormone derivatives tested, only 3,3′-diiodothyronine(3,3′-T_2) cross-reacted significantly (10%) with rT_3-binding sites on the antiserum, while thyroxine (T_4) 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 rT_3 in ethanolic extracts of serum. The dose-response curves of inhibition of the binding of [^{125}I]rT_3 to antibody obtained by serial dilutions of serum extracts were essentially parallel to the standard assay curve. Recovery of nonradioactive rT_3 added to serum before extraction averaged 93%.

Serum rT_3 concentrations were found to be (mean ±SD) 41±10 ng/100 ml in *27* normal subjects, 103±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 T_3 was detected regularly in normal or supranormal concentrations in serum of 12 hypothyroid patients rendered euthyroid or mildly hyperthyroid by treatment with synthetic T_4. It is suggested that serum rT_3 values noted here should be taken to reflect the relative changes in serum rT_3 rather than its absolute values in health and thyroid disease. True serum rT_3 may be somewhat different because: (*a*) D,L-rT_3 employed in the standard curve and L-rT_3 present in human serum may react differently with anti-D,L-rT_3. (*b*) Even though 3,3′-T_2, which cross-reacted 10% in rT_3 RIA, has been considered unlikely to be present in human serum, it may circulate in low levels. (*c*) Cross-reaction of T_4 in rT_3 RIA of 0.06% although small, could contribute to RIA estimates of rT_3; the effect of T_4 would be particularly important in case of serum of hyperthyroid patients. Thus, serum rT_3 concentration in hyperthyroid patients averaged 89±48 μg/100 ml after correction for cross-reaction effects of T_4; this value was about 14% lower than that before correction (see above).

Serum rT_3 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 T_4 concentration in the newborn cord sera was moderately higher than normal and about one-half that in hyperthyroid patients, whereas serum T_3 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 μg/mg protein of rT_3, T_4, and T_3, respectively. The various data suggest that: (*a*) rT_3 is a normal component of human serum and thyroglobulin: (*b*) peripheral metabolism of T_4 is an important source of the rT_3 present in serum; (*c*) peripheral conversion of T_4 to T_3 and rT_3 may not necessarily be a random process.

INTRODUCTION

On the basis of recent studies of the metabolism of radioactive thyroxine (T_4), 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 (T_3),[1]

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[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; D,L-rT_3, 3,3′5′-D,-L-triiodothyronine (reverse T_3); L-rT_3, 3,3′,5′-L-triiodothyronine; T_1, L-monoiodothyronine; T_2, L-diiodothyronine; T_3, 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 T_3, rT_3), when monodeiodination occurs first in the tyrosyl ring (1). While several studies have provided data which have clearly established that T_4 is indeed an important source of the body pool of T_3 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. rT_3 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 rT_3 in the rat and in man. Thus, whereas the urinary and fecal excretion of injected radioactive rT_3 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 rT_3 has led to the consideration that rT_3 may not exist naturally in man (12). Because of the apparent importance of these issues to an understanding of the metabolism of T_4 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 rT_3 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 T_4 is an important source of rT_3 in serum.

METHODS

Reagents. D, L-rT_3 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}I]rT_3 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_3-HSA conjugates. rT_3 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 D,L-rT_3 dissolved in a mixture of 2 ml of dimethylformamide and two drops of 0.4 M sodium hydroxide, 1 ng of [^{125}I]T_3, and 15 mg of morpho-CDI. The pH of the solution was adjusted to 7.5 with dilute HCl. 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 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 [^{125}I]T_3 added to the reaction mixture had been incorporated into the conjugate. Radioactive T_3 rather than radioactive rT_3 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 rT_3. 37% of the tracer [^{125}I]rT_3 was incorporated into the conjugate at this time.

Preparation of rT_3-binding antiserum. Five New Zealand rabbits were immunized with rT_3-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 rT_3 was detected by incubation of 40 pg of [^{125}I]rT_3 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 [^{125}I]rT_3. 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 rT_3, the lowest threshold of detection of unlabeled rT_3 in a displacement assay (see below) as well as minimal or no interference in its binding of [^{125}I]rT_3 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 [^{125}I]rT_3 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 rT_3 for standard curve. 100 μl of various solutions of rT_3 was added to place 5 pg–3 ng rT_3 in tubes for a 10–12 point standard curve. rT_3 had been dissolved and diluted to a concentration of 10 μg/ml in 0.01 M sodium hydroxide. Subsequent dilutions and those used in the standard curve were made in barbital buffer. (*d*) 100 μl of 1:25 diluted rT_3-binding antiserum. (*e*) approximately 10,000–15,000 cpm of [^{125}I]rT_3 (30–40 pg rT_3) in 100 μl 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 [^{125}I]rT$_3$ 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$_3$. Subsequent steps of separation of bound from free radioactivity, correction for nonspecific binding or trapping of [^{125}I]rT$_3$ in the precipitate, and plotting of the standard curve were undertaken as described previously for RIA of T$_3$ (18). The rT$_3$ content in 100 μl of test serum was read off the standard curve and results were expressed in nanograms of D,L-rT$_3$ 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-μl aliquot of the supernate was used directly for rT$_3$ 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 ^{131}I uptake, and elevated serum T$_4$ and T$_3$ concentrations as determined by RIAs (19, 20). Hypothyroidism was diagnosed on the basis of clinical examination, low serum T$_4$, and elevated serum thyrotropin (TSH) (21). Sera were also obtained from 12 hypothyroid patients when they received treatment with synthetic T$_4$ (Synthroid, Flint, Eaton & Co., Div. of Baxter Laboratories, Inc., Morton Grove, Ill.). Serum T$_4$ concentration in these patients varied between 7.5 and 25 μg/100 ml. Sera of five patients with elevated serum concentration of thyroxine-binding 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 rT$_3$ levels were tested in four Graves' disease patients receiving treatment with propylthiouracil. Additionally, serum rT$_3$ 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 T$_3$ (Cytomel, Smith, Kline & French Laboratories, Philadelphia, Pa.), 75 and 100 μg. 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 rT$_3$ 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 rT$_3$ content of the extract was determined by the RIA described above. T$_4$ and T$_3$ content of this Tg was determined by RIA (26, 27).

RESULTS

Specificity. Results of studies showing the ability of nonradioactive rT$_3$, T$_4$, T$_3$, 3,3′-T$_2$, and monoiodothyronine (3-T$_1$) to inhibit the binding of [^{125}I]rT$_3$ to antibody are shown in Fig. 1. Addition of all of these agents resulted in progressive reduction in the proportion of [^{125}I]rT$_3$ bound to antibody with roughly similar dose-response curves. Significant inhibition of the binding of [^{125}I]rT$_3$ to antibody occurred with as little as 10 pg of rT$_3$, 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 rT$_3$ 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}I]rT$_3$ to antibody. 3,3′-T$_2$ demonstrated the most cross-reactivity with rT$_3$-binding sites on the antiserum. T$_4$, T$_3$, and the various other thyroid hormone derivatives, as well as iodide, demonstrated little or no effect

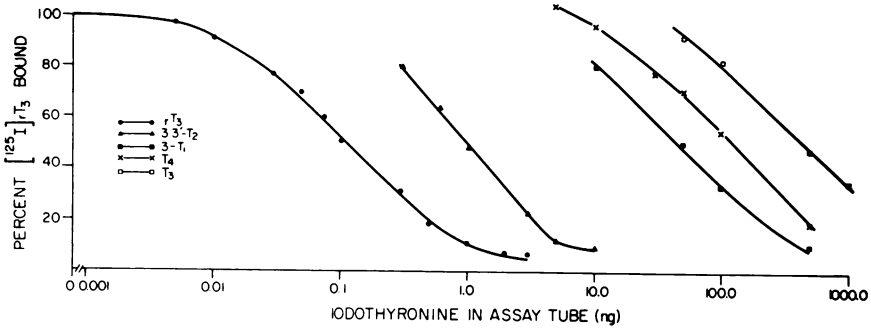

FIGURE 1 Dose-response curves. Inhibition of the binding L-[^{125}I]rT$_3$ to D,L-rT$_3$ antibody by increasing quantities of D,L-rT$_3$, 3,3′-L-T$_2$, 3-L-T$_1$, L-T$_4$, and L-T$_3$ is shown on a semilogarithmic plot.

TABLE I

Relative Reactivity of Various Thyroid Hormone Derivatives with rT_3-Binding Antibody

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-T_4	0.06
D-T_4	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 [^{125}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 T_4 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 T_4 on the binding of [^{125}I]rT_3 to antibody was checked especially carefully. Thus, the two batches of reagent T_4 and three lots of proprietary T_4 (Synthorid), tested in four assays, showed a cross-reaction varying between 0.06 and 0.09%; variation in cross-reactivity of different batches of T_4 suggests that at least part of the cross-reaction must be a result of contamination of commercial T_4 with rT_3. The smallest dose of T_4 which appreciably reduced the binding of [^{125}I] rT_3 to antibody varied between 15 and 20 ng/assay tube (Fig. 1). This amount of T_4 would be expected in 100 μl of serum, tested routinely, only when the serum T_4 concentration is 15 μg/100 ml or higher.

Comparison of samples with standards. The dose-response curves of inhibition of the binding of [^{125}I]rT_3 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 rT_3 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 rT_3. It was probably related to the observation that the binding of [^{125}I]rT_3 to antibody in zero-rT_3 tubes was about 10% less in the absence of ethanol than in its presence. The reason for the higher binding of [^{125}I]rT_3 to antibody in presence of ethanol than in its absence is not clear. It was not attributable to higher

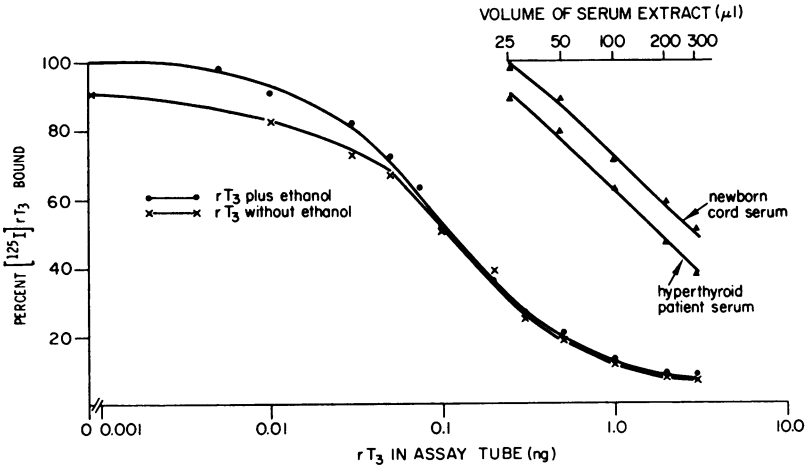

FIGURE 2 Comparison of the standard assay curve and the pattern of inhibition of L[^{125}I]rT_3 binding to D,L-rT_3 antibody produced by different doses of ethanol extracts of newborn cord serum and serum of a hyperthyroid patient. A dose response curve, obtained with unlabeled D,L-rT_3 when ethanol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

nonspecific binding (or trapping) of [^{125}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$_3$ concentration. To evaluate the effect on the RIA of ethanol extracts of serum with reduced rT$_3$ content, rT$_3$ was measured in extracts of sera before and after treatment of the sera with activated charcoal (28). Pilot experiments with four sera, using [^{125}I]rT$_3$, 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$_3$ remains in sera after charcoal treatment.

Effect of addition of T$_4$ on serum rT$_3$ concentration. Although cross-reaction studies had shown that T$_4$ would have little interference in the rT$_3$ assay, the effect of addition of T$_4$ to serum on immunoassayable rT$_3$ was studied. A T$_4$ preparation, which had shown 0.06% cross-reactivity in the rT$_3$ assay, was added in concentrations varying between 15 and 100 μg/100 ml to sera with variable rT$_3$ concentration. The data on serum rT$_3$ levels measured in these sera are shown in Table III. It is apparent that addition of this T$_4$ to serum does not increase the rT$_3$ 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$_3$ added to 10 sera was 89±1.5% [2] (range 84–94). Recovery did not vary systematically in sera from different classes of patients. In separate experiments, mean recovery of nonradioactive rT$_3$ added to ethanol extracts of a pooled serum from hospitalized patients in amounts ranging from 50 to 500 ng/100 ml was 96±0.5% for 50 ng ($n=4$), 101±1.5% for 100 ng ($n=4$), 99±6% for 300 ng ($n=4$), and 106±3.2% for 500 ng ($n=3$). The mean recovery of all 15 experiments was 101±2%. The rT$_3$ concentration in the serum extract was determined to be 54 ng/100 ml. The recovery of nonradioactive rT$_3$ added to the same pooled serum before ethanol extraction was 96±2% for 50 ng ($n=4$), 91±4% for 100 ng ($n=4$), 91±4% for 300 ng ($n=4$), and 93±3% for 500 ng ($n=4$). The mean recovery for all 16 experiments was 93±1%.

[2] The data here and subsequently are presented as mean ±SE unless specified otherwise.

TABLE II

Effect of Charcoal Treatment on Serum rT$_3$ Concentration

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

* Serum rT$_3$ concentration, mean±SD (n).

Reproducibility. Both intra- and interassay reproducibility was examined in samples with variable rT$_3$ concentration. The mean coefficient of variation (SD/mean × 100) for 28 samples varying in rT$_3$ concentration from 28 to 240 ng/100 ml, each of which was tested in duplicate or quadruplicate in the same assay, was 5.6±0.8%. The mean coefficient of variation of four specimens varying in rT$_3$ concentration from 50 to 100 ng/100 ml and assayed in duplicate two to six times in different assays was 10.3±0.8%. No systematic differences in reproducibility were noted in serum rT$_3$ 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$_3$, T$_4$, and T$_3$ 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-

TABLE III

Effect of Addition of T$_4$ on Serum rT$_3$ Concentration

Experiment	T$_4$* added, (μg/100 ml)				
	0	15	25	50	100
			ng/100 ml		
1	<10±0 (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)

* Tested separately, this T$_4$ cross-reacted 0.06% in rT$_3$ assay.
‡ Serum rT$_3$ concentration, mean±SD (n).

TABLE IV

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

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 Graves' disease	rT_3	22	103¶	48.6	54–230
	T_4	22	23.3¶	5.7	17–38
	T_3	22	744¶	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, *μU/ml*	11	137	60	16–>200
Hypothyroidism treated with T_4**	rT_3	12	54.8‖	18.6	20–90
	T_4	12	14.4¶	6.3	7.5–25
	T_3	12	174‖	74	80–336
Patients with elevated serum TBG	rT_3	5	54.2§	6.5	40–64
	T_4	5	15.0¶	6.4	8.4–22
	T_3	5	195‡	126	72–328
	TBG, *mg/100 ml*	5	4.6¶	0.5	4.1–5.4
Newborn (term fetus cord serum)	rT_3	7	136¶	29	100–180
	T_4	7	11.9¶	2.4	6–15.5
	T_3	7	24¶	9.7	<15–40

* Results expressed in terms of weight equivalent of D,L-rT_3.

‡ Cf. normal $P < 0.02$.

§ Cf. normal $P < 0.01$.

‖ Cf. normal $P < 0.005$.

¶ Cf. normal $P < 0.001$.

** Mean (±SD) dose of synthetic T_4 was 0.25±0.07 mg/day.

mal men and women had comparable levels of serum rT_3, and for this reason values in both sexes have been combined. The serum rT_3 concentration of 103 ng/100 ml in 22 hyperthyroid patients was significantly higher than in normal subjects ($P < 0.001$). When the serum rT_3 values in individual hyperthyroid patients were corrected for the cross-reaction effects of the T_4 in their serum, the mean ±SD range value of serum rT_3 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 rT_3 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 rT_3 in 12 hypothyroid patients treated with synthetic T_4 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 T_4 and T_3 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 rT_3 concentration of 54.2 ng/100 ml, which was significantly higher than the corresponding normal value ($P < 0.01$). The mean serum rT_3 concentration of 136 ng/100 ml in seven newborn cord sera was also significantly higher than that in normal adults. Serum T_4 concentration in the newborn was modestly higher than that in the adult. On the other hand, the mean newborn serum T_3 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 rT_3 from serum of two healthy laboratory workers who received a bolus of 2 mg of D,L-rT_3 intravenously. The baseline serum rT_3 concentration in these subjects was 44 and 33 ng/100 ml. It increased to 41.4 and 43.2 μg/100 ml, respectively, 10 min after the injection and steadily fell thereafter. The rate of disappearance of nonradioactive rT_3 from serum of these patients was similar to that reported previously for the similar racemic mixture of radioactive rT_3 (12). rT_3 (% dose/liter) remaining in serum 1 h after injection of rT_3 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 rT_3 from serum in the first few hours makes it difficult to estimate the half-life of rT_3 in the circulation or its volume of distribution with any degree of accuracy.

Serum rT_3 concentration was measured for a day in hypothyroid patients receiving commercially available preparations of T_4 and T_3 for 2 wk or longer. Serum rT_3 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 T_4. Serum rT_3 levels were 12 ng/100 ml or less during a day in two patients taking 75 and 100 μg of T_3/day; serum T_3 concentration in these patients was 495 and 1,050 ng/100 ml, respectively, 4 h after administration of T_3, and gradually decreased thereafter. Serum levels rT_3, T_4, and T_3, 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 T_4 three days before study were markedly elevated; the values decreased modestly to 196 ng/100 ml, 72 μg/100 ml, and 2,200 ng/100 ml, respectively, 24 h after the initial study.

Reverse T_3, T_4, and T_3 in normal Tg. Reverse T_3, T_4, and T_3 content of Tg prepared from pooled normal thyroid glands of 10 subjects was 0.042, 3.0, and 0.16 μg/mg protein, respectively. The T_4/rT_3 ratio in this Tg was 71.4, whereas the T_4/T_3 ratio was 18.7.

DISCUSSION

The present studies have demonstrated that it is feasible to prepare a highly specific rT_3-binding antiserum with an affinity suitable for detection of picogram quantities of nonradioactive rT_3. 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_4 showed little cross-reaction with the rT_3 antibody. In this respect, the experience with rT_3 is very similar to the previous experience with T_3-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

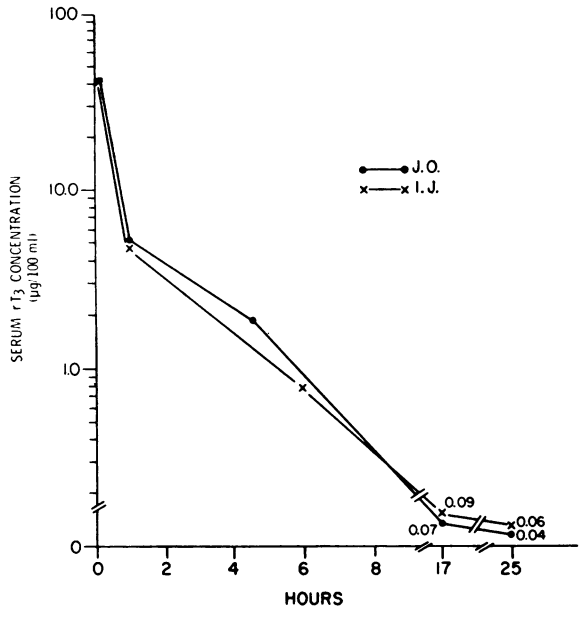

FIGURE 3 Disappearance of immunoassayable rT_3 from serum of two normal subjects, each of whom received 2 mg of D,L-rT_3 i.v. Serum rT_3 concentration is plotted on the y-axis on a logarithmic scale and time in hours on x-axis on an arithmetic scale. Zero h represents the time of injection of D,L-rT_3.

the 5′ position, in case of rT_3, to the 5 position, in case of T_3.

Of various thyroid hormone derivatives studied, only 3, 3′-T_2 cross-reacted significantly (10%) with rT_3 antibody. This ought to be considered, however, the maximal estimate of the cross-reaction. The possibility that 3, 3′-T_2 was contaminated with rT_3 cannot be excluded. Significant interference of 3,3′-T_2 in rT_3 assay may also be related to the possible presence of some 3,3′-T_2 antibody in the serum of rabbits immunized with rT_3-HSA conjugate; this could happen if the 5′ iodine of rT_3 were lost during the process of conjugation or if the rT_3 used to prepare rT_3-HSA conjugate were contaminated with 3,3′-T_2. 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′-T_2, the effect of the cross-reaction with T_4 is clearly impotrant in the rT_3 assay because of large amounts present in serum compared to rT_3. However, the remarkably low cross-reaction of T_4 with rT_3 antibody held the effect of T_4 on the rT_3 measurement to an acceptably low level. Thus, in the case of normal subjects, whose serum T_4 concentration averaged 8.6 μg/100 ml (Table III), the cross-reaction of T_4 with rT_3 anti-

body of 0.06% would be expected to increase average normal serum rT_3 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 rT_3 in man, we expected to find, if any, only small quantities of rT_3 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 rT_3 from serum and that antisera can be generated that allow an accurate and precise measurement of rT_3 in ethanol extracts of serum. The experience with rT_3 was similar in these respects to that previously reported for RIA of T_3 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 rT_3 is indeed a normal constituent of human serum. The observed mean serum rT_3 concentration of 41 ng/100 ml could not be attributed to a cross-reaction of T_4 in the rT_3 assay; this value was about seven times greater than what could be expected merely from cross-reaction of T_4 (see above). However, since 3,3′-T_2 cross-reacted 10% in the rT_3 RIA, one could consider the estimated normal mean serum rT_3 concentration of 41 ng/100 ml to be a result of the 410 ng/100 ml of 3,3′-T_2 in serum. However, this appears highly unlikely. The half-time of disappearance of 3,3′-T_2 has been estimated to be 1 h or less (32). Even if 3,3′-T_2 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′-T_2 of about 205 μg/day, which is over twice that of T_4. This could occur if 3,3′-T_2 were much more abundant in the human thyroid than T_4. 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,L-rT_3 used in the standards is different from the L-rT_3 assumed to be present in man, it is possible that the true rT_3 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_3 and T_4 have been noted to react with T_3 and T_4 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_3 reacted with one anti-T_3 with a potency only 39% that L-T_3 (35). In any case, even if the D- and L-isomers of rT_3 were to react differently with rT_3 antibody, this would only alter the true serum concentration of L-rT_3 but would not limit the interpretation of relative changes in serum rT_3 in health and disease presented in this report. Serum rT_3 values presented here should be interpreted to reflect relative changes in various situations rather than as absolute (true) serum rT_3 values also because of (*a*) some (0.06%) cross-reaction of T_4 in rT_3 RIA and (*b*) 3,3′-T_2, which cross-reacted 10% in rT_3 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 T_4. Reverse T_3 was detected in the serum of each of these patients, and this could not be attributed to contamination of proprietary T_4 with rT_3. The cross-reaction of proprietary T_4 with rT_3 antibody was found to be no more than that observed with reagent T_4, 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 T_4 (1), that just as in the case of T_3, peripheral metabolism of T_4 may also be an important source of rT_3 in serum of man.

Study of Pronase digest of human Tg revealed the presence in it of rT_3-like immunoreactive material in an amount approximately 1.4% that of T_4. If 3,3′-T_2 were present in human Tg in quantities approximately 14% that of T_4, it could explain the finding of the observed amount of rT_3 in human Tg. However, until later studies can prove this to be the case, it would appear that in addition to peripheral metabolism of T_4 (see above), thyroidal secretion may also be a source of rT_3 in human serum.

The changes in serum rT_3 values noted in hyper- or hypothyroid patients appeared similar to those observed in the case of T_4 and/or T_3 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 rT_3 in these patients may signify the influence of the availability of serum T_4 for conversion to rT_3, a change in thyroidal activity, or a combination of these factors. The serum rT_3 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 T_4 and T_3, rT_3 may also be bound to TBG. The situation in which changes in serum rT_3 deviated strikingly from those expected from serum T_4 and T_3 values was in the newborn. Thus, serum rT_3 values in the term fetus cord serum were higher than or in the range of values seen in hyperthy-

roid adults. The mean serum T_4 in newborns, however, was only about one-half that in hyperthyroid patients (Table IV). As against serum rT_3, serum T_3 concentration in the newborn was comparable to that in hypothyroid patients. The serum T_3 and T_4 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 rT_3 levels in the newborn are intriguing. They could signify that the peripheral metabolism of T_4 may be different in the newborn than in the adult. It has already been suggested that peripheral conversion of T_4 to T_3 may be less in the newborn than in the adult (36). The serum rT_3 and T_3 values in the present study would suggest that conversion of T_4 to rT_3 or to T_3 may not occur randomly in the fetus, as has been suggested to be the case in the adult (1) and that conversion of T_4 to rT_3 may be a major pathway of degradation of T_4 at the time of birth. This in turn raises the question whether nonrandom conversion of T_4 to rT_3 and T_3 might also occur under some conditions in adults. Obviously, studies in the newborn of the peripheral degradation and the thyroidal content of rT_3 are necessary to validate these considerations. However, if the characteristics of the peripheral metabolism of T_4 or T_3, as well as the thyroidal content of T_4 and T_3, in the newborn are any indication of the changes in these parameters in the case of rT_3, one could predict that the elevated cord serum rT_3 is due mainly to a difference in metabolism of T_4 in the newborn compared to the adult. Thus, the thyroidal content of T_4 and T_3 in the human term fetus is very similar to that in the adults (36), and the peripheral degradation rate of T_4 and T_3 in the fetal sheep is not lower but much greater than that in the adult (37).

Although the physiological significance of the conversion of T_4 to T_3 (2–8), a biologically more potent iodothyronine (38), can be readily appreciated, the physiological function, if any, of the conversion of T_4 to rT_3, a relatively inert iodothyronine (39, 40), remains unclear. rT_3 has been shown to be capable of inhibiting the calorigenic effects of T_4 as well as slowing its disappearance from the circulation (41–43). These considerations lead one to suspect that rT_3 may not be just an inactivation product of T_4, but that it may be involved in physiological regulation of metabolism and biological action of T_4. 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 D_3 (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 D_3 (25-OH-D_3) to either biologically more active 1,25$(OH)_2$-D_3 or to a relatively biologically inert 24,25-$(OH)_2$-D_3. Generally, when 1,25-$(OH)_2$-D_3 production is diminished, 24,25-$(OH)_2$-D_3 production is increased (44–46). In this respect, the findings of the present study, i.e., that serum rT_3 is markedly elevated in the newborn whose serum T_3 is markedly low, are similar. However, further study is needed to ascertain whether there is indeed an analogy between the metabolism of T_4 and 25-OH-D_3.

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