A Multiple Ligand-Binding Radioimmunoassay of Diiodotyrosine

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ABSTRACT A radioimmunoassay has been developed for the measurement of 3,5-diiodo-L-tyrosine (DIT) in serum. DIT was coupled to porcine thyroglobulin (PTg) with a molar ratio of 205:1. Rabbits were immunized with 1 mg of immunogen emulsified in complete Freund's adjuvant. Sera were screened for their ability to bind trace amounts of [^{125}I]DIT. A serum that bound 40% of the tracer at a final dilution of 1:1,750 was used in the assay.

Assay specificity was improved by the use of thyroxine (T_4)-binding globulin as a second ligand-binding protein to decrease T_4 and triiodothyronine (T_3) cross-reactivity with the antibody. Double antibody and polyethylene glycol radioimmunoassays were compared. DIT present in the second antiserum shifted the double antibody assay standard curve and altered estimates of assay specificity and assay sensitivity.

By using the polyethylene glycol system and butanol: ethanol extracts of serum, DIT was measured in human serum. In 35 apparently healthy young adult controls DIT levels averaged 156 ng/100 ml. Random DIT levels averaged 158 ng/100 ml in 11 untreated hyperthyroid patients and 84 ng/100 ml in 15 untreated primary hypothyroid patients. No diurnal pattern in DIT levels could be demonstrated. Thyroid-stimulating hormone administration led to a variable but small rise in DIT levels, but short term T_3 suppression was not associated with a measurable fall in DIT concentrations.

Paired serum samples from the carotid artery and thyroid vein of 10 euthyroid goiter patients and one patient with a toxic solitary adenoma all showed a positive transthyroidal gradient indicating the thyroidal release of DIT in each patient. Measurable DIT levels of 45, 47, 68, and 80 ng/100 ml, respectively, were found in four fasting athyrotic patients indicating that the thyroid is not the only source of serum DIT.

INTRODUCTION

Thyroid hormone synthesis involves the iodination of tyrosine residues within the thyroglobulin molecule to form monoiodotyrosine (MIT) [1] and diiodotyrosine (DIT) followed by the coupling of two iodotyrosines to form either triiodothyronine (T_3) or thyroxine (T_4). Thyroid hormone secretion involves the proteolysis of thyroglobulin to release free iodoaminoacids within the thyroid. The free iodothyronines, T_3 and T_4, are secreted into the circulation, but the free iodotyrosines, MIT and DIT, are deiodinated within the thyroid epithelial cell by a specific iodotyrosine dehalogenase thereby making their iodine available for reentry into the pathway of thyroid hormone synthesis (1). This thyroidal deiodination of iodotyrosines is thought to be so complete that no iodotyrosines are released from the thyroid and, therefore, none are present in the circulation (1). The total organically bound iodine in plasma is, however, greater than the concentration of hormonal iodine (2), and the total thyroidal iodine release is greater than the iodine content of thyroid hormones secreted (3). This raises the possibility that the iodotyrosines could be released from the thyroid.

Numerous efforts have been made to measure iodotyrosines in serum or plasma, and the literature dealing

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[1] *Abbreviations used in this paper:* DIT, diiodotyrosine (3,5-diiodo-L-tyrosine); ethyl CDI, 1-ethyl-3(3-dimethylamino-propyl)-carbodiimine HCl; MIT, monoiodotyrosine; NRS, normal rabbit serum; PBS, 0.01 M phosphate-buffered 0.15 M saline; PTg, porcine thyroglobulin; T_3, triiodothyronine; T_4, thyroxine; TBG, thyroxine-binding globulin; TSH, thyroid-stimulating hormone.

with this subject has been reviewed by Rhodes (4). Some investigators have failed to find measurable levels of iodotyrosines in the circulation while others, reporting the presence of measurable levels, disagree widely in their estimates of iodotyrosine concentration (4–7). As a result, there is confusion regarding the presence or absence of iodotyrosines in the circulation and their role in extrathyroidal iodine metabolism. Existing methods for the measurement of iodotyrosines in serum or plasma are tedious, relatively insensitive, and subject to analytical errors (4, 5), and these methodological problems may account for the present disagreement. The recent development of radioimmunoassays for the measurement of T_3 (8–12) and T_4 (13, 14) has shown that these methods, with their great sensitivity and specificity, can be applied to the measurement of iodoaminoacids. The development of radioimmunoassays of the iodotyrosines might lead to new information regarding the question of their presence in the circulation and the nature of the nonhormonal iodine released from the thyroid. This is a report of the development of a radioimmunoassay of DIT.

METHODS

Materials. Porcine thyroglobulin (PTg), 3,5-diiodo-L-tyrosine (DIT), and 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl (ethyl CDI) were obtained from Sigma Chemical Company, St. Louis, Mo. Pyridine and polyethylene glycol were obtained from J. T. Baker Chemical Company, Phillipsburg, N. J., and dioxane was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. ^{125}I-labelled DIT with specific activities of 160–380 μCi/μg was obtained from Abbott Laboratories, Chemical Marketing Div., North Chicago, Ill. 4-hydroxy-3,5-diiodophenyl-pyruvic acid was a gift of Mr. Fred Jeffreys, Abbott Laboratories. Human thyroglobulin was a gift of Robert P. Uller, M. D., UCLA School of Medicine. All water was deionized but not distilled.

Immunogen. Ptg, 300 mg, was dissolved in 20 ml of pyridine: H_2O, 1:10 (vol:vol) at pH 5.5. 468 mg of DIT were dissolved in 35 ml of pyridine: dioxane: H_2O, 2:15:18 at pH 5.5 to which approximately 0.5 μCi [^{125}I]DIT was added. After the PTg and DIT were combined, a solution of 470 mg of ethyl CDI in 5 ml of pyridine: H_2O, 1:10 pH 5.5 was added dropwise with constant stirring at room temperature. The reaction mixture was incubated at 4°C for 24 h. During this time the mixture turned yellow, and a precipitate formed. The pH was then raised to 12.0 with concentrated NaOH, and the precipitate was redissolved. The precipitate remained in solution while the pH was lowered to 10.6 with concentrated and 1 N HCl. An additional 470 mg of ethyl CDI was dissolved in 2 ml of pyridine: H_2O, 1:10 pH 10.0 and added to the reaction mixture. A second 24-h incubation at 4°C was followed by dialysis at 4°C against 4 liters of 0.005 M phosphate-buffered 0.075 M saline pH 7.5. The dialysate was changed at 6–24 h intervals until the radioactivity in the dialysate reached background levels. The ^{125}I retained within the dialysis bag was used to calculate the conjugation of DIT to PTg. The conjugate had an estimated molar ratio of PTg to DIT of 1:205.

Immunization. Young, adult, female New Zealand albino rabbits received 1 mg of immunogen in 1 ml of 0.01 M phosphate-buffered 0.15 M saline (PBS) emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and injected in multiple subcutaneous sites. The initial immunization consisted of six injections at 10-day intervals. Booster injections were given monthly thereafter. The animals were bled by cardiac puncture 10–14 days after the initial immunization and after each booster injection.

Antisera screening. Sera were screened for the presence of DIT-binding antibodies in a reaction mixture containing 100 μl of diluted serum, 50 μl of [^{125}I]DIT containing 10,000–20,000 cpm of ^{125}I, 100 μl 0.1 M EDTA in PBS, and 750 μl of 2% normal rabbit serum (NRS) in PBS. After a 24-h incubation at 4°C a previously determined amount (100 μl) of goat anti-rabbit gamma globulin (Antibodies Inc., Davis, Calif.) was added, and the mixture was incubated for a second 24 h at 4°C. Following centrifugation for 30 min at 2000 *g* at 4°C the bound radioactivity was determined by measuring the radioactivity in the precipitate after aspiration of the supernate. At a final dilution of 1:1,000 four of the five animals receiving PTg-DIT bound between 35–60% of the labelled DIT. The serum from one rabbit was pooled and used for further studies.

Polyethylene glycol radioimmunoassay. 100 μl of first antibody diluted 1:100 (initial dilution) in 1% NRS in PBS, pH 7.0; 50 μl of [^{125}I]DIT (50–100 pg); 100 μl of 0.1 M EDTA in 2% NRS in PBS; 0–400 μl of standard or sample; and 350–750 μl of 2% NRS in PBS, pH 7.0, were vortexed in 10 × 75-mm glass culture tubes (Scientific Products, Santa Ana, Calif.). The first antibody incubation was carried out at 4°C for 4 h. The effect of varying concentrations of polyethylene glycol (15) on the precipitation of bound [^{125}I]DIT is compared with the nonspecific counts precipitated in the absence of DIT antiserum in Fig. 1. In subsequent assays 1 ml of 26% (or 0.5 ml of 39%) polyethylene glycol was added to 1 ml of the incubation mixture to give a final polyethylene glycol concentration of 13%. No incubation period was required after the addition of polyethylene glycol except for the time required for thorough vortexing and centrifugation. When varying amounts of unlabelled DIT were added a

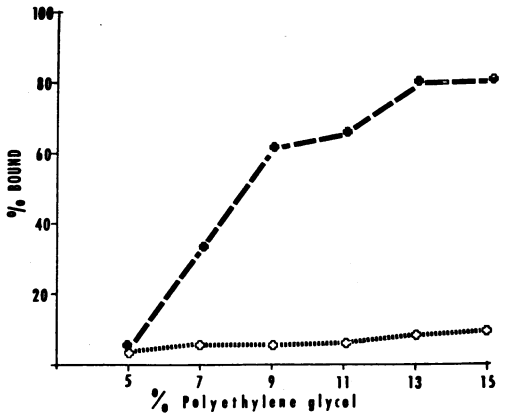

FIGURE 1 The effect of varying final concentrations of polyethylene glycol on the nonspecific counts and the bound radioactivity precipitated. Closed symbols, total radioactivity precipitated; open symbols, nonspecific counts precipitated.

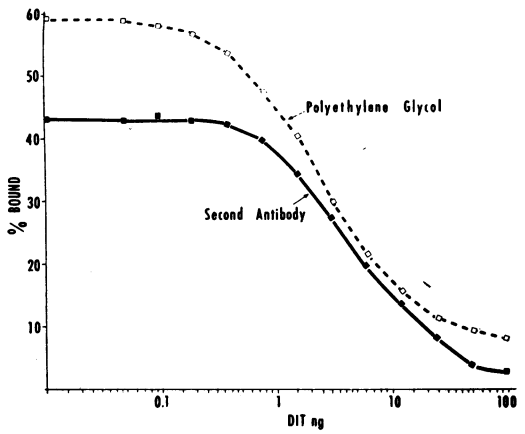

FIGURE 2 Comparison of the standard curves in the double antibody and polyethylene glycol assays (first antibody dilution 1 : 1,000 in both assays).

standard curve was obtained. The effect of the pH of the incubation mixture was studied over the range of pH 3.5–8.5. The optimum pH was 6.8.

DIT-free serum. Pooled normal human serum was treated in batches with Rexyn 202 (Fisher Scientific Company, Fair Lawn, N. J.) using 1 lb of resin to 1 liter of serum. The treatment was repeated until $< 1\%$ of DIT remained as judged by the removal of a tracer of [^{125}I]-DIT. This treatment markedly reduced the HCO_3^-, Cl^-, and pH with smaller reductions in Na^+, K^+, and Ca^{++}. The total protein concentration and serum protein electrophoretic pattern were unchanged, and the resin-T_3 uptake was only slightly reduced. The total T_4 was decreased by approximately 50%. Before use, the electrolyte changes were corrected by reconstitution to bring the electrolytes into the normal range. Smaller amounts of goat anti-rabbit gamma globulin serum were similarly treated to give iodotyrosine-free second antiserum.

Comparison of polyethylene glycol and double antibody assays. When anti-rabbit gamma globulin was used to precipitate immunoglobulins in place of polyethylene glycol, the standard curve obtained was different as shown in Fig. 2. This difference was due to the presence of DIT in the second antiserum and was absent when DIT-free second antiserum was used. Because the percent radioactivity bound was greater in the polyethylene glycol system the first antiserum could be further diluted giving 40% bound in the "zero" tubes at final dilutions of 1 : 1,750. With this further dilution of first antibody, assay sensitivity improved (Fig. 3).

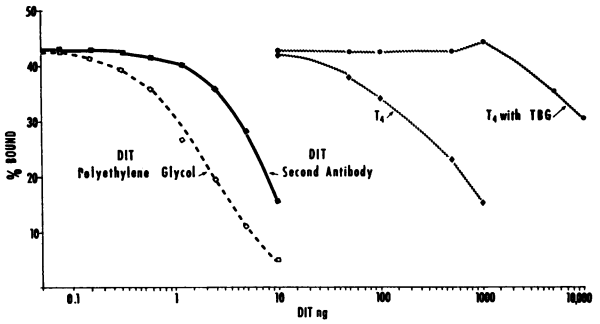

FIGURE 3 A comparison of T_4 cross-reactivity curves in the presence of TBG and the absence of TBG (in the polyethylene glycol assay) with the standard curves in the polyethylene glycol assay (first antibody dilution 1 : 1,750) and the second antibody assay (first antibody dilution 1 : 1,500).

Specificity. The specificity of the DIT antiserum was examined in cross-reactivity studies. The results are expressed as the relative potency, on a weight basis, of each substance compared to DIT in displacing 50% of bound radioactive DIT. Initial studies performed in the double antibody system gave falsely high cross-reactivity estimates since the DIT content of the second antiserum was not considered in the calculations (Fig. 3). True cross-reactivity estimates from the polyethylene glycol assay are shown in Table I. All other substances tested had $< 0.01\%$ cross-reactivity. These included porcine thyroglobulin; tetraiodothyroformic acid; 3,5,3′-triiodothyroformic acid; 3,5,3′-triiodothyroacetic acid; 3,5,3′-triiodothyropropionic acid; D-T_3; 3,5-diiodothyropropionic acid; 3,5-diiodothyroacetic acid; 3-iodothyronine; thyronine; L-tyrosine; L-tyramine; L-phyenylalanine; L-dopamine; L-epinephrine; L-norepinephrine; bilirubin; EDTA; and sodium iodide.

The multiple ligand-binding system. Thyroxine-binding globulin (TBG) avidly binds iodothyronines but not iodotyrosines (2, 7). TBG added to the incubation mixture should bind any T_4 or T_3 present and thereby diminish their availability for cross-reaction with the DIT antibody. When DIT-free, thyroxine-poor, normal human serum was added to the incubation mixture (400 μl/ml) the cross-reactivity of T_4 was appreciably diminished (Fig. 3). Amounts of T_4 up to 600 ng/tube (150 μg/100 ml of serum using a 400-μl serum sample) have no effect on this assay, whereas, without TBG, amounts above 10 ng/tube or 2.5 μg/100 ml of serum would cross-react. The use of TBG as a second ligand-binding protein also decreased the cross-reactivity of T_3, but this effect was less than the effect on T_4 as would be expected from the knowledge that TBG binds T_4 more avidly. In this system T_3 and T_4 cross-reactivity were both reduced to 0.06% at 50% displacement of label.

Recovery studies. If either the concentration or avidity of serum protein binding sites for DIT were to vary significantly from sample to sample this might be expected to influence potency estimates of DIT in unknown serum samples. To test for this possibility three types of recovery

TABLE I

Cross-reactivity in the Polyethylene Glycol Assay without TBG

	Relative potency
	%
4-Hydroxy-3,5-diiodophenyl-pyruvic acid	6.90
T_4	0.55
MIT	0.34
3,5-diiodotyramine	0.22
T_3	0.11
Human thyroglobulin	0.06
Tetraiodothyroacetic acid	0.02
3,5-diiodothyronine	0.01

experiments were run. In each, the total amount of serum per tube was held constant at 400 μl by adding 400 μl DIT-free serum to the standard curve tubes and by adding DIT-free serum to any unknown samples that were smaller than 400 μl in volume.

When varying amounts of DIT were added to different aliquots of DIT-free serum and DIT content of these sera was measured in the polyethylene glycol radioimmunoassay system, the amount of DIT measured was a direct linear function of the amount of DIT added. When a single aliquot of DIT-free serum was enriched with DIT to 50 ng/ml and the DIT content of 25, 50, 100, 200, and 400 μl of this serum was measured, the recovery curve obtained was virtually identical. When, however, fresh serum samples from healthy hospital personnel were similarly studied the results varied. Some but not all sera gave improper recovery curves in which the amount of DIT measured was not a direct function of the volume of serum sample assayed. This indicated either DIT-binders or other, as yet unknown, factors nonspecifically interfered with the assay. Therefore, attention was turned to an iodoaminoacid extraction of the serum samples.

Serum extraction. Iodotyrosines and iodothyronines were extracted from 2 ml of serum with 3.0 ml of *n*-butanol: ethanol 1:1 in 17 × 100-mm plastic test tubes (Falcon Plastics, Division of Becton Dickinson Laboratories, Inc., Oxnard, Calif.). After thorough vortexing, the tubes were placed on a rotary shaker for 2 h, then vortexed again and centrifuged at 2000 *g* at 4°C for 5 min. Extraction recovery was determined by the addition of [^{125}I]DIT (3,000–4,000 cpm) to a separate aliquot of the sample. After incubation for 30–60 min at room temperature this aliquot was extracted in the same way, and the percent extraction was calculated as the percent radioactivity present in the supernate. The percent recoveries from 78 serum samples ranged from 79–100% with a mean of 86%. There was no significant difference ($P > 0.2$) between the mean recoveries from sera of normal subjects, and patients with hyperthyroidism, hypothyroidism, or Hashimoto's thyroiditis. This extraction recovers an average of 82% of serum T_4.

Radioimmunoassay of serum extracts. When butanol: ethanol extraction of serum samples preceded DIT measurements the assay recoveries of DIT from DIT-enriched, DIT-free serum ranged from 86–108% and averaged 98%. Furthermore, the nonspecific effects of serum, present in some of the unextracted serum samples, were not encountered. The DIT in these extracts was entirely ultrafilterable through a Millipore Pellicon filter with a molecular weight cutoff of 1,000 and exclusion limits of 750–1,250 mol wt.

The possibility of iodothyronine conversion to iodotyrosines during the extraction step was studied in two ways. When random normal serum samples that gave proper linear recovery curves in the unextracted assay were measured in that system the mean DIT concentration was not significantly different from that measured after extraction. When T_4 was added to a pool of normal serum with a T_4 concentration of 8 μg/100 ml the mean of triplicate DIT determinations was the same over the range of 0–15 μg/100 ml of T_4 added or a total T_4 concentration of 8–23 μg/100 ml.

The assay finally employed used 1 ml of a butanol: ethanol extract of serum and DIT standards dissolved in butanol: ethanol. Both standards and unknowns were run in triplicate, and all samples from one subject were run in the same assay. Both were dried down in 10 × 75-mm glass culture tubes in a 40°C water bath under compressed air.

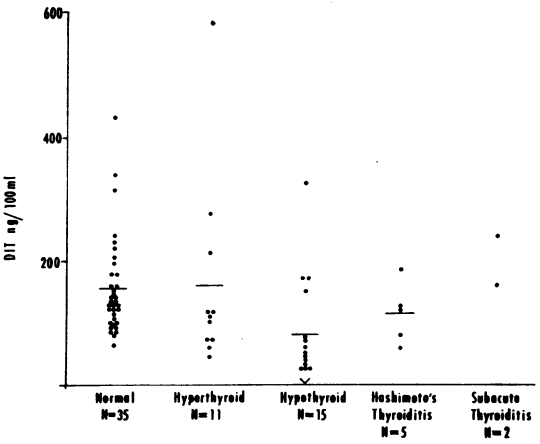

FIGURE 4 Concentrations of DIT in human serum.

Both were redissolved in 450–650 μl PBS and 200–400 μl of DIT-free serum (as a source of TBG) by vortexing, incubating for 30 min at 40°C in a water bath, and revortexing. 50–100 pg of [^{125}I]DIT in 50 μl of PBS and 100 μl of DIT antiserum diluted 1:150 in PBS were added bringing it to a total volume of 1 ml. The tubes were thoroughly vortexed, then incubated at 4°C for 2 h (the time needed for the DIT-antiDIT reaction to reach equilibrium). After the addition of polyethylene glycol, the tubes were centrifuged at 2000 *g* for 30 min at 4°C. The supernate was decanted, and the radioactivity in the precipitate was counted in a Nuclear Chicago 1185 automatic well type gamma counter (Nuclear Chicago Corp., Des Plaines, Ill.). The standard curves are linear on a logit-log plot, and the assay results could be analysed by computer using the program of Rodbard, Bridson, and Rayford (16). The counts were simultaneously printed and punched out on paper by a Teletype model 8470 (Teletype Corporation, Skokie, Ill.) connected directly to the gamma counter. By using the paper tape, the standard curve was drawn, quality control data were calculated, and unknown values were interpolated (16) with an EMR 6130 computer (EMR-Instruments, Sarasota, Fla.) and a Calcomp 565 plotter (California Computer Products, Inc., Anaheim, Calif.). All standards and unknowns were run in triplicate. During patient perturbations all samples were run in the same assay.

Assay precision, including the extraction step, was such that the intraassay coefficient of variation among 12 replicates was 8.7 at 1,000 pg/tube and 3.6 at 4,000 pg/tube, and the interassay coefficient of variation among 16 replicates was 15.7 at 1,000 pg/tube. Assay sensitivity was calculated from the mean±2 SD of the counts bound in 10 replicates of the "zero" tubes. Sensitivity has averaged 140 pg/tube or 35 ng/100 ml of serum and has been as low as 40 pg/tube or 10 ng/100 ml in the best runs.

RESULTS

The DIT concentrations found in human serum are shown in Fig. 4. 35 apparently healthy young adult controls had random DIT levels ranging from 63–432 ng/100 ml with a mean of 156 ng/100 ml. The random levels in 11 untreated hyperthyroid patients ranged

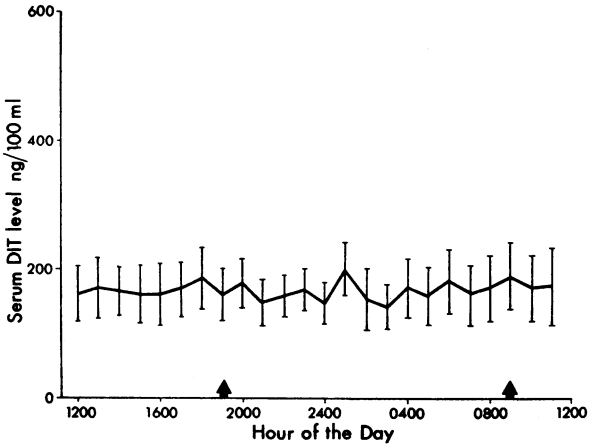

FIGURE 5 Hourly DIT levels in control subjects. The arrows indicate meals.

from 44–580 ng/100 ml. Some were higher, and others were lower than the control values, but the mean of 158 ng/100 ml was not significantly different from controls. Among 15 patients with untreated, primary hypothyroidism the random values ranged from undetectable (<24 ng/100 ml) to 324 ng/100 ml with a mean of 84 ng/100 ml which was significantly lower ($P<0.01$) than the controls. Five patients with biopsy-proven, untreated, euthyroid Hashimoto's thyroiditis had random DIT levels of 63–184 ng/100 ml with a mean of 114 ng/100 ml. This was not significantly different from either control levels or from hypothyroid levels ($P>0.05$). When DIT was measured hourly for 24 h in five healthy volunteers there was no detectable diurnal pattern (Fig. 5). This was equally true when each individual's DIT levels were expressed as a percent deviation from that individual's mean level. The wide standard error of the mean is due to the individual variation in DIT levels (78–316 ng/100 ml).

When paired serum samples were obtained from both carotid artery and thyroid vein blood at the time of surgery from eleven patients undergoing thyroidectomy, the DIT concentrations found are shown in Table II. All had higher DIT concentrations in the venous effluent indicating thyroidal release of DIT. DIT concentrations in four fasting thyroid carcinoma patients after total thyroidectomy were 45, 47, 68, and 80 ng/100 ml at a time when they were not taking thyroid hormone, providing evidence that the thyroid gland is not the only source of circulating DIT. When 10 mg of DIT in aqueous solution were administered orally to three fasting healthy volunteers serum DIT rose promptly from base line levels of 200–420 ng/100 ml (mean 320) to peak levels of 11,200–13,100 ng/100 ml (mean 12,200) 60 min later. The three curves were very similar and the mean values (±SE) are shown in Fig. 6. After 60 min the DIT levels fell exponentially with a half-time of 102 min. Since the DIT levels in the athyrotic patients were obtained during fasting and hence many DIT half-lives after the last meal, ingestion of iodoaminoacids seems unlikely to account for the DIT in their serum. The relative unimportance of food intake as a direct source of circulating DIT is also shown by the absence of any significant postprandial rise in DIT levels during the diurnal pattern study (Fig. 5).

The effect of stimulation of the thyroid with thyroid-stimulating hormone (TSH) was studied in five healthy volunteers. Three base-line levels were measured at hourly intervals before TSH administration. Each subject received 15 U bovine TSH i.m. Serum levels were measured at 2, 8, 24, 48, 72, 96, and 120 h thereafter. Four of the five subjects showed a similar mono-

TABLE II
Thyroid Arterial-Venous DIT Differences

	Arterial	Venous	Difference
	ng/100 ml	*ng/100 ml*	*ng/100 ml*
Hashimoto's thyroiditis	141	344	203
Multiadenomatous goiter	59	731	672
Multiadenomatous goiter	282	662	380
Multiadenomatous goiter	450	535	85
Multiadenomatous goiter	250	370	120
Multiadenomatous goiter	30	1,113	1,083
Solitary cold nodule	<37	980	947
Solitary cold nodule	380	820	440
Solitary cold adenoma	296	323	27
Solitary toxic adenoma	816	3,786	2,970
Follicular carcinoma	94	11,500	11,406

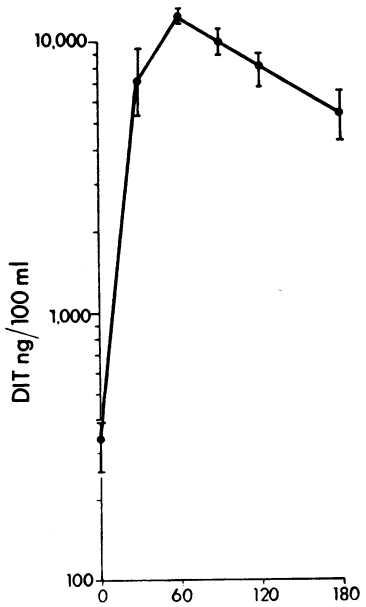

FIGURE 6 Serum DIT levels after DIT administration.

phasic response with a small rise in DIT levels after TSH which became apparent at 8 h. One subject showed a biphasic response with a fall in DIT at 8 h followed by a rise at 24 h. Fig. 7 shows the mean of the four similar responses (*middle*) and the one biphasic response (*top*) as well as mean T_4 responses (*bottom*), which were similar in all five subjects. The 2-h levels, which are not shown, were not different from the basal levels. Because the mean basal levels vary so widely (63–216 ng/100 ml) the before-TSH and after-TSH levels for the group of four subjects were not significantly ($P > 0.05$) different. However, when the mean of the three before-TSH DIT levels for each subject were compared with the mean of the 8-, 24-, and 48-h DIT levels after TSH for that same individual the differences were all significant ($P < 0.05$).

T_3 suppression with 100 μg P. O. daily was begun on the 5th post TSH day and continued for 12 days. Though serum DIT had returned to basal levels by day 5 it did not fall below basal levels during T_3 administration despite a fall in mean T_4 from 8.5 to 5.0 μg/100 ml.

DISCUSSION

Ever since Trevorrow in 1939 (17) found two organic iodine fractions in blood, one resembling T_4 and the other resembling DIT, the question of whether or not iodotyrosines are present in the circulation has remained unsettled (1, 4, 5, 7). Up to the present time, the methods used to measure iodotyrosines in serum or plasma have raised question regarding the acceptability of the reported results (4, 5, 7). The radioimmunoassay of DIT provides far greater sensitivity and specificity than existing methods for measuring DIT in biologic fluids and its readily applicable to large numbers of samples.

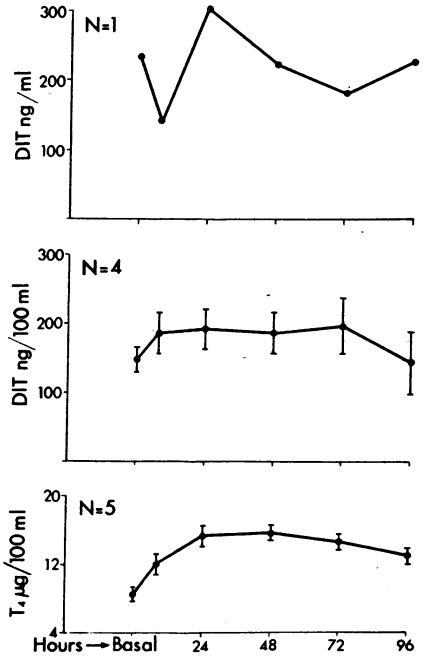

FIGURE 7 Serum DIT and T_4 levels after TSH injection.

Assay specificity has been made even greater than the inherent DIT antibody specificity by using a unique multiple ligand-binding system in which TBG was used as a second binding protein to combine with free T_4 and T_3 in the incubation mixture thereby reducing their cross-reaction with the DIT antibody. This increase in specificity made it possible to measure low concentrations of DIT in the presence of the high concentrations of T_4 found in serum. None of the antisera produced possess less than 0.5% (1–200) T_4 cross-reactivity. Since the terminal iodinated phenolic ring of both T_4 and DIT possess the same molecular configuration, it is unlikely that antibody specificity alone would allow the measurement of DIT in the presence of up to 1,000-fold greater T_4 concentrations. This multiple ligand-binding principle would seem to have general application for improving radioimmunoassay specificity for closely related molecules.

The presence of measurable DIT levels in all but one of the 78 sera tested is convincing evidence that this iodotyrosine is ordinarily present in the human circulation. The fact that the concentration was decreased in thyroid failure and athreosis, increased after TSH stimulation in normals, and higher in thyroid vein blood than in arterial blood provides conclusive evidence for thyroidal release of iodotyrosines as the source of some of the iodotyrosine in the circulation.

The mean DIT concentration of 156 ng/100 ml in the control subjects represents 90 ng/100 ml (0.09 μg/100 ml) of DIT iodine. This is in reasonable agreement with the report of Weinert, Masui, Radichevich, and Werner (5) who employed a complex and arduous system of extraction, filtration, reduction, and chromatography to measure DIT iodine in two normal human sera and found mean concentrations of 0.30 and 0.39 μg/ml, respectively. It is close to the mean of 0.16 μg/100 ml of DIT iodine in 10 normal subjects reported by Arosenius (6) using ion exchange resin extraction of serum iodoaminoacids followed by acid elution from the resin and separation of the iodoaminoacids by high-voltage paper electrophoresis.

Since only 60% of serum DIT is TCA-precipitable, DIT iodine would account for approximately 0.05 μg/100 ml of the protein-bound iodine, indicating that DIT iodine contributes little to the circulating nonhormonal protein-bound iodine. The possibility that some of the extractable DIT measured in serum might be bound in very small polypeptide molecules (mol wt < 750) instead of "free," is a subject for further study.

The presence of measurable levels of DIT in four athyrotic subjects provides evidence for extrathyroidal sources of circulating DIT. Ingestion of iodotyrosines (or iodoproteins subsequently digested to iodotyrosines) is a possible nonthyroidal source of circulating DIT as shown by the striking rise in serum DIT after DIT administration. The rapid removal of this DIT from the circulation and the absence of measurable postprandial DIT elevations in the 24-h study suggest that this is, at best, a minor source of DIT in serum.

DIT rose slightly after TSH stimulation but not to the degree that T_4 rose, and 12 days of T_3 suppression did not significantly lower DIT levels despite a fall in T_4. This raises the question of the role of TSH in controlling thyroidal DIT release. TSH stimulates thyroglobulin hydrolysis and the release of free iodoaminoacids within the thyroid (1). TSH also increases the activity of thyroidal iodotyrosine deiodinase (1). These two effects of TSH would exert opposite influences on thyroidal DIT release. If the former predominated DIT release would increase, but if the latter were predominant DIT release would diminish. Furthermore, the level of DIT in the peripheral blood is the result of both DIT release into and DIT removal from the circulation. There is evidence that the thyroid takes up DIT from the circulation (18), and it is possible that this thyroidal uptake of iodotyrosines may be altered by TSH thereby introducing still another variable in the TSH effect on circulating DIT levels. The variable pattern of DIT response to TSH administration seen in this study suggests that the interplay of these factors may be different from one subject to another. The small change in DIT concentration after TSH administration and the absence of a measurable change after TSH suppression with T_3 are in striking contrast to the changes in T_4. This implies that either opposing TSH responsive processes largely counterbalance each other or that TSH is not as important a factor in the control of circulating iodotyrosine concentrations as it is for the iodothyronines.

The finding of iodotyrosine in the serum of athyrotic and hypothyroid humans confirms the findings of Row, Webster, Ezrin, Johnston, and Volpe (19) and provides a possible explanation for the fact that attempts to measure iodotyrosines in serum by methods that detect chemical iodine have generally given higher estimates of serum concentrations than methods which rely on in vivo labelling of iodotyrosines after the administration of radioactive iodine (4).

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REFERENCES

1. Tong, W. 1971. Thyroid hormone synthesis and release. *In* The Thyroid. S. C. Werner and S. H. Ingbar, editors. Harper & Row, Publishers, New York. 3rd edition. 24.
2. Oppenheimer, J. H., and M. I. Surks. 1971. Nature, transport in plasma, and metabolism of thyroid hormones. *Ibid.* 52.
3. Fisher, D. A., T. H. Oddie, and C. S. Thompson. 1971. Thyroidal thyronine and non-thyronine iodine secretion in euthyroid subjects. *J. Clin. Endocrinol. Metab.* **33**: 647.
4. Rhodes, B. A. 1968. The circulating iodotyrosines. *Acta Endocrinol. Suppl.* **127**: 5.
5. Weinert, H., H. Masui, I. Radichevich, and S. C. Werner. 1967. Materials indistinguishable from iodotyrosines in normal human serum and human serum albumin. *J. Clin. Invest.* **46**: 1264.
6. Arosenius, K. E. 1968. Studies on the iodo-amino acids in blood serum. *Acta Soc. Med. Ups.* **68**: 215.
7. Bismuth, J., M. Castay, and S. Lissitzky. 1971. Sephadex filtration of serum iodocompounds and protein-binding of iodotyrosines. *Clin. Chim. Acta.* **35**: 285.
8. Gharib, H., W. E. Mayberry, and R. J. Ryan. 1970. Radioimmunoassay for triiodothyronine: a preliminary report. *J. Clin. Endocrinol. Metab.* **31**: 709.
9. Chopra, I. J., D. H. Solomon, and G. N. Beall. 1971. Radioimmunoassay for measurement of triiodothyronine in human serum. *J. Clin. Invest.* **50**: 2033.
10. Mitsuma, T., N. Nihei, M. C. Gershengorn, and C. S. Hollander. 1971. Serum triiodothyronine: measurements in human serum by radioimmunoassay with corroboration by gas-liquid chromatography. *J. Clin. Invest.* **50**: 2679.
11. Lieblich, J., and R. D. Utiger. 1972. Triiodothyronine radioimmunoassay. *J. Clin. Invest.* **51**: 157.
12. Larsen, P. R. 1972. Direct immunoassay of triiodothyronine in human serum. *J. Clin. Invest.* **51**: 1939.
13. Chopra, I. J., D. H. Solomon, and R. S. Ho. 1971. A radioimmunoassay of thyroxine. *J. Clin. Endocrinol. Metab.* **33**: 865.
14. 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.
15. Desbuquois, B., and G. D. Aurbach. 1971. Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays. *J. Clin. Endocrinol. Metab.* **33**: 732.
16. Rodbard, D., W. Bridson, and P. L. Rayford. 1969. Rapid calculation of radioimmunoassay results. *J. Lab. Clin. Med.* **74**: 770.
17. Trevorrow, V. 1939. Studies on the nature of iodine in blood. *J. Biol. Chem.* **127**: 737.
18. Haibach, H. 1971. Comparison of the intrathyroidal fate of iodotyrosine-derived and newly entering iodide in the rat. *Endocrinology.* **89**: 184.
19. Row, V., B. R. Webster, C. Ezrin, W. Johnston, and R. Volpé. 1964. Circulating iodotyrosines in severe myxedema and cretinism. *Canad. Med. Assoc. J.* **90**: 486. (Abstr.).