

# A Radioimmunoassay for Serum Rat Thyroglobulin

## PHYSIOLOGIC AND PHARMACOLOGICAL STUDIES

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**ABSTRACT** A double antibody radioimmunoassay has been developed to measure thyroglobulin in rat (RTg) serum. The lowest detectable quantity measurable was 5.0 ng/ml. Specificity was documented by: (a) fall in serum RTg to undetectable levels after thyroid ablation; (b) the fact that L-thyroxine, D-thyroxine, L-triiodothyronine, D-triiodothyronine, triiodothyroacetic acid, tetraiodothyroacetic acid, triiodothyropropionic acid, monoiodotyrosine, diiodotyrosine, and human thyroglobulin (HTg) in concentrations up to 40,000 ng per tube did not cross-react in the assay; (c) the demonstration that constant levels of serum RTg were observed while varying amounts of serum (criterion of parallelism) were introduced in the assay. The mean RTg concentration in tail vein blood of adult Sprague-Dawley rats was  $101.5 \pm 13.0$  ng/ml (SEM) ( $n = 21$ ); values ranged from 12.0 to 258.0 ng/ml. Chronic administration of a high-iodine diet (HID) did not affect serum thyroglobulin levels. Chronic administration of a low-iodine diet (LID) and propylthiouracil (PTU) led to a statistically significant increase in serum RTg that was accompanied by a significant rise in serum thyrotropin (rTSH). Serum thyroxine ( $T_4$ ) administered to normal rats for 14 days ( $20 \mu\text{g/day}$  subcutaneously) depressed serum RTg concentration from a mean level of  $119.4 \pm 17.5$  ng/ml ( $n = 19$ ) to a mean of  $35.0 \pm 0.27$  ng/ml ( $n = 19$ ) ( $P < 0.001$ ). While rats were on continuous  $T_4$  suppression, bovine thyroid-stimulating hormone (bTSH) given intravenously (2 IU) resulted in a mean maximal increment of RTg of  $332.0 \pm 81.5$  ng/ml ( $n = 6$ ) at 24 h. IgG-(LATS) long-acting thyroid stimulator injected intravenously resulted in a mean maximal increment of RTg concentration at 96 h of  $87.2 \pm 14.3$  ng/ml ( $n = 5$ ). Normal IgG had no sta-

tistical significant effect on RTg levels at any time after the injection.

## INTRODUCTION

The measurement of human thyroglobulin (HTg)<sup>1</sup> in the circulation has been made possible by the development of a double antibody radioimmunoassay for this protein (1). However, because of the restrictions imposed on human studies, a specific and sensitive radioimmunoassay for measurement of serum thyroglobulin in the rat (RTg) has been developed which permitted detailed studies of the magnitude and the significance of this protein in the circulation under various physiologic and experimental conditions. A radioimmunoassay for RTg previously reported by British investigators required extensive purification of the antisera to obtain the required sensitivity and specificity (2). We have studied a simplified assay method which provides sensitivity and specificity, and the level of RTg in the serum of the Sprague-Dawley rat under physiologic and experimental conditions is the subject of the present report.

## METHODS

### Buffer

Unless otherwise mentioned the buffer used throughout the assay was 0.15 M NaCl-0.0035 M phosphate-buffered sa-

<sup>1</sup> *Abbreviations used in this paper:* bTSH, bovine thyroid-stimulating hormone; DIT, diiodotyrosine; D- $T_3$ , D-triiodothyronine; D- $T_4$ , D-thyroxine; HID, high-iodine diet; HTg, human thyroglobulin; LATS, long-acting thyroid stimulator; LID, low-iodine diet; L- $T_3$ , L-triiodothyronine; L- $T_4$ , L-thyroxine; MIT, monoiodotyrosine; NID, normal iodine diet; PBS, phosphate-buffered saline; PTU, 6-n-propyl-2-thiouracil; RB, RTg antiserum from rabbits; RTG, rat thyroglobulin; rTSH, rat thyroid-stimulating hormone;  $T_4$ , serum thyroxine; TETRAC, tetraiodothyroacetic acid; TRIAC, triiodothyroacetic acid; Triprop, triiodothyropropionic acid.

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line (PBS), pH 7.0. No preservatives were added since pilot studies had indicated that the absence of an antibacterial agent during the incubation gave comparable results with an assay where 0.01% sodium azide was added to the buffer.

### RTg source

Purified RTg was extracted from the thyroid glands of normal Sprague-Dawley rats. The glands were removed immediately after sacrifice of the animals while still attached to the trachea; they then were dissected from the tracheal structure and surrounding tissues and kept at  $-20^{\circ}\text{C}$  until further processing. The thyroids were minced when a sufficient number was collected, and 1.0 ml of PBS was added per gram of thyroid tissue. The total mixture was subsequently homogenized by an all-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 2,500 rpm for 10 min at  $4^{\circ}\text{C}$  to remove cellular debris and particles. The supernate (5 ml) was treated with an equal amount of a saturated ammonium sulfate solution, and the resulting precipitate was centrifuged, redissolved in PBS, and extensively dialyzed against several changes of this buffer. The thyroglobulin solution thus obtained was chromatographed on a  $35 \times 2.5\text{-cm}$  Sephadex G-200 column (Pharmacia, Uppsala, Sweden) using PBS as the eluent. Fractions from the first protein peak, as measured by optical density of the fractions at 280 nm, were combined and concentrated by negative pressure dialysis against PBS. Because of the consistent presence of a small shoulder on the ascending slope of this breakthrough peak, the concentrated first peak was rechromatographed on a  $45 \times 2.1\text{-cm}$  Sepharose-4B column (Pharmacia) to remove larger constituents. Under these chromatographic conditions, purified RTg appeared in the second protein peak (OD 280 nm). The fractions constituting this second peak were reconcentrated by negative pressure dialysis against PBS, and protein determinations were performed by the method of Lowry et al. (3). A total of 1.85 g of rat thyroid tissue was processed and yielded a purified thyroglobulin preparation (2.0 ml) with a concentration of 10.2 mg/ml.

The RTg preparation appeared to be pure as judged by the presence of a single band in the interalpha region on acetate electrophoresis and the absence of precipitation with rabbit antiwhole rat serum (Arnel Products Corp., Brooklyn, N. Y.) in a double immunodiffusion system (see Results section). The purified RTg was used for the production of antisera and for the preparation of the tracer.

### Preparation of antiserum

Anti-RTg antiserum was produced by initial immunization of young white male New Zealand rabbits with purified RTg by the lymph node technique of Newbould (4). Two animals were first injected bilaterally into the popliteal lymph nodes with 3.25 mg of purified RTg (250  $\mu\text{l}$ ) per animal suspended in equal parts (250  $\mu\text{l}$ ) of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). 2, 4, and 8 wk later each animal was reinjected with an additional emulsion containing 1.64 mg of RTg in complete Freund's adjuvant via bilateral dorsal subcutaneous injection sites. 4 mo after the last immunization the animals were bled, and the serum was separated and stored at  $-20^{\circ}\text{C}$  until further use. The present study was carried out totally with an antiserum from one animal referred to as serum RB.

### Iodination of RTg

$[^{131}\text{I}]\text{RTg}$  was obtained by labeling purified RTg according to the method of Greenwood et al. (5). The  $[^{131}\text{I}]\text{RTg}$  was immediately separated from free radioactive iodine by means of a  $13 \times 100\text{-mm}$  Sephadex G-200 column. The  $[^{131}\text{I}]\text{RTg}$  was eluted by discarding an initial 3.5 ml fraction and then collecting 350  $\mu\text{l}$  fractions. The top fraction of the first radioactive peak (protein peak) was selected as tracer since this fraction contained the least amount of damaged  $[^{131}\text{I}]\text{RTg}$ . To remove molecules with a tendency to combine nonspecifically to antigen-antibody complexes, 0.5 ml of a 0.2 mg/ml ovalbumin solution (Sigma Chemical Co., St. Louis, Mo.) and 0.5 ml of a rabbit antiovalbumin serum (Arnel Products Corp.) at equivalence were added to the tracer preparation. The mixture remained at  $4^{\circ}\text{C}$  overnight and then was centrifuged at 3,000 rpm for 20 min; the supernate was subsequently used as the tracer preparation after appropriate dilution in 0.05 M EDTA in PBS. The tracer solution contained  $[^{131}\text{I}]\text{RTg}$  with a specific activity of approximately 250 mCi/ $\mu\text{g}$  and displayed predominantly a 19S pattern with a moderate amount of 13.3S material on sucrose density gradient centrifugation. The latter sedimentation coefficient was calculated using purified human IgM in the same run as a 19S marker (Fig. 1). The TCA precipitability of labeled  $[^{131}\text{I}]\text{RTg}$  was 93.3%, and the immunoprecipitability was 87.8% in the presence of excess first antibody (RB 1:100).

### Radioimmunoassay procedure

The standards for the RTg assay consisted of purified RTg appropriately diluted in RTg-free serum (1:10 in PBS) and will be referred to as the diluent. The RTg-free serum was obtained from animals surgically thyroidectomized followed by radioablation (2 mCi  $^{131}\text{I}$ ). Standard RTg solutions (100  $\mu\text{l}$ ) ranging from 2.5 to 1,000 ng/ml (0.25–100 ng/tube) were added to disposable glass tubes (Becton-Dickinson and Co., Rutherford, N. J.). 50  $\mu\text{l}$  of the test sera were added to 50  $\mu\text{l}$  of diluent in glass tubes. Pilot studies demonstrated no significant difference in serum

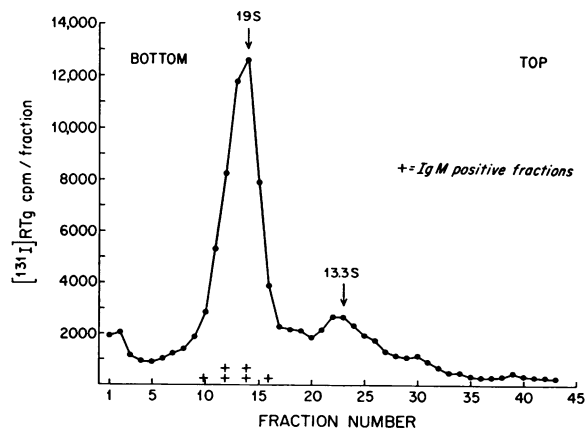


FIGURE 1 Sucrose density patterns of  $[^{131}\text{I}]\text{RTg}$ . Tracer  $[^{131}\text{I}]\text{RTg}$  and purified IgM (19S marker) were layered on top of a 5–20% linear sucrose density gradient. Centrifugation was carried out in a Spinco SW 41 rotor at 27,000 rpm for 24 h at  $4^{\circ}\text{C}$ . A symmetrical radioactive peak is observed in the same fractions where IgM was detected by radial immunodiffusion.

RTg concentrations when either 50  $\mu$ l or 100  $\mu$ l serum were used in the assay. 50- $\mu$ l samples were used because of the limited amount of serum available in small animals. The first antibody (RB, RTg antiserum from rabbits) diluted 1:80,000 in PBS (200  $\mu$ l) (final dilution 1:200,000) bound approximately 60-75% of the [<sup>125</sup>I]RTg in the absence of cold, competing RTg. 200  $\mu$ l of tracer [<sup>125</sup>I]RTg containing 3,000 cpm were added to all tubes, and after vortexing the mixture was incubated for 5 days (optimal incubation time) at room temperature. Subsequently 100  $\mu$ l of rabbit serum (Colorado Serum Co., Denver, Colo.) diluted 1:200 in PBS were added as a carrier, and 200  $\mu$ l of goat antirabbit gamma globulin (Antibodies Inc., Davis, Calif.) in a 1:32 dilution (PBS) were added to all tubes. The tubes were vortexed and incubated for an additional 18 h at room temperature and subsequently spun at 3,000 rpm for 20 min at room temperature. The supernates and precipitates were separated, and both were counted in a well-type gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.). For each standard curve and all serum samples, nonspecific trapped radioactivity was measured by using a control tube omitting the first antibody. The percentage of bound radioactivity was calculated after correction for the nonspecific bound radioactivity. The latter represented not more than 8% of the total radioactive counts added. All values obtained on serum samples (when 50  $\mu$ l of serum were used) were multiplied by a factor of two at the end of the calculations.

### Serum samples

All sera samples assayed had been stored at -20°C for periods up to 2 yr without measurable loss of RTg immunoreactivity. The latter was established in pilot studies. All studies reported were performed on Sprague-Dawley rats; the sera were ordinarily obtained by tail vein punctures after heating the tail briefly (30 s) in warm water (52°C).

### Additional procedures and materials

Serum thyroxine (T<sub>4</sub>) was determined according to a radioimmunoassay described by Chopra (6). Rat thyroid stimulating hormone (rTSH) was measured with the NIAMD kit (National Institute of Arthritis and Metabolic Diseases, rat pituitary hormone program, National Institutes of Health). The rTSH reference preparation for the radioimmunoassay NIAMD/rTSH·RP-1 had a biologic potency equivalent to 0.22 USP (bovine) TSH U/mg (McKenzie assay).

Purified IgG was obtained by the method of Miayi and Werner (7) and was quantitated by the radial immunodiffusion technique (Immunoplate, Hyland Laboratories, Costa Mesa, Calif.). The presence of the long-acting thyroid stimulator (LATS) was determined by the Snyder et al. modification (8) of the McKenzie bioassay. The following chemicals were used for displacement analysis: L-thyroxine (L-T<sub>4</sub>), D-thyroxine (D-T<sub>4</sub>), L-triiodothyronine (L-T<sub>3</sub>), D-triiodothyronine (D-T<sub>3</sub>), tetraiodothyroacetic acid (TETRAC), triiodothyroacetic acid (TRIAC), triiodothyropropionic acid (Triprop), L-thyronine, 6-n-propyl-2-thiouracil (PTU) (Sigma Chemical Co.) and monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Calbiochem, San Diego, Calif.).

### Experimental studies

The effect of different pharmacologic agents on the release of RTg, rTSH, and T<sub>4</sub> was studied in male adult Sprague-

Dawley rats. All samples obtained from animals belonging to a single study were assayed in one radioimmunoassay to exclude interassay variation. Statistical analysis was performed using Student's *t* test (two tailed). Experimental designs were as follows:

#### LOW-IODINE DIET (LID)

A group of seven rats was fed a Remington iodine-deficient diet (General Biochemicals, Chagrin Falls, Ohio) and distilled water for 8 wk. Before the onset of this diet the animals were bled via the tail vein, and serial tail vein samples were subsequently obtained at intervals of 2 wk for the measurement of RTg, rTSH, and T<sub>4</sub> in serum.

#### HIGH-IODINE DIET (HID)

A group of seven rats was fed for 8 wk on regular laboratory food with addition of potassium iodide (50 mg/liter) to their drinking water (estimated intake 20-30 ml/day). Serum samples were obtained by tail vein puncture, and the same functional parameters were studied at identical intervals as for the LID animals. A control group of seven rats, fed laboratory food and distilled water (NID, normal iodine diet), was carried throughout the study, and sampling was spaced as for the LID and HID groups. At the end of 8 wk all animals were killed and their thyroids removed and carefully weighed.

#### PTU TREATMENT

In this study Sprague-Dawley rats (*n* = 7) were fed regular laboratory food, and PTU was added to the drinking water (0.01%). The animals were bled via the tail vein before the start of the study twice a week for the first 2 wk and then at 2-wk intervals. All sera were analyzed for RTg, rTSH, and T<sub>4</sub>. A control group of animals (*n* = 6) was fed laboratory food and distilled water, and blood samples were obtained at the same intervals as indicated for the PTU-treated animals; the same parameters were analyzed as in the PTU-treated group. At the end of this study the animals were killed, and their thyroids were removed and carefully weighed.

#### ADMINISTRATION OF THYROID STIMULATORS

*Thyroid-stimulating hormone (TSH).* Bovine TSH (bTSH), 2 IU (Thyropar, Armour Pharmaceutical Co., Chicago, Ill.), was injected intravenously into rats whose thyroids were suppressed with T<sub>4</sub> (*n* = 6). Suppression was achieved by subcutaneous injection of L-T<sub>4</sub>, 20  $\mu$ g daily, for 14 days before the injection of bTSH and then daily throughout the sampling period. Blood samples were obtained before bTSH injection and at 2, 8, 24, 48, 72, 96, 120, and 144 h. All samples were then introduced into one single RTg assay.

*Normal IgG and IgG-LATS administration.* Purified IgG was prepared by the method of Miayi and Werner (7) from a normal subject and from a patient with Graves' disease (R. V.) with a strongly positive LATS titer (0.15 mg of IgG-LATS injected in the bioassay mice elicited a response index of 388) (8). The serum of patient R. V. did not contain human thyroid-stimulating hormone (< 2.0  $\mu$ U/ml) or antithyroglobulin antibodies (tanned red cell agglutination test) and had an HTg concentration of 46.0 ng/ml. The IgG concentration for both preparations was adjusted to 8.0 mg/ml before testing in the rat.

Six Sprague-Dawley rats were suppressed as for the bTSH-injected animals. After 14 days of thyroxine administration the animals were intravenously injected with

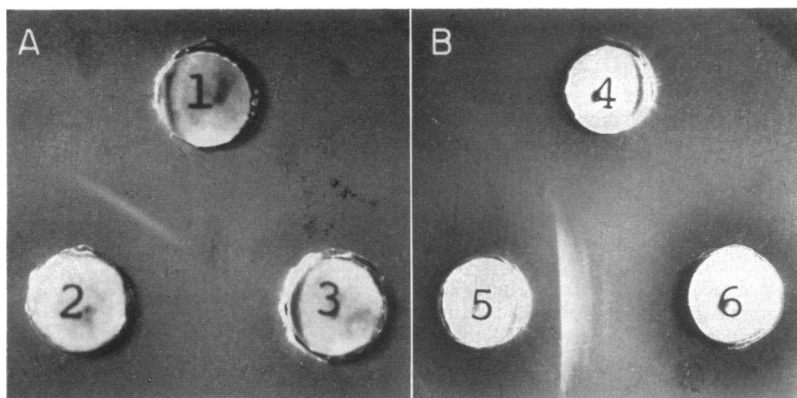


FIGURE 2 Double immunodiffusion studies. In Fig. 2A and B, well no. 1 and 4 contain purified RTg, well no. 3 and 6 contain normal rat serum. Well no. 2 in Fig. 2A contains rabbit anti-RTg (RB) used in the assay. A precipitation line with RTg in well no. 1 is visible, but not with rat serum in well no. 3. Well no. 5 in Fig. 2B contains rabbit anti-rat serum. This reacted with normal serum components in well no. 6, but not with purified RTg in well no. 4.

1 ml of normal human IgG (8.0 mg/ml IgG).  $T_4$  administration was continued during the remainder of the study. Blood samples were obtained via the tail vein at 0, 2, 8, 24, 48, 72, 96, 120, and 144 h after injection. In addition, five rats that underwent an identical period of  $T_4$  suppression were injected with IgG-LATS (8.0 mg/ml IgG). Sampling was performed via the tail vein at the following intervals: 0, 8, 24, 48, 72, 96, 120, 144, 168, 192, and 216 h. RTg was measured in all samples in one single assay.

## RESULTS

**Antiserum.** Antiserum RB was tested in an Ouchterlony double immunodiffusion system (Fig. 2A B) (9). Under these circumstances it did not cross-react with any serum component, but gave a strong precipitation line with purified RTg (Fig. 2A). Fig. 2B indicates that rabbit anti-whole rat serum did not lead to precipitation lines with the purified RTg preparation but gave,

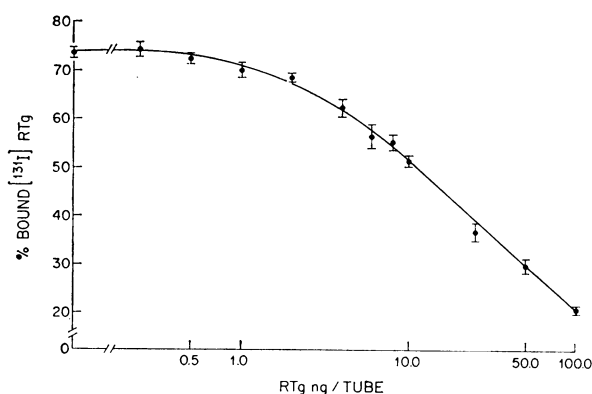


FIGURE 3 Inhibition of  $[^{125}\text{I}]\text{RTg}$  by unlabeled RTg. Each point represents an average of quadruplicate determinations.

as expected, precipitation lines with several unidentified components in rat serum (Fig. 2B). The potency of the present antiserum was such that a final dilution of 1:200,000 in the radioimmunoassay incubation mixture could be used.

**Assay characteristics.** Fig. 3 shows a typical standard curve for RTg in the range of 0–100 ng/tube (0–1,000 ng/ml). The lower sensitivity of the assay varied from 5 to 10 ng/ml.

A number of specificity studies were carried out. Recovery studies showed an average recovery rate of 101.3% (Table I). Fig. 4 indicates that the concentrations of serum RTg found in a given serum are inde-

TABLE I  
RTg Recovery

Experiment	Serum RTg			Recovery
	Initial*	Added	Final*	
	ng/ml	ng	ng/ml	%
1	$27.7 \pm 2.3$	50	$74.5 \pm 11.7$	95.8
		100	$111.2 \pm 16.0$	87.1
		250	$277.5 \pm 15.0$	99.9
2	$68.0 \pm 14.1$	50	$121.6 \pm 7.6$	103.9
		100	$172.5 \pm 9.5$	102.6
		250	$381.2 \pm 23.2$	119.8
3	$159.0 \pm 6.8$	50	$200.0 \pm 20.0$	95.7
		100	$293.0 \pm 30.5$	113.2
4	$164.0 \pm 5.4$	50	$187.5 \pm 21.0$	87.6
		100	$283.3 \pm 25.1$	107.3

\*  $\pm$ SD.

TABLE II  
*Serum Thyroglobulin (RTg), T<sub>4</sub>, and Serum Thyrotropin (rTSH) in Control (NID), High-Iodine Diet (HID), and Iodine-Deficient (LID) Rats*

	RTg ng/ml	T <sub>4</sub> μg/100 ml	rTSH ng/ml
Normal diet (NID), <i>n</i> = 7*			
Base line	94.4±18.3‡	5.4±0.31	567.1±121.3
2 wk	119.8±21.5 (NS)§	5.8±0.37 (NS)	822.6±187.8 (NS)
4 wk	105.0±22.4 (NS)	4.3±0.47 ( <i>P</i> < 0.05)	1,147.1±315.0 ( <i>P</i> < 0.05)
6 wk	83.7±19.2 (NS)	4.6±0.51 (NS)	884.2±227.2 (NS)
8 wk	59.7±13.4 ( <i>P</i> < 0.05)	5.4±0.23 (NS)	768.5±247.4 (NS)
High-iodine diet (HID), <i>n</i> = 7*			
Base line	117.2±31.3	4.9±0.43	691.4±85.7
2 wk	115.6±32.5 (NS)	5.6±0.33 ( <i>P</i> < 0.05)	707.1±196.4 (NS)
4 wk	100.0±30.9 (NS)	5.5±0.32 (NS)	940.0±235.6 (NS)
6 wk	86.1±21.5 (NS)	5.1±0.54 (NS)	948.5±192.9 (NS)
8 wk	96.8±31.2 (NS)	5.8±0.35 ( <i>P</i> < 0.02)	828.5±208.6 (NS)
Low-iodine diet (LID), <i>n</i> = 7*			
Base line	82.5±17.4	6.0±0.55	697.1±127.3
2 wk	98.5±18.8 (NS)	6.3±0.32 (NS)	854.2±184.7 (NS)
4 wk	104.2±13.9 (NS)	4.2±0.30 ( <i>P</i> < 0.025)	801.4±101.6 (NS)
6 wk	135.4±18.2 ( <i>P</i> < 0.005)	3.3±0.39 ( <i>P</i> < 0.01)	1,060.0±78.4 ( <i>P</i> < 0.05)
8 wk	147.4±21.5 ( <i>P</i> < 0.01)	2.9±0.43 ( <i>P</i> < 0.005)	1,564.0±169.0 ( <i>P</i> < 0.001)

\* Number in parentheses indicate number of animals in each group.

‡ Mean±SEM.

§ Significance was determined using Student's *t* test analysis for paired data. All mean serum concentrations were compared with the mean base-line level for that group (*P* value < 0.05 was considered significant).

pendent of the quantity of serum used which further adds to the specificity of this assay.

The specificity of the assay was also analyzed by testing the cross-reactivity of a number of thyroid compounds. No cross-reactivity was observed for the following 10 thyroid compounds when added in amounts of up to 40,000 ng/tube: D-T<sub>3</sub>, L-T<sub>3</sub>, D-T<sub>4</sub>, L-T<sub>4</sub>, TETRAC, TRIAC, MIT, DIT, Triprop, and L-thyronine.

During the present studies the effect of the intravenous injection of human IgG-LATS on the release of RTg was studied. To validate the results of these studies the lack of cross-reactivity in the RTg assay of HTg as well as antithyroglobulin antibodies frequently present in the sera had to be tested. Indeed, if substantial cross-reactivity existed at low concentrations with one of these components, erroneous conclusions could be reached by injection of serum containing these factors. It was demonstrated in separate experiments (not shown) that addition of 100,000 ng of HTg per tube cross-reacted in the assay similar to a solution of RTg containing 10 ng/ml. A concentration of 100,000 ng/ml of HTg has thus far never been observed in pathologic human sera (1). The serum of a patient with an antithyroglobulin

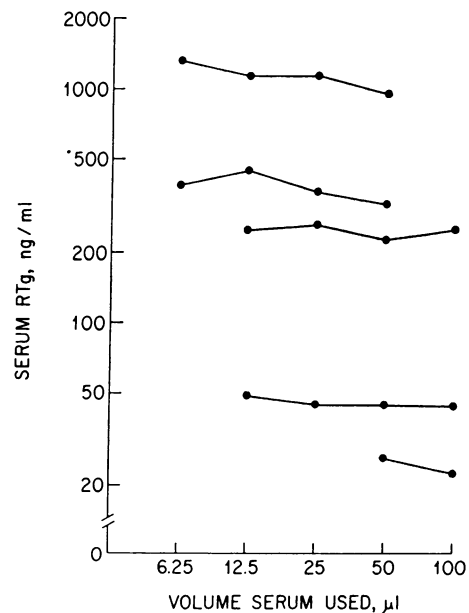


FIGURE 4 The effect of introduction of different volumes of serum in the assay on serum RTg concentration.

antibody titer greater than 1:4,500,000 (tanned red cell agglutination technique) had no substantial effect on [<sup>125</sup>I]RTg binding by the RTg antibody (RB). This human serum did not bind [<sup>125</sup>I]RTg in absence of RB.

Further evidence of specificity comes from studies carried out in rats that underwent surgical and radioablation of the thyroid (2 mCi of <sup>131</sup>I). In no instances could RTg be detected in the serum of these animals. These sera were consequently used as a diluent for all test samples and standard curves (see Methods section).

**Reproducibility.** The reproducibility of the serum RTg determinations was evaluated by calculation of the coefficient of variation (10). The coefficient of variation within one single assay was 11.0%, calculated on the basis of duplicate determinations on 12 samples ranging in concentration from 16.0 to 148.0 ng/ml. The interassay coefficient of variation, based on repeated determination of 10 samples in two different assays, was 15%.

**Normal serum RTg levels.** Serum thyroglobulin levels in tail vein blood of normal adult Sprague-Dawley

rats was 101.5±13.0 ng/ml (SEM) (n = 21). The values ranged from 12.0 to 258.0 ng/ml. In no instances were undetectable RTg levels encountered. To establish whether or not important variation of serum RTg levels occurred during an experimental period (12 h), tail vein blood was obtained from a group of six normal rats at 8 a.m. and 8 p.m. The mean serum RTg level at 8 a.m. was 157.6±31.0 ng/ml and was 136.3±20.6 ng/ml at 8 p.m. These mean values were not significantly different. A study of arterial (carotid) and venous (tail vein) blood obtained in adult rats did not reveal statistically significant differences.

**Effect of NID, LID, and HID on serum thyroglobulin concentration.** Results of these studies are represented in Table II. When sera of rats on NID were analyzed no systematic significant changes in RTg, T<sub>4</sub>, or rTSH occurred, based on 2 weekly interval determinations up to 8 wk. A significant fall in serum T<sub>4</sub> and an increase in rTSH were observed in the control (NID) group 4 wk after starting the study. A significant fall at 8 wk in serum RTg was also observed in this group. Rats on a HID diet failed to reveal significant changes in RTg and serum rTSH levels throughout the study. However, a significant fall in serum T<sub>4</sub> at 2 and 8 wk was observed. In contrast, in the LID group a rise in mean RTg levels was observed reaching statistical significance (P < 0.005) at 6 wk. Of interest is a statistically significant depression of serum T<sub>4</sub> observed as early as 4 wk (P < 0.025) after initiation of the diet and a significant rise of rTSH at 6 and 8 wk (P < 0.05 and P < 0.001, respectively). Depression of serum T<sub>4</sub> (P < 0.005) and elevation of serum rTSH (P < 0.001) remained significantly different from base line at the termination of the study (8 wk). When the mean thyroid weight of the LID group (34.3±3.7 mg) (n = 7) was compared with the thyroid weight of the NID control group (22.7±1.6 mg) (n = 7), a statistically significant difference (P < 0.01) was observed. No significant difference was observed between the mean thyroid weights in the NID and the HID group.

**Effect of PTU treatment on serum RTg concentrations.** PTU administration, as 0.01% in the drinking water, elicited a statistically significant increase (P < 0.01) in serum RTg levels as early as 1 wk after initiation of therapy (Fig. 5). This increment persisted until the end of the study at which time a doubling of the base-line RTg level was observed. Serum T<sub>4</sub> levels, determined in parallel, disclosed a dramatic and expected fall in the PTU-treated group (P < 0.001) to near undetectable levels as early as 7 days after starting the treatment. A concomitant and expected rise in rTSH became significant 4 days after the initiation of PTU therapy, and this elevation persisted throughout the study. No statistically significant change in serum rTSH

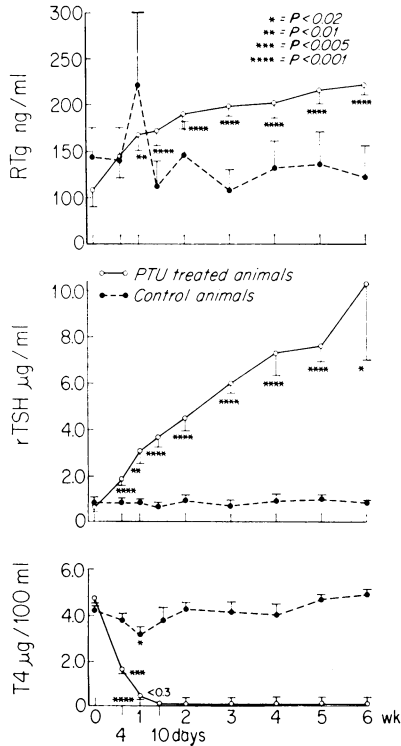


FIGURE 5 Effect of PTU on serum RTg, rTSH, and T<sub>4</sub> levels. The mean serum levels (±SEM) for RTg, rTSH, and T<sub>4</sub> is shown for control (●--●) (n = 6) and PTU (○--○) (n = 7)-treated rats. The significance level of the observed changes for the respective parameters in the PTU-treated animals is indicated in the top panel. No persistent statistical significant changes were observed in the normal control group.

and RTg levels were observed in the control group. A transient but significant fall in  $T_4$  levels ( $P < 0.02$ ) was observed in these control animals 7 days after initiation of the study. The mean thyroid weight in the control animals,  $24.3 \pm 1.2$  mg ( $n = 6$ ), was significantly different from that in PTU-treated rats,  $89.9 \pm 8.5$  mg ( $n = 7$ ) ( $P < 0.001$ ).

**Correlations between log serum RTg, log serum rTSH and log thyroid weight.** It was reasoned that if the observed serum RTg at the end of the study was a result of rTSH stimulation then a positive correlation should exist between these two parameters, independently whether an enhancement of a normal existing stimulatory process was present or not. Fig. 6A demonstrates such a positive correlation between the log of serum RTg and the log serum rTSH in control and experimentally-treated animals (HID, LID, PTU) combined ( $r = 0.448$ ,  $P < 0.008$ ). A positive correlation between log serum rTSH and log thyroid weight also was present, the results of these studies are shown in Fig. 6B ( $r = 0.838$ ,  $P < 0.001$ ). Finally a positive correlation between log serum RTg and log of thyroid weight in all groups is shown in Fig. 6C ( $r = 0.672$ ,  $P < 0.001$ ). Separate analysis of the subgroups was not carried out because of the paucity of data in the respective subgroups.

**Effect of  $T_4$  administration.** The administration of  $L-T_4$ , 20  $\mu$ g subcutaneous/day for 14 days, resulted in a highly significant drop in RTg levels ( $P < 0.001$ ) from a mean of  $119.4 \pm 17.5$  ng/ml ( $n = 19$ ) to  $35.0 \pm 8.2$  ng/ml ( $n = 19$ ).

**Effect of bTSH, IgG-LATS, and normal IgG.** The intravenous injection of bTSH (2.0 IU) resulted in a statistically significant increase in serum RTg levels 24 h after the injection ( $P < 0.02$ ) (Fig. 7). In contrast, IgG-LATS after intravenous injection, resulted in a steady increase in RTg levels reaching a peak at 96 h ( $P < 0.005$ ) and remaining significantly elevated until the 8th day after injection. The injection of normal IgG in an amount equal to IgG-LATS did not result in a significant increase of RTg for the length of the study (144 h).

## DISCUSSION

Although substantial information was available regarding the thyroglobulin concentration in cervical and thyroidal lymph of different experimental animals (2, 11-13), the presence of this protein in peripheral blood of rats was studied by only one group of investigators (2). This group, however, was unable to quantitate the protein in all experimental animals under study. The present report describes a simple, precise, and specific

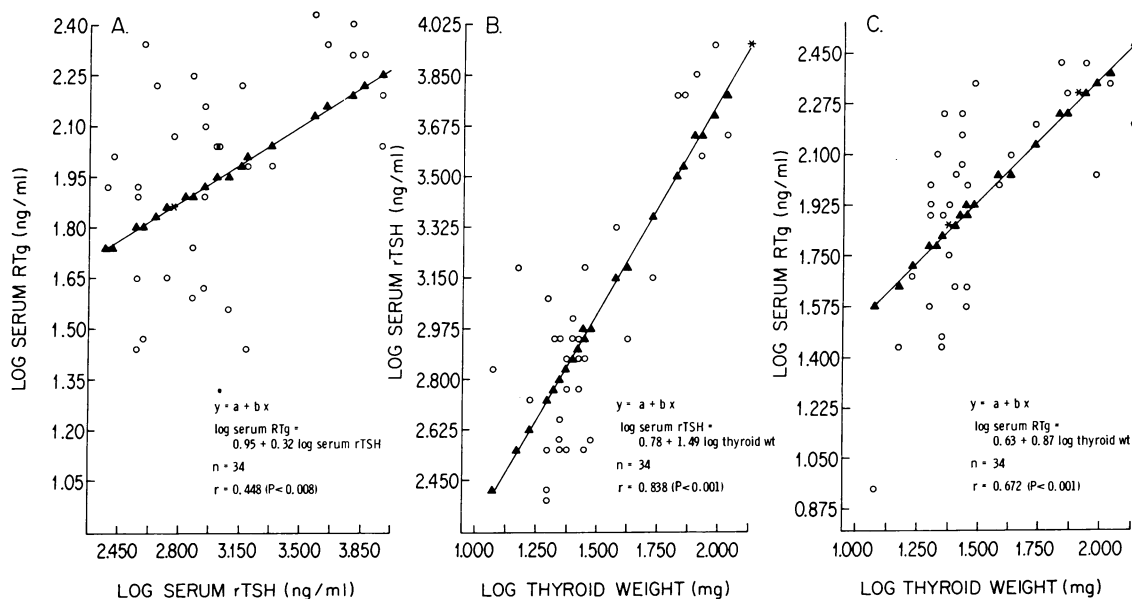


FIGURE 6 Relationship between log serum RTg, log serum rTSH, and log of thyroid weight. 6A represents the relationship between log serum RTg and log serum rTSH. 6B represents the relationship between log rTSH and log thyroid weight. 6C represents the relationship between log serum RTg and log thyroid weight.  $\circ$ , single observations;  $\blacktriangle$ , values predicted from the relationship between two parameters; \*, points where  $\circ$  and  $\blacktriangle$  were superimposed. A good to excellent correlation was observed for the parameters under analysis (see  $r$  values on figure).

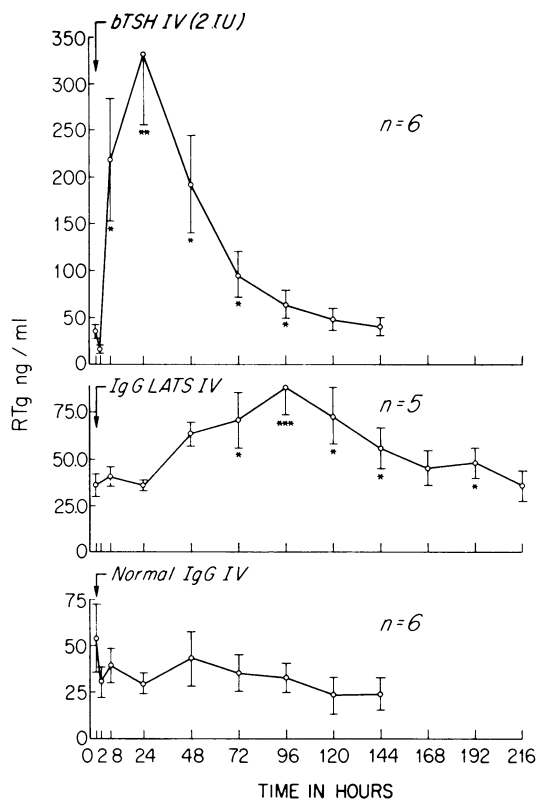


FIGURE 7 Effect of bTSH, IgG-LATS, and normal IgG on rat serum RTg levels. The mean serum RTg levels ( $\pm$ SEM) after intravenous injection of bTSH, IgG-LATS, and normal IgG are shown. The mean serum RTg concentrations were elevated above the levels at time zero at various intervals for bTSH and IgG-LATS-injected animals. The significance of the observed rise is indicated by asterisks (\* $P < 0.05$ , \*\* $P < 0.02$ , \*\*\* $P < 0.005$ ). Note that peak serum RTg levels were observed 24 h after injection of bTSH and 96 h after IgG-LATS. No significant changes in serum RTg levels were observed in the group treated with normal IgG. 14 days before time 0 and during the whole study all animals received daily 20  $\mu$ g of thyroxine subcutaneously.

radioimmunoassay for the measurement of thyroglobulin in the peripheral blood of the rat. The study also indicates that the thyroid is responsible for the release of this protein because of the absence of RTg in the circulation of rats after surgical and radioablation of this organ.

The sensitivity of the present assay was 10.0 ng/ml in all instances. The reasons for the discrepancy between detectability of RTg in the sera of all Sprague-Dawley rats in our series and only 28% detectability reported by Daniel et al. (2) remains obscure. RTg levels in the rat serum are not subject to large fluctuations, as indicated by serial RTg determinations in control animals of the different pharmacologic studies. In addition, the

mean serum RTg concentration is relatively high; it is 20 times higher than the mean level observed in humans using a specific radioimmunoassay for HTg (1).

Anions such as perchlorate and iodine are known to affect thyroid hormone production or release (14-20). In the present study animals given a NID or HID displayed no statistically significant persistent changes in  $T_4$ , rTSH, or RTg. An occasional fall and/or rise in serum  $T_4$ , rTSH, and RTg were observed in the control animals; however, these changes were never persistent. In contrast, the LID led to a significant and persistent fall in serum  $T_4$  levels and a significant increase in RTg levels at 6 and 8 wk after the start of the diet. A parallel increase in rTSH was also noted. The fall in  $T_4$ , although less dramatic than in the study reported by Abrams and Larsen (21), presumably triggers a pituitary TSH release which in turn is responsible for thyroid hyperplasia and a proportional increase in RTg release into the circulation. Indeed when correlations between log serum RTg and log serum rTSH or log thyroid weight were carried out for control and treated animals (HID, LID, PTU), a positive correlation was found. A positive correlation between log serum rTSH and log thyroid weight also coexisted.

To further clarify the relationship of thyroid hyperplasia and TSH levels to RTg release, the effect of PTU administration was studied. These studies revealed a rapid fall in serum  $T_4$  levels as early as 4 days after PTU administration accompanied by a prompt and sustained rise in rTSH levels. The rise in RTg became significant 1 wk after initiation of the PTU administration. These data support the concept that irrespective of whether serum rTSH is present in physiologic or supraphysiologic levels, RTg is released in the circulation in proportion to the glandular weight achieved by this stimulator. The assumption that rTSH is the mediator in the RTg release is further supported by more acute experiments indicating a depression of RTg levels after  $T_4$  administration and the prompt (24-h peak) release of RTg after bTSH. A large dose of bTSH was used to study RTg release since it is a well-known observation that stimulation of the rat thyroid gland is difficult to achieve.

To test whether or not serum RTg release is a sensitive bioassay system, still another thyroid stimulator, namely LATS, was utilized. A highly potent LATS positive IgG fraction led to a release of RTg that was maximal and significant at 96 h. No release of RTg was seen when an equal amount of normal IgG was injected into test animals. These observations confirm the data of Purves and Adams (22), which show that the thyroid stimulator present in serum of patients with Graves' disease causes stimulation of the thyroid gland in rats and adds the important observation that in this stimulation process thyroglobulin, an organ-specific



protein, is released in a delayed fashion when compared with bTSH.

The present study establishes the radioimmunoassay for RTg as a valuable tool in the quantitation of serum RTg in physiological and experimental conditions. In addition, it qualifies RTg as a normal secretory product of the thyroid gland in the rat. Indeed the effect of T<sub>4</sub> and TSH on RTg release suggests that this circulatory protein is under pituitary control and universally present in the rat species studied. The observation that RTg is released into the serum after IgG-LATS injections in T<sub>4</sub>-suppressed animals further confirms that the IgG fraction of some patients with Graves' disease can serve as a potent stimulator of the rat thyroid.

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