《ノート》

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

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1. INTRODUCTION

Radioimmunoassay (RIA) of 3, 3′, 5′-triiodo-L-thyronine (reverse T_3 or rT_3) has been reported by various investigators which mentioned many clinical data about human serum levels of rT_3 (1–9). We have also developed a sensitive, specific and reproducible RIA system for the measurement of rT_3 in unextracted human serum using specific antiserum against rT_3 and very high specific radioactivity ^{125}I-rT_3.

2. MATERIALS AND METHODS

Reagents. Reverse T_3 was kindly supplied from Dr. H. J. Cahnmann of National Institutes of Health, Bethesda, Md. L-thyroxine (T_4), 3, 3′, 5-triiodo-L-thyronine (T_3) and 3, 5-diiodo-L-thyronine were obtained from Sigma. 3-monoiodo-L-thyronine (3-T_1), 3′-monoiodo-L-thyronine (3′-T_1), and 3, 3′-diiodo-L-thyronine (3, 3′-T_2) were obtained from Henning Berlin GMBH. Bovine serum gamma globulin and bovine serum albumin (BSA) were obtained from Miles Laboratories, Inc. 8-anilino-1-naphthalen-sulfonic acid (ANS), polyethylene glycol (PEG)-6,000 and charcoal (Norit A) were obtained from Eastman Kodak Co., Wako Pure Chemical Industries Ltd. and American Norit Company, Inc., respectively. Na^{125}I was obtained from Radio Chemical Center, Amersham.

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Preparation of antiserum. Reverse T_3 was conjugated to BSA by a modifications of coupling method of T_3 to proteins (10). Ten mg of BSA was dissolved in 5 m*l* of distilled water, and 6 mg of "Morpho CDI" (1-cyclohexyl-3 (2-morpho-linoethyl)-carbodiimide metho-p-toluenesulfonate) was added to it. Four mg of rT_3 dissolved in 5 m*l* of dimethylformamide was then added to the solution dropwise under stirring. The pH was adjusted to 5.5 with 0.1 N HCl or 0.1N NaOH. The solution was kept at room temperature under constant stirring in dark place for 20 hours. The reaction mixture was then dialyzed against distilled water for 72 hours and stored at −20°C. In order to calculate the coupling yield, about 10,000 cpm (approximately 0.3 pg) of ^{125}I-rT_3 was added to the rT_3 solution and the recovery was calculated by counting the total activities of the final products, and the coupling yield was found to be about 80%. One hundred μg of rT_3 coupled with BSA was suspended in 2 m*l* of distilled water and emulsified with 2 m*l* of complete Freund's adjuvant. This emulsion was injected into toe-pade and neck of each rabbit. The immunization were repeated at the time intervals of one month. Blood for antiserum was drawn at 7 days after the third injection.

Preparation of ^{125}I-rT_3. (a) Preparation of ^{125}I-rT_3 of high specific activity by radioiodination. High specific activity ^{125}I-rT_3 was prepared by a modification of the method of Weeke and Örskov (11). Two hundred ng of 3-T_1 dissolved in 10 μl of 0.01N NaOH was added to 3 mCi of Na^{125}I. The pH was adjusted to 7.5 with 0.5 M phosphate buffer. Twenty second after the addition of 25 μl of chloramine-T (3.5 mg/m*l*) the reaction was stopped by adding 100 μl of sodium metabisulfite (3.5 mg/m*l*).

Key Words: Radioimmunoassay, Reverse T_3, ^{125}I-rT_3, Polyethylene glycol (PEG), 8-anilino-1-naphthalen-sulfonic acid (ANS)

A sephadex G-25 (fine) column (1×30 cm) was used for the separation of ^{125}I-rT$_3$ (9). The column was eluted with 0.01 N NaOH. The purity of the labeld rT$_3$ was checked by thin-layer chromatography using acetone: water: ammonia (35:4:1) as the developer. The Rf values of 3-T$_1$, rT$_3$, T$_3$ and T$_4$ in this solvent system were 0.43. 0.07, 0.55 and 0.38, respectively.

(b) Preparation of ^{125}I-rT$_3$ of low specific activity by isotopic exchange reaction. Two hundred ng of rT$_3$ was used instead of 3-T$_1$, and prepared and purified with the same method as described in the preparation of ^{125}I-rT$_3$ of high specific activity. The specific activity of the ^{125}I-rT$_3$ was calculated by the self-displacement method (11).

Preparation of rT$_3$-free serum. Reverse T$_3$-free serum was prepared by the method by Mitsuma for T$_3$ and T$_4$ (10). One hundred m*l* of normal human serum was incubated with 100 g of Norit A charcoal for 3 hours at 25°C, and then the mixture was centrifuged at 20,000 g. More than 99% of rT$_3$ was removed from the human serum. This was confirmed by using ^{125}I-rT$_3$ as a tracer.

Incubation system. One hundred μl of standard rT$_3$ (dissolved in rT$_3$-free serum) or of unknown sample and ^{125}I-rT$_3$ (diluted with 0.1 M borate buffer pH 8.6 containing 0.1% BSA) were added to each reaction tube. Then 400 μl of anti-rT$_3$ serum (diluted with 0.1 M borate buffer pH 8.6 containing 7.5 mg/m*l* of bovine serum gamma globulin and 0.9 mg/m*l* of ANS) were then added to the reaction tube. The mixture was incubated for 20 hours at 4°C. The bound and free rT$_3$ were separated by PEG method (12). One m*l* of 25% PEG was added to the incubation mixture and the mixture was centrifuged at 2,200 g for 15 minutes at 25°C and the radioactivity of the precipitates was counted in a well scintillation counter.

Sources of sera. Serum was obtained from 59 nomal subjects, 15 untreated hyperthyroid patients with Graves' disease and 10 patients with primary hypothyroidism. Sera were also obtained from 10 normal pregnancy, 3 complete starvation and 30 anorexia nervosa. The heparinized and EDTA treated (1 μU and 1 mg/m*l*) plasma samples were collected from four normal subjects, to see the effect of heparin and EDTA.

3. RESULTS

Specific activity and sensitivity. The specific activity of ^{125}I-rT$_3$ which is obtained by diiodination of phenolic ring of 3-T$_1$ is calculated to be 6,600 μCi/μg theoretically and calculated to be 4,500–5,500 μCi/μg by self displacement method. When high specific activity of ^{125}I-rT$_3$ was used for RIA, a significant inhibition of ^{125}I-rT$_3$ binding to antibody could be detected at rT$_3$ concentrations as low as 1 pg/tube, and linear dose-response curve was obtained from 6 pg/tube to 200 pg/tube (Fig. 1). On the other hand, when low specific activity of ^{125}I-rT$_3$ (400–500 μCi/μg) was used, we could not detect rT$_3$ less than 10 pg/tube.

Specificity. The cross-reactivities of the anti-rT$_3$ serum with other thyroid hormone analogues are shown in Table 1. The relative reactivities of various compounds were calculated as the amounts that gave 50% inhibition of the binding of ^{125}I-rT$_3$ to antibody. We found that T$_4$ and T$_3$ which present in large quantities in the serum showed no significant effect on the binding of ^{125}I-rT$_3$ to the antibody. But in the various thyroid hormone analogues, 3′-T$_1$ cross-reacted significantly.

Effect of ANS. In order to inhibit the binding of rT$_3$ to serum protein, ANS, which is known as a inhibitor of T$_3$ and T$_4$ to TBG, was added to the

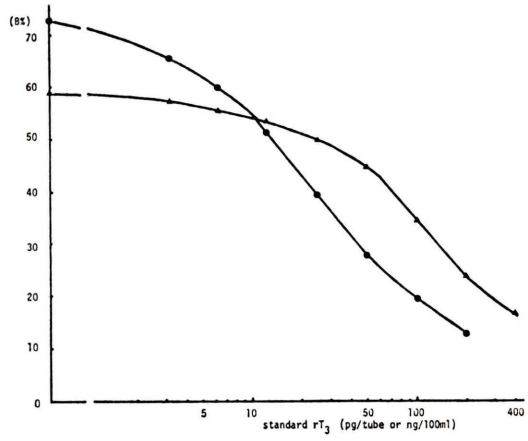

Fig. 1 Comparison of standard curve, using high specific activity (4,500–5,500 μCi/μg) —●— and low specific activity (400–500 μCi/μg) —▲— as a tracer, and final dilution of antiserum were 1:70,000 and 1:2,500, respectively.

incubation mixture. Various concentrations of ANS ranging from 90 to 1444 μg/tube were added to different quantities of TBG. Fig. 2 showed that the binding percent of ^{125}I-rT_3 to antibody was maximum at ANS concentration of 360 μg/tube in all samples.

Effect of dilution experiments. Effect of serum dilution on the measurement of rT_3 was studied with serum diluted with rT_3-free serum using the high and low specific activity of ^{125}I-rT_3. When high specific activity of ^{125}I-rT_3 was used, a good linearity was obtained. But when low specific activity was used, the good linearity was not observed (Fig. 3, a)

Effect of concentration of serum protein. In

Table 1 Relative reactivity of various iodinated compounds with rT_3 antibody*

Compounds	Relative Reactivity
L-rT_3	100
L-T_4	0.009
L-T_3	<0.0001
3, 5-L-T_2	<0.0001
3, 3′-L-T_2	0.009
3-L-T_1	0.0009
3′-L-T_1	0.12
L-MIT	<0.0001
L-DIT	<0.0001

* The relative reactivities of various compounds were calculated as the amounts that resulted in 50% inhibition of the binding of labeled hormone to antibody.

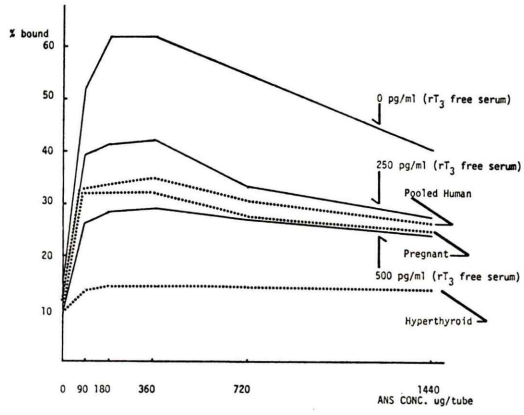

Fig. 2 Effect of addition of increasing quantities of ANS to different serum samples on the binding of ^{125}I-rT_3 antiserum.

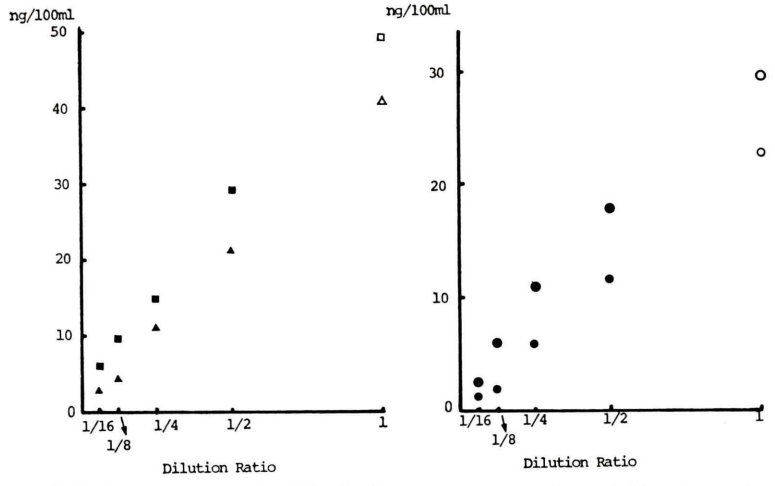

Fig. 3a Dilution serum sample with rT_3 free serum were evaluated rT_3 value, using both high specific activity and low specific activity as a tracer. □ and △, ○ and ○ are indicated same sample. □, ○; sample before dilution, measured with low specific activity of ^{125}I-rT_3 as a tracer. △, ○; sample before dilution, measured with high specific activity of ^{125}I-rT_3 as a tracer. ■, ●; sample after dilution, measured with low specific activity of ^{125}I-rT_3 as a Tracer. ▲, ●; sample after dilution, measured with high specific activity of ^{125}I-rT_3 as a tracer.

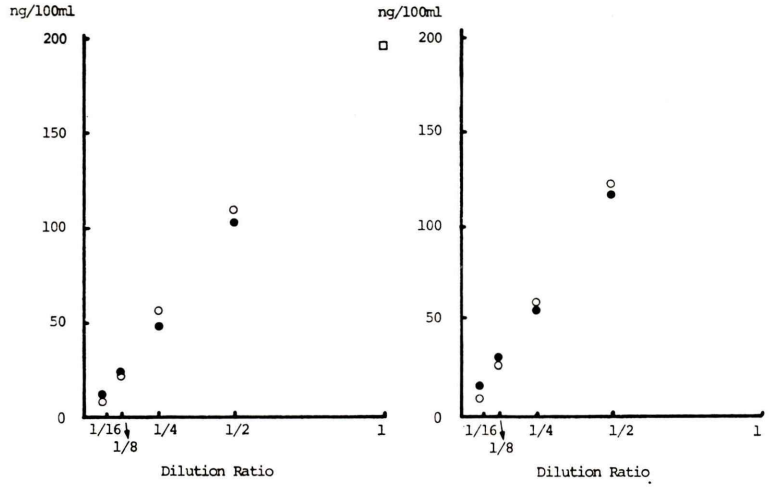

Fig. 3b Effect of serum protein on the measurement rT_3 value. □; sample before dilution. ●; sample after diluted with rT_3 free serum. ○; sample after diluted with 0.9% NaCl.

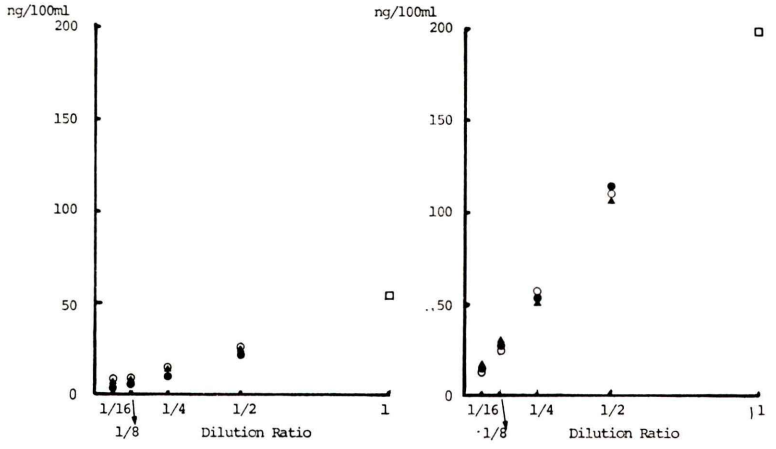

Fig. 3c ●, ▲ and ○ show rT_3 value diluted with rT_3 free serum enriched 10, 20 and 40 mg/m*l* of human gamma globulin, respectively.

order to clarify the effect of serum protein on the measurement of rT_3, test sera were diluted with 0.9% NaCl or rT_3-free serum. The results obtained with both diluents were essentially identical and good quantitativities were demonstrated by good linearity of the curve as is shown in Fig. 3, b. In the experiment where sera diluted with rT_3-free serum containing different concentrations of human gamma globulin were used, the similar results were obtained (Fig. 3, c).

Recovery experiments. Reverse T_3 were added to sera from euthyroid, hyperthyroid and hypothyroid patients to contain 6.25 to 100 ng/100 m*l*. These samples were assayed using both high and low specific activity of ^{125}I-rT_3. The recovery of rT_3 was good with high specific activity of ^{125}I-rT_3, while poor with low specific activity of ^{125}I-rT_3 (Table 2).

Reproducibility. The within assay precision was evaluated by ten replicates determinations. Three different samples were measured and these rT_3 values were 20.0±2.0, 37.5±2.6 and 131±1.7 ng/

100 m*l* (mean±SE), respectively. The reproducibility between assay was also evaluated by 15 times determinations using the same samples, and found to be 18.3±0.5, 36.7±0.6 and 149.3±0.34 ng/100 m*l* (mean±SE), respectively.

Effec of heparinized and EDTA treated plasma on the measurement of rT_3. Reverse T_3 levels of serum, heparinized plasma, and EDTA treated plasma from same 4 normal subjects were measured, and rT_3 values were 27.4±0.9, 26.9±0.8 and 28.3±1.1 ng/100 m*l* (means±SE), respectively.

Table 2 Recovery of rT_3 added to the sera from the subjects with various thyroidal states and measured with both high specific activity and low specific activity of ^{125}I-rT_3 as a tracer.

rT_3 Added (ng/100 m*l*)	Initial Serum rT_3 (ng/100 m*l*)		Recovery (%)	
	High Specific Activity	Low Specific Activity	High Specific Activity	Low Specific Activity
6.3	7.6	9.1	107.8	129.4
12.5			102.2	119.6
25.0			105.6	115.5
50.0			116.2	107.3
100.0			177.0	117.6
6.3	10.1	18.3	105.3	128.7
12.5			105.5	118.3
25.0			119.9	116.4
50.0			118.7	105.8
100.0			121.4	107.7
6.3	22.8	29.8	107.3	117.9
12.5			105.7	116.2
25.0			117.8	110.3
50.0			103.8	101.5
100.0			99.8	98.2
6.3	41.3	49.8	96.7	111.8
12.5			103.8	108.2
25.0			114.2	103.0
50.0			120.5	108.9
100.0			112.4	97.1

Serum rT_3 levels in various states. Table 3 presents data on serum rT_3 concentration in various states. The values for rT_3 in 59 normal subjects averaged 27.9±0.9 ng/100 m*l* (mean±SE) and ranged from 17.6 to 52.3 ng/100 m*l*. Serum rT_3 concentration significantly increased in patients with Graves' disease (168.1±13.8 ng/100 m*l*, from 104 to 267.0 ng/100 m*l*), normal pregnancy, complete starvation, and anorexia nervosa, and decreased significantly in untreated primary hypothyroidism (9.8±1.0 ng/100 m*l*, from 3.3 to 13.0 ng/100 m*l*).

4. DISCUSSION

We have developed a RIA system for the measurement of serum rT_3 by using a highly specific antiserum and ^{125}I-rT_3 with high specific activity. Theoretically, two atoms of radioactive iodine can be tagged to one phenolic ring of thyronine, and therefore high specific activity can be more easily obtained with rT_3 than T_3. The highest possible specific activity of ^{125}I-rT_3 that can be obtained for diiodinated to phenolic ring is 6,600 μCi/μg, if one assumes an isotopic abundance of 100% for ^{125}I (17.2 μCi/μg). Gavine (8), Burman (9) and Mainhold (5) have previously reported that ^{125}I-rT_3 was prepared from the iodination of 3,3′-T_2, and specific activity was 300, 500 and 3,300 μCi/μg, respectively. In this study, however, ^{125}I-rT_3 was prepared from diiodination of 3-T_1, therefore it is theoretically expected that ^{125}I-rT_3 with highest specific activity can be obtained by chloramin-T method. The high sensitivity of our assay method was mainly due to the high

Table 3 Serum rT_3, T_3 and T_4 concentrations in various thyroidal states

Group	n	rT_3 (ng/100 m*l*)	T_3 (ng/100 m*l*)	T_4 (ng/100 m*l*)
Normal	59	27.9± 0.9*	123± 3	9.6±0.4
Hyperthyroidism	15	168.1±13.8	526±37	23.4±1.8
Hypothyroidism	10	9.8± 1.0	44± 4	3.9±0.4
Normal Pregnancy	10	36.4± 2.2	180± 5	14.5±1.5
Complete Starvation	3	38.0± 9.8	70± 8	**
Anorexia Nervosa	30	41.6± 4.8	84± 5	**

* Means±SE ** Not examined

specific activity of the ^{125}I-rT$_3$ used. The very high specific activity of the ^{125}I-rT$_3$ enable us to use only 0.2–0.3 pg of ^{125}I-rT$_3$ per assay tube which permits us to detect 1 to 200 ng/100 m*l* of rT$_3$ in the serum. From our experiment, when ^{125}I-rT$_3$ of low specific activity (400–500 μCi/μg) was used, we could not obtain good results both in the serum dilution test and recovery test from serum at rT$_3$ concentration of 7–20 ng/100 m*l*. Since the concentration of rT$_3$ in serum is considered to be lower than that of T$_3$ because of its rapid metabolism (1, 4), high sensitive assay is required for the rT$_3$ RIA.

The assay procedure described here satisfies requirements for the measurement of rT$_3$ in the presence of serum protein: a) by adding ANS, binding of rT$_3$ to serum proteins were blocked in various kinds of serum such as those from normal, pregnant, hypothyroid and hyperthyroid, b) rT$_3$ added to sera at widely different concentrations were quantitatively recovered, and the serum dilution test showed that human or animal sera containing gamma globulin at different concentrations can be measured quantitatively, c) due to the remarkably low cross-reaction of T$_3$ and T$_4$ with the rT$_3$ antibody, T$_3$ and T$_4$ in serum do not affect on the measurement of rT$_3$. d) the precision within an assay and reproducibility between assays were excellent. Accordingly, we conclude that our RIA system described here is the most sensitive, reproducible and quantitative method for the measurement of rT$_3$ level in serum.

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ABSTRACT

A sensitive, specific and reproducible radioimmunoassay (RIA) for the measurement of 3, 3′ 5′-triiodothyronine (rT$_3$) in serum without extraction is described. A highly specific rT$_3$ binding antiserum was prepared by immunization of rabbits with rT$_3$-bovine serum albumin conjugate A method for the preparation of ^{125}I-rT$_3$ of very high specific activity which was iodinated from 3-monoiodothyronine was developed. Utilizing this RIA, a mean ($\pm$SE) serum rT$_3$ level of normal subject was 27.9$\pm$0.9 ng/100 m*l* (n=59). Serum rT$_3$ was found to be increased in hyperthyroidism (168.1$\pm$13.8 ng/100 m*l*, n=13), normal pregnancy

(36.4±2.2 ng/100 m*l*, n=10), complete starvation (58.0±9.8 ng/100 m*l*, n=3), anorexia nervosa (41.6±4.8 ng/100 m*l*, n=30). And the serum concentration was decreased in hypothyroidism (9.8±1.0 ng/100m*l*, n=10). The method described here satisfies requirments for measurement of rT_3 by RIA.

要　　旨

3′, 3′, 5′-トリヨードチロニン (rT_3) のラジオイムノアッセイ

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血中 3, 3′, 5′-トリヨードチロニン (rT_3) のラジオイムノアッセイ (RIA) による測定法を検討した．抗 rT_3 抗体は，ウシ血清アルブミンと結合させた rT_3 を，家兎に投与して得た。3, 5, 3′-トリヨードチロニン (T_3) 及びチロキシン (T_4) との交叉反応は，それぞれ，0.0001％以下，0.009％であり，本法による，rT_3 測定値は，T_3 及び T_4 の影響をほとんど受けていないと考えられる．

^{125}I-rT_3 は，3-モノヨードチロニンをクロラミン T 法で標識し，4,500～5,500 μCi/μg の高比放射能を得た．本法の測定によって得た正常者 59人の血中 rT_3 値は，27.9±0.9 ng/100m*l* (Mean±SE) であった．又，甲状腺機能亢進症，正常妊婦，絶食者および，神経性食思不振症の血中 rT_3 値も，正常者に比較し，高値を示し，甲状腺機能低下症において，正常より低値を示した．