

Radioimmunoassay for Measurement of Thyroglobulin in Human Serum

A. J. Van Herle, ... , N. L. Matthews, Josiah Brown

J Clin Invest. 1973;52(6):1320-1327. <https://doi.org/10.1172/JCI107303>.

Research Article

A specific and reproducible double antibody radioimmunoassay for the measurement of thyroglobulin (HTg) in human serum has been developed. Since antithyroglobulin autoantibodies combine with the [¹³¹I] HTg tracer, antibody-positive sera were rejected for measurement. Specificity is demonstrated in that thyroid analogues such as thyroxine (T₄), triiodothyronine (T₃), monoiodotyrosine (MIT) and diiodotyrosine (DIT) did not crossreact. Sera previously reacted with anti-HTg-Sepharose contained no immunoassayable HTg. Finally, sera obtained from patients after total thyroid ablation for thyroid carcinoma did not contain demonstrable HTg. The sensitivity of the assay is 1.6 ng/ml, and HTg was detectable in 74% of 95 normal subjects. The mean concentration was 5.1 ng/ml ±0.49 SEM (range <1.6-20.7 ng/ml). Day to day variation in HTg levels is large in some euthyroid subjects and nearly absent in others. HTg was detectable in 90% of the sera obtained in 23 pregnant women at delivery in whom a mean concentration of 10.1 ng/ml ±1.3 SEM was observed. The mean level for the corresponding newborn infants at birth was 29.3 ng/ml ±4.7 SEM a value significantly higher than the mean maternal HTg concentration (*P* <0.01). A group of 17 thyrotoxic individuals all had elevated HTg levels; the mean for this group was 344.8 ng/ml ±90.7 SEM. In the acute phase of subacute thyroiditis HTg was also elevated in all of 12 patients, and [...]

Find the latest version:

<https://jci.me/107303/pdf>



Radioimmunoassay for Measurement of Thyroglobulin in Human Serum

A. J. VAN HERLE, R. P. ULLER, N. L. MATTHEWS, and JOSIAH BROWN

From the Department of Medicine, Division of Endocrinology, UCLA School of Medicine, Los Angeles, California 90024

ABSTRACT A specific and reproducible double antibody radioimmunoassay for the measurement of thyroglobulin (HTg) in human serum has been developed. Since antithyroglobulin autoantibodies combine with the [¹²⁵I] HTg tracer, antibody-positive sera were rejected for measurement. Specificity is demonstrated in that thyroid analogs such as thyroxine (T₄), triiodothyronine (T₃) monoiodotyrosine (MIT) and diiodotyrosine (DIT) did not crossreact. Sera previously reacted with anti-HTg-Sepharose contained no immunoassayable HTg. Finally, sera obtained from patients after total thyroid ablation for thyroid carcinoma did not contain demonstrable HTg. The sensitivity of the assay is 1.6 ng/ml, and HTg was detectable in 74% of 95 normal subjects. The mean concentration was 5.1 ng/ml ± 0.49 SEM (range < 1.6–20.7 ng/ml). Day to day variation in HTg levels is large in some euthyroid subjects and nearly absent in others. HTg was detectable in 90% of the sera obtained in 23 pregnant women at delivery in whom a mean concentration of 10.1 ng/ml ± 1.3 SEM was observed. The mean level for the corresponding newborn infants at birth was 29.3 ng/ml ± 4.7 SEM a value significantly higher than the mean maternal HTg concentration ($P < 0.001$). A group of 17 thyrotoxic individuals all had elevated HTg levels; the mean for this group was 344.8 ng/ml ± 90.7 SEM. In the acute phase of subacute thyroiditis HTg was also elevated in all of 12 patients, and the mean for this group was 136.8 ng/ml ± 74.6 SEM.

INTRODUCTION

In the past cumbersome and semiquantitative methods based on tanned red cell hemagglutination inhibition

This paper was presented in part at the IV International Congress of Endocrinology held in Washington, D. C., 18 June 1972.

Dr. Uller is a Special Fellow of National Institute of Arthritis and Metabolic Diseases (AM 51377).

Received for publication 6 May 1972 and in revised form 24 November 1972.

(1, 2) or electrophoretic immunoretention (3) were used to measure a "thyroglobulin-like" substance in blood of human beings. More recently Roitt and Torrigiani (4) described a radioimmunoassay for this large protein. Elaborate purification of their antisera was required to obtain the reported sensitivity and specificity of their assay. We wish to describe a simplified, sensitive, and specific radioimmunoassay for human serum thyroglobulin. The levels of thyroglobulin in the sera of normal subjects and in patients with hyperthyroidism and subacute thyroiditis are also described in this report.

METHODS

Buffer

The buffer used throughout unless otherwise specified, was 0.15 M NaCl-0.0035 M phosphate buffer pH 7.0, referred to as PBS.¹

Purification of antigen

Thyroid tissue was obtained from subjects who underwent subtotal thyroidectomy for hyperthyroidism due to Graves' disease. Extraction and purification of human thyroglobulin (HTg) was performed as follows: a mixture of 2.0 ml of PBS per gram of minced tissue was stirred overnight at 4°C, and the coarse debris was then removed by gauze filtration; the filtrate was centrifuged at 1,000 × *g* for 10 min at 4°C to remove cellular particles. 4 ml of the supernate thus obtained was applied to a 35 × 3.5 cm Sephadex G-200 (Pharmacia, Uppsala, Sweden) column and eluted with PBS, and 3.7 ml fractions were collected. The first peak, as indicated by optical density of the fractions at 280 nm, was concentrated by negative pressure dialysis against PBS. The concentrated protein solution thus obtained was rechromatographed on a 45 × 2.1 cm Sepharose 4B (Pharmacia) column in order to remove larger and smaller constituents. Elution was performed with PBS, and 3.7 ml fractions were collected. Purified HTg appeared in the second peak. The fractions constituting the second peak

¹ Abbreviations used in this paper: CV, coefficient of variation; DIT, diiodotyrosine; HCT, human chorionic thyrotropin; HTg, human thyroglobulin; Huc, HTg antiserum of one rabbit; IgG, immunoglobulin G; MIT, monoiodotyrosine; PBS, phosphate-buffered saline; T₃, triiodothyronine; T₄, thyroxine; TSH, thyroid stimulating hormone.

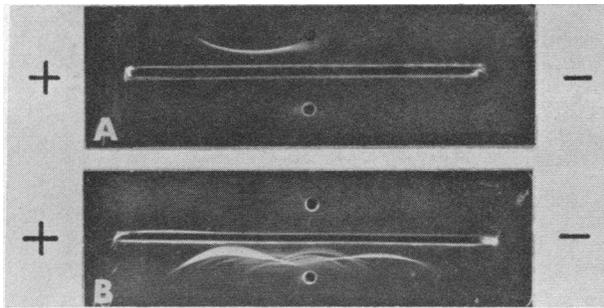


FIGURE 1 Immuno-electrophoretic patterns. In Fig. 1 A and B the top wells contained purified HTg (employed in the assay), the bottom wells reference serum GA. The central trough contained HTg antiserum (Fig. 1 A) and anti-whole human serum (Fig. 1 B). A well defined precipitation arc developed with HTg (top well Fig. 1 A) no precipitation line developed with reference serum GA (bottom well Fig. 1 A). In Fig. 1 B no precipitation arc developed with purified HTg (top well), a classical serum electrophoretic pattern developed with material in bottom well.

were combined and concentrated by negative pressure dialysis. Homogeneity of this HTg solution was established by cellulose acetate electrophoresis (single band in the inter-alpha region) and immuno-electrophoresis. Immuno-electrophoresis in agar was carried out in barbital buffer at pH 8.6 as previously described (5). Purified human thyroglobulin and serum GA were used as source of HTg and serum, respectively. Serum from a thyrotoxic patient with a HTg concentration of 25,000 ng/ml is referred to as reference serum GA. Antihuman HTg antiserum obtained from one rabbit (Huc) and anti-whole human serum obtained from Meloy Laboratory Inc., Biological Products, Springfield, Va. Batch A-122-3 were used as antibody sources. HTg antiserum (Huc) reacted only with purified HTg, and absence of precipitation arcs with serum GA was noted (Fig. 1A). Anti-whole human serum on the other hand reacted only with human serum, and absence of precipitation arcs with purified HTg was noted (Fig. 1B).

The protein concentration in the thyroglobulin solution was determined by the Lowry method (6), by Kjeldahl determination, and by dry weight after extensive dialysis against distilled water and subsequent lyophilization. There was very good agreement between values obtained by these three techniques. The HTg solution obtained was employed for construction of standard curves, tracer preparation, and immunization procedure. Reference serum GA was also used to construct standard curves.

Preparation of antiserum

Antisera to HTg were obtained by immunization of two New Zealand white rabbits with HTg which was purified as described above. An emulsion of equal parts of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and a purified HTg solution was prepared. Each animal received three doses of the emulsion containing 8.26 mg/ml of antigen per injection. The first dose was administered into the popliteal lymph nodes. Subsequent injections were given subcutaneously 2 and 5 wk after the initial injection. 2 wk after the third injection, the animals were bled, and the sera obtained were stored at -20°C . The totality of the present study was carried out with the antiserum of one rabbit (Huc).

Preparation of labeled antigen

$[^{125}\text{I}]$ HTg was obtained by labeling purified HTg according to the method of Greenwood, Hunter, and Glover (7). The $[^{125}\text{I}]$ HTg was immediately separated from free radioactive iodine by filtration of the iodination mixture through a 13×100 mm Sephadex G-200 column, collecting 350 μl fractions after discarding an initial fraction of 3.0 ml. The first peak of radioactivity as determined with a small radiation monitor corresponds to the labeled protein peak. The second fraction on the descending slope of the protein peak was selected as tracer, since this fraction had been previously determined by immunologic methods to contain the least amount of damaged $[^{125}\text{I}]$ HTg. In order to remove molecules with a tendency to combine nonspecifically with antigen antibody complexes, a precipitate in situ was performed by addition to the tracer of amounts of ovalbumin and antiovalbumin serum at equivalence (Arnel Products Corp., Brooklyn, N. Y.). The mixture was incubated overnight at 4°C , and after centrifugation the precipitate was discarded. The solution thus obtained contained $[^{125}\text{I}]$ HTg with a specific activity of approximately 250 $\mu\text{Ci}/\mu\text{g}$. Predominance of $[^{125}\text{I}]$ HTg of the 19S variety is shown by sucrose density centrifugation (Fig. 2) using a purified IgM preparation as 19S marker.

Radioimmunoassay procedure

Reagents were added, and the various steps were carried out in the following order in 10×75 mm disposable glass culture tubes (Becton-Dickinson and Co., Rutherford, N. J.):

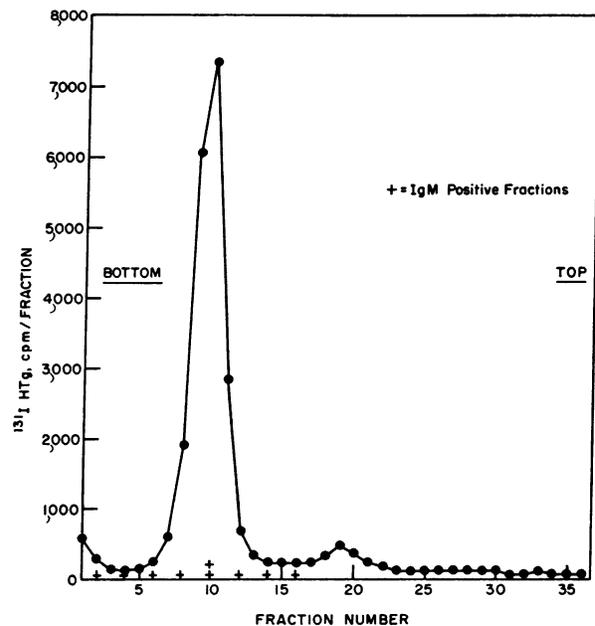


FIGURE 2 Sucrose density pattern of $[^{125}\text{I}]$ HTg. Tracer $[^{125}\text{I}]$ HTg and purified IgM (19S marker) were layered on top of a 5%–20% sucrose density gradient. Centrifugation was carried out in a Beckman SW 41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.), 27,000 rpm, 24 h, 4°C . A symmetrical radioactive peak is observed in the area where IgM was detected by radial immunodiffusion.

Unlabeled HTg. A 200 μ l sample of standard HTg, reference serum GA, or test serum was added to the tube. Using dilutions of purified HTg a 13 point standard curve ranging from 5–300 ng/ml (1–60 ng/tube) was constructed. Goat serum diluted 1:10, (Pentex Inc., Division of Miles Laboratories, Kankakee, Ill.) was used as diluent in order to reduce tracer damage. Using dilutions of reference serum GA, a 10 point standard curve ranging from 0.8–250.0 ng/ml (0.16–50.0 ng/assay tube) was constructed in diluted goat serum (1:10). Standards and samples were always assayed in duplicate; the mean of duplicate determinations is reported.

Tracer (200 μ l). [125 I] HTg solution was diluted in 0.05 M EDTA Na in PBS so that 3,000 cpm were added to all tubes, yielding a final count after 5 days of 2,000 cpm per tube.

First antibody (100 μ l). Antiserum Huc was used in two different dilutions. Ordinarily a dilution of 1:100,000 (1:500,000 final) which bound 60–70% of [125 I] HTg in the absence of unlabeled antigen was employed. In addition, when an increase in sensitivity of the assay was required a dilution of 1:300,000 (1:1,500,000 final) was used. With this antibody dilution 30–40% of [125 I] HTg was bound in the absence of unlabeled antigen.

First incubation. Tubes were vortexed and incubated for 4 days at 4°C, an interval which was found to be optimal for the present assay.

Carrier (100 μ l). Normal rabbit serum (Colorado Serum Co., Denver, Colo.) diluted 1:200 was then added as a carrier to obtain suitable precipitates from which supernates could be decanted with ease.

Second antibody (200 μ l). An excess of goat antirabbit gamma globulin (Pentex, Inc., Division of Miles Laboratories) was used. The optimal dilution was found to be 1:8.

Second incubation. The tubes were vortexed and incubated overnight (18 h) at 4°C.

Centrifugation and counting. After the second incubation all tubes were centrifuged for 20 min at 1,000 \times g at 4°C; supernatants and precipitates were separated and counted in an automatic spectrometer (Nuclear-Chicago Corp., Des

Plaines, Ill). The concentration of thyroglobulin in samples was calculated by reading the percent of radioactivity bound from a standard curve that was constructed with reference serum GA. To evaluate nonspecific binding in the precipitate, a control tube containing no first antibody was set up for each sample, and the appropriate correction was made.

Affinity chromatography

Affinity chromatography was carried out by a modification of the method of Cuatrecasas (8). We prepared purified IgG from a human serum containing a high titer of anti-thyroglobulin antibodies by chromatography on *O*-(diethylaminoethyl)-Sephadex (DEAE-Sephadex A50) (Pharmacia). Purified IgG thus obtained was then coupled to Sepharose-4B. After extensive washing, this was packed in a siliconized Pasteur pipet plugged with glass wool. A gel bed of 5 cm length was used in all studies. These small columns were again extensively washed with diluted phosphate buffer (PBS 1:10). After 100 ml of the diluted buffer had passed through the columns an 8.0 ml fraction (control fraction 0) was obtained before application of 1 ml of the test serum. After the application of the test serum, two additional 8.0 ml fractions were collected (fractions 1 and 2). These fractions were lyophilized, and each fraction was subsequently diluted in 1.0 ml of distilled water. The first fraction after the application of the serum contained the serum proteins. All fractions were assayed for their concentration of HTg. The same serum was applied to a control column containing Sepharose-4B that had been exposed in a coupling reaction to an identical concentration of IgG obtained from a serum of a normal individual free of antithyroglobulin antibodies. A solution containing a total of 15 mg. IgG was exposed in both of these coupling reactions to 20 ml of activated Sepharose-4B. IgG was measured by radioimmuno-diffusion technique (Hyland Div., Travenol Laboratories Inc., Costa Mesa, Calif.).

Serum sample

All serum samples to be assayed were tested for the presence of HTg autoantibodies by the tanned red cell agglutination technique using a commercial kit (Burroughs Wellcome, Greenville, N. C.). Samples with positive titers of 1:5 or greater were rejected as being unsuitable for measurement of HTg. Sera could be stored at least 3 yr at –20°C without any measurable loss of HTg immunoreactivity.

RESULTS

Standard curve

Fig. 3 shows a typical standard curve obtained with purified HTg and with reference serum GA at two different dilutions of the antiserum Huc. The smallest amount of thyroglobulin detectable using a final dilution of 1:500,000 was usually 6.2 ng/ml. Further dilution of the antibody (1:1,500,000 final) allowed concentrations as low as 0.8 ng/ml to be measured.

Specificity studies

Cross-reactivity of different thyroid analogues. This was investigated in the present assay system because human thyroglobulin has been used (9–11) as an immuno-

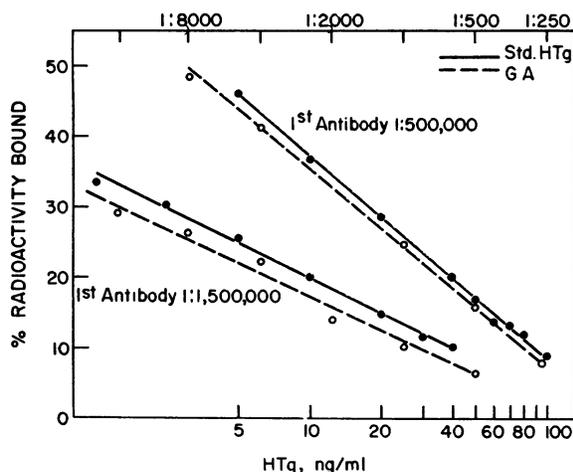


FIGURE 3 Dose response curve. Inhibition of binding of [125 I] HTg by unlabeled HTg present in standards or in various dilutions of reference serum GA (serum dilutions at top of figure). Two different dilutions of first antibody were used as described in the text.

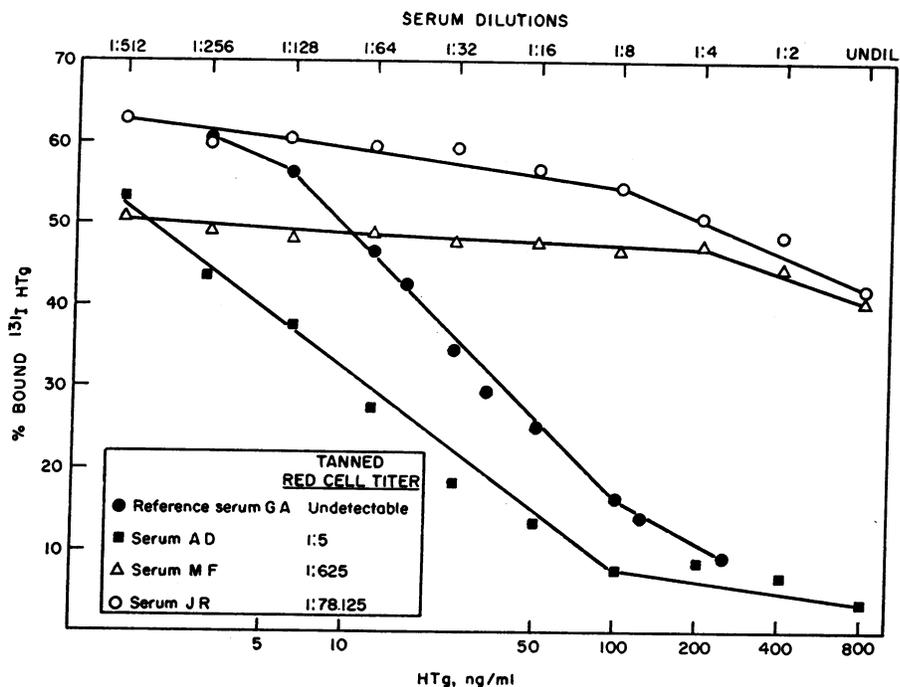


FIGURE 4 The effect of HTg antibody-positive sera on binding of $[^{125}\text{I}]$ HTg. A standard curve constructed with reference serum GA (absolute HTg concentration at the bottom of the figure), is compared with the curves obtained when antibody positive sera are diluted (serum dilutions at top of figure).

gen to develop antibodies against T_4 and T_3 for radioimmunoassay of these compounds. Amounts of T_4 , T_3 , MIT, and DIT up to 100,000 ng per tube did not interfere with the binding of $[^{125}\text{I}]$ HTg.

These amounts greatly exceed the T_3 and T_4 levels encountered in thyrotoxic individuals. However, when the same antibody was diluted 1:200 a distinct binding with $[^{125}\text{I}]T_4$ was present, and displacement with cold T_4 was observed.

Antithyroglobulin autoantibodies. Since antithyroglobulin autoantibodies were reported to interfere with the double antibody radioimmunoassay (4) the effect of antibody-positive sera on tracer-binding was further studied. Different dilutions of antibody-positive sera were therefore introduced in the assay (Fig. 4). Dilutions of antibody-positive sera all yielded curves which are nonparallel with reference serum GA. In addition purified human HTg-autoantibodies obtained by the method of Metzger and Edelhoch (12) were introduced in the assay instead of the antigen (HTg). Serial dilutions of this purified autoantibody bound $[^{125}\text{I}]$ HTg in a linear fashion. However, parallelism with the standard curve was not observed in other studies. Therefore serum samples with an autoantibody titer of 1:5 or greater were rejected for measurement of HTg concentrations. Under these conditions HTg levels were

detectable in 74% of the sera obtained from a normal population.

Further suggestive evidence of specificity came from data obtained in two patients lacking thyroid tissue. Thyroidectomy had been performed for thyroid carcinoma more than 10 yr ago followed by several therapeutic doses of ^{131}I . There was no detectable HTg in the sera of these patients.

Additional evidence. Additional evidence for specificity comes from the fact that the slope of the standard curve constructed with reference serum GA consistently parallels the slope of a standard HTg dose-response curve, irrespective of the concentration of first antibody used (Fig. 3). This by itself does not constitute proof of identity of the measured substance but can be used as an operational criterion for specificity (13).

Final and strongest evidence of specificity was obtained through affinity chromatography studies. Indeed it was reasonable to assume that a column that was constituted of Sepharose 4B-IgG antithyroglobulin should remove the majority if not all of the HTg present in the serum and that a control column packed with Sepharose 4B-IgG normal should retain little or none of the HTg present in the same test sample. When identical sera with varying concentrations of HTg were subjected to both column systems the results in Table I confirm that

TABLE I
Affinity Column Chromatography with Sepharose-4B
on Serum HTg Concentrations

| Sample | HTg concentration in original serum | HTg in fractions after passage through column IgG-Sepharose | | HTg in fractions after passage through antithyroglobulin Sepharose | |
|--------|-------------------------------------|---|-----------|--|---|
| | ng/ml | ng/ml | | ng/ml | |
| 987 | 330.0±0.0 | Fraction 0 | U | Fraction 0 | U |
| | | Fraction 1 | 330.0±0.0 | Fraction 1 | U |
| | | Fraction 2 | U | Fraction 2 | U |
| 1,201 | 193.5±6.5 | Fraction 0 | U | Fraction 0 | U |
| | | Fraction 1 | 220.0±8.0 | Fraction 1 | U |
| | | Fraction 2 | U | Fraction 2 | U |
| 1,643 | 6.6±0.09 | Fraction 0 | U | Fraction 0 | U |
| | | Fraction 1 | 5.5±1.9 | Fraction 1 | U |
| | | Fraction 2 | U | Fraction 2 | U |

The data presented are the mean (±SEM) of duplicate determinations, for details of experiment see Method section. U stands for undetectable this is <3.1 ng/ml in the assay.

human autoantibodies removed HTg and that the antithyroglobulin antibody negative IgG coupled to Sepharose-4B did not do so.

Precision and reproducibility

The precision of HTg measurement was evaluated by calculating the coefficient of variation (CV) of results of duplicate determinations around their means, according to the following formula (14):

$$CV = \sqrt{\frac{\sum d^2}{2n}}$$

where

$$d = \left[\frac{\text{Higher value of each pair}}{\text{Lower value of each pair}} - 1 \right] \times 100.$$

Variation, both intra- and inter-assay, was comparable to that reported for other radioimmunoassay systems (15). In an assay in which the final dilution of antibody (Huc) was 1:500,000, the CV was 5.6%, calculated on the basis of values ranging from 272.5 to 90.0 ng/ml obtained on 11 samples. In an assay in which the final dilution of antibody was 1:1,500,000, the CV was 8.3%, calculated on the basis of values ranging from 3.5 to 21.5 ng/ml obtained on nine samples. When

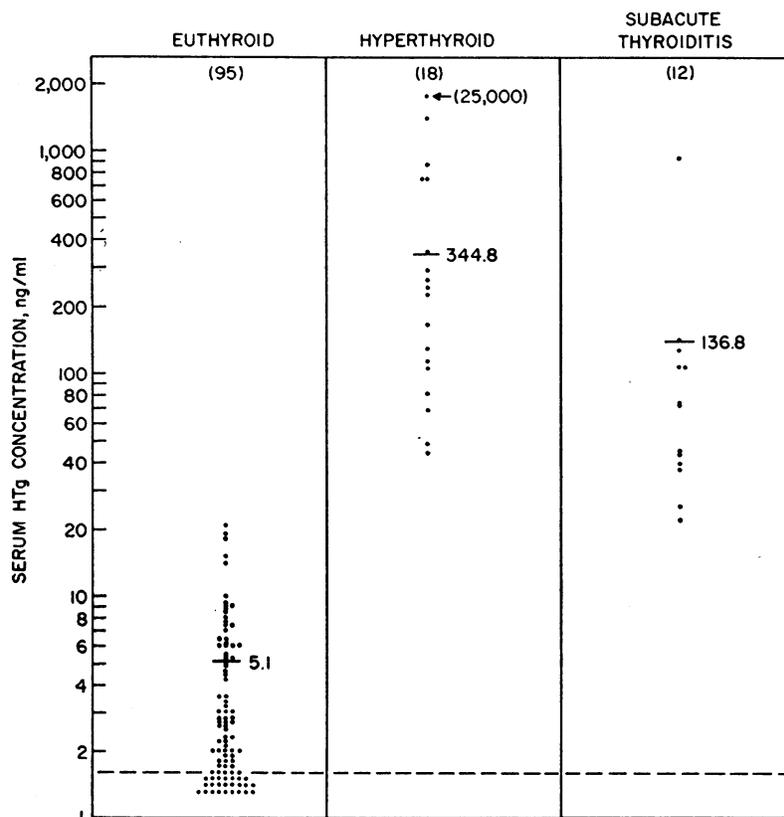


FIGURE 5 HTg concentrations in human sera. The broken horizontal line represents the normal threshold of detectability (1.6 ng/ml). The solid horizontal lines represent the mean serum HTg concentration for that particular group of patients. The value of 25,000 ng/ml was not used in the calculation of the mean for the hyperthyroid group. For conditions of sampling in patients with subacute thyroiditis (see Results section).

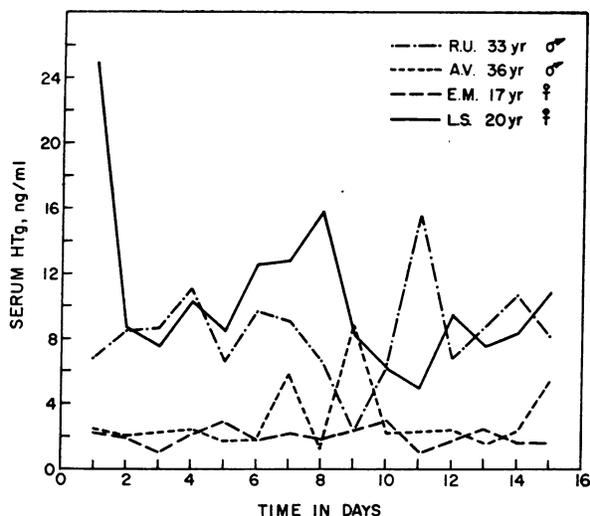


FIGURE 6 Concentration of HTg in four euthyroid individuals. Serum samples were obtained on 14 consecutive days between 8 and 10 A.M.

calculated on the basis of repeated determinations on 20 samples in two separate assays, and at both concentrations of antibody, the CV was 14.5%.

Thyroglobulin levels

Normal test sera. Determinations of HTg concentration in 95 antibody-negative samples selected from male and female blood bank donors revealed detectable HTg levels in 74% of the subjects so tested (Fig. 5). The mean of the detectable samples was 5.1 ng/ml \pm 0.49 SEM with a range from 1.6 to 20.7 ng/ml. There was a significant difference between males and females in this group ($P < 0.005$). Of 37 males 64% had detectable levels; the mean of the detectable levels was 3.4 ng/ml \pm 0.42 SEM with a range of 1.6–8.0 ng/ml ($n = 24$). Of 58 females 81% were detectable with a mean of 6.0 ng/ml \pm 0.68 SEM ($n = 47$) and a range from 1.6 to 20.7 ng/ml. No correlation with age was found.

Determinations on samples collected daily for 15 days in two adult euthyroid males and two adult euthyroid females revealed fluctuations in three of the four subjects which were, except for one sample, within the range demonstrated in the group of 95 normals (Fig. 6).

HTg levels in sera of pregnant women at delivery and in cord sera. HTg concentrations determined on 23 samples obtained from pregnant women at delivery revealed detectable HTg levels in 90% with a mean level of 10.1 ng/ml \pm 1.3 SEM and a range from 3.1 to 22.0 ng/ml (Fig. 7). The mean for this group was significantly higher than the mean of the detectable samples in the nonpregnant control group ($P < 0.02$). Assay of the cord bloods of the newborn infants revealed detectable

levels in 95%, and the mean concentration of HTg was 29.3 ng/ml \pm 4.7 SEM and ranged from 4.1 to 88.0 ng/ml. The mean for the total group assigning a value of 3.0 ng/ml to the undetectable samples was significantly different from the mean of the mothers ($P < 0.001$). In only two instances was the HTg value for the baby lower than the respective mother.

Effect of thyroid hormone administration in euthyroid subjects. Four euthyroid adult obese male subjects were studied before and after the oral administration of T_4 (Table II). Suppression of the serum HTg levels was observed in each of these individuals.

Thyrotoxicosis. Determinations on samples from 17 thyrotoxic patients revealed a mean HTg concentration of 344.8 ng/ml \pm 91.0 SEM with a range of 44.0–1400.00 ng/ml (Fig. 5). Determinations on samples collected daily on one such thyrotoxic individual showed the elevated levels to be very constant.

Subacute thyroiditis. Serum samples were collected on 12 adult women with classic symptoms of subacute thyroiditis without hyperthyroidism. Total serum thyroxine concentrations were within normal limits, and antithyroglobulin antibodies were undetectable. The samples were

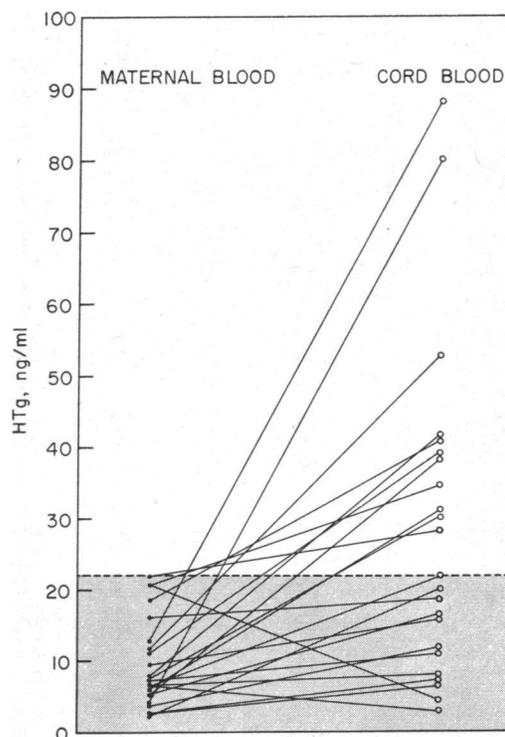


FIGURE 7 HTg concentrations in maternal and cord blood sera. HTg concentrations in 23 maternal and cord blood sera obtained at delivery are shown. Serum HTg levels in the mother are connected with the level in their respective infants. The shaded area represents the range for normal adult females.

TABLE II
Effect of T_3 (225 $\mu\text{g/day}$) on Serum HTg (ng/ml)
Concentration in Obese Subjects

| | Period | | |
|-------|---------|---------|----------------------|
| | Control | Placebo | T_3 administration |
| G. I. | 6.9 | 8.5 | 3.4 |
| L. P. | 5.7 | 5.5 | 3.1 |
| C. E. | 6.2 | — | 3.6 |
| B. A. | 14.2 | 11.7 | 4.2 |

The values given are the mean of duplicate determinations, 3.1 ng/ml was the lowest detectable quantity. Each period consisted of 4 wk, values obtained at the end of this time interval are reported except for subject C.E. where the value of 3.6 ng/ml was obtained after 2 wk of T_3 administration.

collected during the acute phase of the disease, and the mean HTg concentration was 136.8 ng/ml \pm 74.6 SEM (range 22.3–950.0 ng/ml). One subject not included in Fig. 5 was studied more than 2 mo after the onset of her symptoms and had a HTg level of 5.5 ng/ml.

DISCUSSION

The simplicity of this method for measuring thyroglobulin in human serum results from the following: (a) special attention was paid to the purification of the antigen, HTg. Thus, intensive purification of antiserum was not required, and at the same time a purified protein for labeling purposes was obtained. (b) Complement was inactivated by the addition of 0.05 M EDTA Na to the incubation mixture instead of heat inactivation of the sera at 56°C. (c) Precipitates were not washed after centrifugation. Specificity of the HTg binding antiserum was demonstrated by a variety of observations and methods. Thyroid analogues, T_4 , T_3 , MIT, and DIT did not cross react with the antibody to HTg. The absence of HTg in the circulation of patients who underwent total thyroidectomy, the parallelism of the standard curve with dilutions of a reference serum, and the dissociation between the high incidence of detectable HTg levels in our normal population and the absence of thyroglobulin autoantibodies in the same population all indicate the specificity of this assay. Further analysis using affinity chromatography showed that human IgG containing HTg autoantibodies removed HTg from the sera tested, while normal IgG did not do so. The sensitivity of the present method was greater than that of the previously reported assay; 1.6 ng/ml compared with 10 ng/ml (4). It seems likely that greater sensitivity accounts for the observation that HTg was detectable in 74% of the normal population instead of 60% in a previous study (4). The reason for the lower mean levels found in the normal population in the present study com-

pared to the data reported by Roitt and Torrigiani (4) in London is a matter of speculation. It may be the result of different binding characteristics of the first antibody and/or purity of the antigen used. Since it has been demonstrated in a previous study that HTg release is mediated by exogenous TSH and endogenous TSH (16) it is possible that differences in endogenous TSH secretion may account for observed differences in the two studies. Systematic studies of TSH levels and HTg levels on samples obtained in the United States and Great Britain and assayed in one single laboratory could help to clarify the differences observed. Such studies have not been conducted to our knowledge. Remarkable steadiness in day to day HTg concentration is observed in one euthyroid individual, in other normal subjects the HTg levels can vary from one day to another without exceeding the normal range. Although no correlation with age is observed, certain sex differences were noted. Not only was the frequency of detectability of thyroglobulin higher in the normal female population than in the male, but the mean HTg levels were also significantly higher in the normal female population than in the male. The mechanism and significance of this finding is not understood at the present time. In the pregnant females at term, HTg levels are detectable in 90%; identical to the 92% reported previously (17). The mean level was statistically higher than a group of nonpregnant females. However, the newborn infants as a group demonstrated an even higher mean HTg level than the corresponding maternal group. Except for two instances, the HTg level in the newborn was invariably higher than its mother's a finding consistent with the data obtained by Hjort and Pedersen (2). This infers that transport of HTg from mother to fetus is unlikely. Lack of transport of ^{125}I rat Tg in either direction across the placenta was also demonstrated by Torrigiani, Doniach, and Roitt (17). Previous studies also have indicated that TSH levels in cord blood are significantly elevated when compared to maternal TSH levels at delivery (18). The higher HTg levels in newborn infants compared to maternal levels may be explained on this basis. The effect of human chorionic thyrotropin (HCT) on the release of HTg in the newborn is speculative, since HCT has not been measured in cord blood and has been reported by only one group of investigators in maternal blood (19). The higher HTg levels in the pregnant subjects might be related to HCT in the maternal circulation; however, this hypothesis remains to be proven. The fact that serum HTg can be raised by exogenous TSH and suppressed by thyroid hormone administration suggests strongly that the release of this protein is TSH-mediated. The elevated HTg levels observed in patients with thyrotoxic Graves' disease are consonant with a stimulatory action of such factors as LATS (20) or a

recently described human thyroid-stimulating substance (21) on thyroglobulin release. Elevated HTg levels, however, cannot be considered a reflection of thyroid hyperfunction, since they are also observed in the active state of subacute thyroiditis where euthyroidism was definitely present. The demonstration of elevated HTg levels in subacute thyroiditis contrasts with the data of Torrigiani et al. (17). The discrepancy between the studies remains unexplained since both patient populations were studied during the acute phase of their disease. At the present time the fate and significance of thyroglobulin in the circulation remains uncertain since degradation and fate of this protein is essentially unknown in man. It is unlikely that the intrinsic T₃ and T₄ content of thyroglobulin makes an important contribution to the total thyroid hormone economy in normal man since the HTg content is so low. Even in thyrotoxic states where extremely elevated values are encountered the possibility of such a contribution to the thyroid hormone economy would be greatly dependent on the degree of iodination of this circulating protein, and this is essentially unknown.

ACKNOWLEDGMENTS

We acknowledge Dr. G. Bray for providing us with serum of T₃-treated subjects, Dr. Rodney Bluestone for a donation of purified IgM, and Dr. G. Holzman for collecting maternal and cord blood samples. We wish to thank Kathy Kellett, Edith Markoff, and Alice Lee for their expert technical assistance and Charlotte Smith for her excellent secretarial assistance. We are grateful to Dr. David H. Solomon for advice and review of this manuscript.

This work was partially supported by U. S. Public Health Service grant CA-13447 and Training Grant AM 5035.

REFERENCES

- Hjort, T. 1961. Determination of serum thyroglobulin by a haemagglutination inhibition test. *Lancet*. 1: 1262.
- Hjort, T., and G. T. Pedersen. 1962. Thyroid antibodies and thyroglobulin in the serum, in pregnant and parturient woman and in newborn infants. *Lancet*. 2: 259.
- Assem, E. S. K. 1964. Thyroglobulin in the serum of parturient women and newborn infants. *Lancet*. 1: 139.
- Roitt, I. M., and G. Torrigiani. 1967. Identification and estimation of undegraded thyroglobulin in human serum. *Endocrinology*. 81: 421.
- Scheidegger, J. J. 1955. Une micro-méthode de l'immuno-électrophorèse. *Int. Arch. Allergy Appl. Immunol.* 7: 103.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265.
- Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ¹²⁵I labelled human growth hormone of high specific radioactivity. *Biochem. J.* 89: 114.
- Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* 245: 3059.
- Chopra, I. J., D. H. Solomon, and R. S. Ho. 1971. A radioimmunoassay of thyroxine. *J. Clin. Endocrinol. Metab.* 33: 865.
- Chopra, I. J., D. H. Solomon, and G. N. Beall. 1971. Radioimmunoassay for measurement of triiodothyronine in human serum. *J. Clin. Invest.* 50: 2033.
- Chopra, I. J., J. C. Nelson, D. H. Solomon, and G. N. Beall. 1971. Production of antibodies specifically binding Triiodothyronine and Thyroxine. *J. Clin. Endocrinol. Metab.* 32: 299.
- Metzger, H., and H. Edelhofer. 1962. Purification of antithyroglobulin antibodies. *Nature (Lond.)*. 193: 275.
- Vivian, S. R., and F. S. LaBella. 1971. Classic bioassay statistical procedures applied to radioimmunoassay of bovine thyrotropin, growth hormone and prolactin. *J. Clin. Endocrinol. Metab.* 33: 225.
- Snedecor, G. W. 1952. Queries. *Biometrics*. 8: 85.
- Rodbard, D., P. L. Rayford, J. A. Cooper, and G. T. Ross. 1968. Statistical quality control of radioimmunoassays. *J. Clin. Endocrinol. Metab.* 28: 1412.
- Uller, R. P., A. J. Van Herle, and I. J. Chopra. 1973. Comparisons of alterations in circulating thyroglobulin (Tg), triiodothyronine (T₃), and thyroxine (T₄) in response to exogenous (bovine) and endogenous (human) thyrotropin (TSH). *Clin. Res.* 21: 206. (Abstr.)
- Torrigiani, G., D. Doniach, and I. M. Roitt. 1969. Serum thyroglobulin levels in healthy subjects and in patients with thyroid disease. *J. Clin. Endocrinol. Metab.* 29: 305.
- Fisher, D. A., W. D. Odell, C. J. Hobel, and R. Garza. 1969. Thyroid function in the term fetus. *Pediatrics*. 44: 526.
- Hennen, G., J. G. Pierce, and P. Freychet. 1969. Human chorionic thyrotropin: Further characterization and study of its secretion during pregnancy. *J. Clin. Endocrinol. Metab.* 29: 581.
- Adams, D. D., and H. D. Purves. 1956. Abnormal responses in the assay of thyrotropin. *Proc. Univ. Otago Med. Sch.* 34: 11.
- Onaya, T., M. Kotani, and T. Yamada. 1972. Evidence for the existence of a human thyroid stimulating substance in the serum of hyperthyroid patients with Graves' disease. IV. International Congress of Endocrinology, Washington, D. C. 193. (Abstr.)