Direct Immunoassay of Triiodothyronine in Human Serum

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ABSTRACT A sensitive and precise radioimmunoassay for the direct measurement of triiodothyronine (T_3) in human serum has been designed using sodium salicylate to block T_3-TBG binding. This assay is sufficiently sensitive to quantitate T_3 accurately in 50–100 μl of normal serum and to measure quantities as small as 12.5 pg in 0.2 ml of hypothyroid serum. The T_3 values observed in euthyroid subjects and in patients with various thyroid diseases are as follows: euthyroid (38) 1.10±0.25 (SD) ng/ml, hypothyroid (25) 0.39 ±0.21 (SD) ng/ml, and hyperthyroid (24) 5.46±4.42 (SD) ng/ml. The levels of T_3 parallel the thyroxine (T_4) concentration in the sera of these subjects. In eight pregnant women at the time of delivery, T_3 concentrations were in the upper normal range (mean 1.33 ng/ml). The levels of T_3 in cord serum obtained at the time of delivery of these patients (mean 0.53 ng/ml) are distinctly lower and close to the hypothyroid mean.

Administration of 10 U of bovine thyroid-stimulating hormone (TSH) to euthyroid subjects causes a twofold increase in serum T_3 levels within 8 hr. At this time, the increase in serum T_4 concentration is only 41%. In two subjects in whom thyroid secretion was acutely inhibited, one after pituitary surgery and another after thyroidectomy, the serum T_3 fell into the hypothyroid range within 1–2 days. Thus, serum T_3 concentrations appear to be a sensitive index of acute changes in thyroid hormone secretion and should prove to be a useful adjunct to both the clinical and physiological evaluation of thyroid function.

INTRODUCTION

Triiodothyronine (T_3)[1] was initially identified in human serum in 1952 by Gross and Pitt-Rivers (1). It was soon recognized that this hormone was more potent than thyroxine (T_4) and had a more rapid onset of action. However, because of the tedious methods required for its quantitation, evaluation of its role in human thyroid physiology and pathophysiology was not possible. In recent years, less cumbersome methods for quantitation of T_3 in human serum have been developed (2, 3). The method described by Sterling and coworkers has allowed new insight into the importance of this hormone (3). It has been estimated that T_3, arising either via primary secretion or via T_4 deiodination, provides greater than 50% of the human thyroid hormone requirement (4). Although the availability of the Sterling method has added new impetus to the investigation of this hormone, careful evaluation of this technique in our laboratory and in others has suggested that it may overestimate the concentration of T_3 in human serum (5–7). The primary cause of this artifact appears to be the unavoidable deiodination of T_4 to T_3 during the paper chromatographic separation of the two hormones after extraction from human serum. This separation was a necessary step due to the fact that thyroxine-binding globulin (TBG), used as the competitive binding protein, has a higher affinity for T_4 than for T_3 (8). Furthermore, relatively large quantities of serum were required for accurate assay of T_3 in normals and in hypothyroid patients. This limited its usefulness in physiologic studies.

Because of these difficulties, there was a need to develop an assay which could measure T_3 accurately in the presence of T_4 in a small volume of serum. To this end, a number of investigators have successfully induced the formation of specific anti-T_3 antibodies by immunization with various T_3-protein conjugates or with thyroglobulin (9–13). While it was possible to develop antibodies with an extreme specificity for T_3 vs. T_4, the competition of the TBG present in human serum with the antibody for T_3 appeared to be a further technical obstacle to the precise quantitation of the hormone in unextracted serum. This problem has been approached by the addition of various compounds (thyroxine [11], tetrachlorthyronine [12], diphenylhydantoin [13]) to the assay system to block T_3-TBG binding.

Received for publication 1 November 1971 and in revised form 22 February 1972.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CDI, carbodiimide; DIT, diiodotyrosine; HSA, human serum albumin; MIT, monoiodytyrosine; T_3, triiodothyronine; T_4, thyroxine; TBG, thyroxine-binding globulin; TSH, thyroid-stimulating hormone.

TABLE I

Procedure for Immunoassay of T_3 in Human Serum

Solutions

Blank solution:	Glycine (0.2 M)–Na acetate (0.13 M), BSA—20 mg/100 ml, sodium salicylate—10 mg/ml.
Tracer solution:	Blank solution plus T_3-^{125}I approximately 0.2 ng/ml.
Antibody solution:	Rabbit anti-T_3 antiserum diluted 1/75,000 in blank solution.
Standards:	0, 0.025, 0.050, 0.10, 0.15, 0.20, 0.30, and 0.40 ng of T_3 in 0.2 ml of T_3-free human serum.

Scheme for Performing Assay

	Volume *ml*
Tubes containing antiserum	
Standard curve: appropriate standard in T_3-free serum	0.200
Standard curve: antibody solution	0.700
	0.900 (Total)
Unknowns: 0.005–0.200 ml. of unknown serum made up to a total volume of 0.2 ml with T_3-free serum	0.200
Unknowns: antibody solution	0.700
	0.900 (Total)
Blanks (tubes with no antiserum)	
Standard curve: T_3-free serum	0.200
Standard curve: blank solution	0.700
	0.900 (Total)
Unknowns: 0.005–0.200 ml of unknown serum made up to a total volume of 0.2 ml with T_3-free serum (corresponds to volume of unknown serum assayed in the tubes containing antiserum)	0.200
Unknowns: blank solution	0.700
	0.900 (Total)

All tubes incubated 2–5 days at 4° C.
0.100 ml tracer solution added to all tubes.
Incubate 2–3 days at 4° C.
Count every 10th tube.
Add 1 ml charcoal-dextran solution, incubate 45 min in an ice bath.
Centrifuge 15 min at 2000 rpm, decant supernate, and count 1 ml.

Calculations

$$\text{Observed per cent bound} = \frac{\text{counts } ^{125}I\text{/ml supernate} \times 2 \times 100}{\text{total initial counts } T_3\ ^{125}I\text{/tube}}$$

for both antibody and blank tubes

$$\text{True per cent bound} = \frac{\text{observed per cent bound (antibody)} - \text{blank per cent bound} \times 100}{100 - \text{blank per cent bound}}$$

The data was then transposed by letting the per cent bound at 0 T_3 concentration equal 100%.
The quantity of T_3 in the unknown is determined by reference to the standard curve and expressed as ng T_3/ml.

We have previously reported evidence that sodium salicylate inhibits binding of T_3 and T_4 to human TBG (14–16). In a recent communication, we have also shown that this property of salicylates can be used to block the binding of T_3 to TBG during the immunoassay of T_3 in unextracted human serum. The drug did not interfere significantly with the binding of T_3 to the antibody (15). This report demonstrated that the use of salicylate in the assay allowed quantitative recovery of T_3 from human serum by direct immunoassay.

The present communication extends these observations and presents results of the direct measurement of T_3 in serum from normal subjects, from patients with thyroid disease, during pregnancy, and in cord serum.

METHODS

Preparation of antigen. Two methods were used for the coupling of T_3 to bovine and human serum albumin. The first was that of Goodfriend, Levine, and Fasman in which Na-3,5,3′ triiodothyronine, 10 mg (plus T_3-^{125}I), was coupled to 20 mg of bovine serum albumin (BSA) using ethyl carbodiimide (17). After termination of the coupling reaction, T_3-^{131}I was added to quantitate removal of unconjugated T_3 from the albumin-T_3 mixture during extensive dialysis (1 wk against approximately 50 liters of 0.1 M phosphate buffer 7.4 at room temperature).

At this time, 20% of the T_3-^{131}I still remained in the protein solution. The quantity of residual unbound T_3-^{125}I was then estimated. Using the original specific activity of the T_3-^{125}I and the protein concentration, it was calculated that 1–2 moles of T_3 were covalently linked per mole albumin in the supernate of this suspension. However, since albumin-T_3 was not separated from unconjugated albumin, the quantity of T_3 conjugated per mole albumin could only be estimated as an average, since variable quantities of T_3/mole of albumin may be present. The insoluble and soluble portions of this mixture were lyophilized together and reconstituted in isotonic saline at a concentration of 2 mg/ml. Though obviously cloudy, this saline suspension was mixed with an equal quantity of complete Freud's adjuvant (Difco Laboratories, Detroit, Mich.) and 1 ml (1 mg) injected in 0.1 ml amounts into the toe pads of four New Zealand white rabbits (designated A, B, C, D). The second antigen, T_3-human serum albumin (HSA), was prepared by the method of Oliver, Parker, Brasfield, and Parker, as modified by Gharib, Mayberry, and Ryan (10, 18). The four rabbits, A–D, received an additional injection of 1 mg of this second antigen 3–4 wk after the injection of the first antigen and again 2 wk later. Antisera were harvested beginning 10 days after the second injection. Two of the initial four rabbits died (B, C). The two remaining rabbits (A, D) have produced antisera with a maximum titer of 1 to 100,000–150,000 for use in the assay described below. Animals immunized with only the T_3-HSA conjugate (E, F), produced antisera of equal affinity, but with usable titers of approximately 1/5000–1/10,000. All of the studies to be reported were performed with antisera labeled A215-71 (rabbit A).

Immunoassay procedure. After extensive experimental evaluation, the immunoassay procedure described in Table I was adopted. The T_3-^{125}I was kindly donated by J. F. Jeffries of Abbott Laboratories, North Chicago, Ill. It had a high specific activity (300–500 mCi/mg). This allowed 5–10,000 counts to be added with a T_3 concentration of less than 0.03 ng of tracer per assay tube.

T_3-free human serum was prepared as follows: tracer T_3-^{125}I, approximately 10,000 counts/ml, was added to pooled human serum. Then, approximately 7.5 g of Norit A decolorizing pharmaceutical charcoal were added per 100 ml of serum and mixed at 4°C for a period of at least 3 hr (although 12–24-hr periods are usually used for convenience). The serum was then centrifuged for three successive 1 hr periods at 25,000 *g* and the clear supernate was removed after each centrifugation. Comparison of quantities of T_3-^{125}I before and after charcoal addition demonstrated that less than 1% of the original T_3-^{125}I remained in the solution. Protein concentration, osmolality, and TBG capacity (by reverse-flow electrophoresis [19]) were unchanged, although

approximately 90% of the T_4 present in the serum was also removed by this technique. Numerous control studies have shown that this serum behaves identically in the immunoassay with serum in which the T_4 concentration has been returned to normal amounts using specially purified T_4 (see below), and with sera of a patient with profound myxedema.

T_3 enrichment of the T_3-free serum to 4 ng/ml was carried out with T_3 dissolved in 0.04 M NaOH. The gravimetric concentration of T_3 in the preparation was verified by its absorbance at 320 mμ using the known molar extinction coefficient previously determined by Gemmill (20). The accuracy of the dilution of the final T_3 preparation to be added to serum was monitored by tracer T_3. Standard serum containing T_3 has been kept for up to 3 wk at −20°C with no significant change in the displacement curve.

The charcoal-dextran solution, prepared as previously described, was diluted two parts charcoal-dextran to three parts glycine-acetate buffer immediately before addition to the assay tube (5). The solution was kept at 4°C until addition and was transferred from a beaker which was constantly stirred with a magnetic stirring bar. In practice, charcoal-dextran is added to the tubes in groups of 30 to 40. This grouping is dictated by the requirement that there should be less than 4 min difference in the duration of exposure of the first and last tubes of the group to the charcoal-dextran. To ascertain that the per cent of T_3-^{125}I bound by the charcoal has not varied during the procedure, two antibody tubes containing no added T_3 are run with each group—one, the first, and the other the last tube to receive charcoal-dextran. The charcoal-dextran is added to each of the standard triplicates in different groups. Unknown samples and corresponding blanks are assayed in duplicate at two dilutions, also in different groups. All assays have been carried out in 16 × 125 mm plastic test tubes obtained from Amersham-Searle Corp., Arlington Heights, Ill.

The per cent tracer bound in the blank (without antibody) in the T_3-free serum (0.2 ml) varied from 7.1 to 10.7 (mean 8.6%) in 13 separate assays with a standard deviation of less than 1.0% within each assay. A "blank" which gives the same per cent bound is also obtained in a tube containing antibody and an excess of unlabeled T_3. Since there was no difference in the blank value in the presence of increasing quantities of T_3, only six blanks containing 0.2 ml of T_3-free serum were used for each standard curve. The blank is slightly greater in unknown tubes from patients with normal TBG levels (mean for 0.2 ml, 9.4±1.27% SD). In serum from pregnant patients and in cord serum with TBG levels about twice normal, the blank is 2–3% higher than that obtained with normal serum. Blanks in the serum enriched with greater than 50 μg T_4/100 ml, are 2–3% lower than those in normal serum simultaneously assayed.

A displacement curve performed using increasing quantities of T_3-^{125}I is superimposable on a standard curve using increasing quantities of unlabeled T_3 up to 0.4 ng of T_3 per assay tube. The per cent bound in the blank is unchanged with addition of as much as 40 times the amount of tracer used in these studies. We have observed that tracer I^-, which may be present in small quantities (less than 2%) in the T_3-^{125}I preparations, is not bound to the charcoal-dextran. This is therefore, eliminated by the formula given in Table I. Sufficient counts in the 1 ml supernate are collected to provide less than 2.5% counting error.

We have chosen to express T_3 concentrations as nanograms per milliliter since it provides a convenient terminology; the normal value being approximately 1 ng/ml of human serum. Secondly, 1 ml closely reflects the quantity of serum assayed.

Enrichment with T_4. Thyroxine (free acid) was subjected twice to paper chromatography in a tertiary amyl alcohol-hexane-ammonia system as previously described (5). After the second chromatography, the T_4 was eluted from the paper with a portion of pooled human serum. This serum was then diluted with T_3-free serum to give a net increase in T_4 concentration of about 50–100 μg of T_4/100 ml as estimated by the Murphy-Pattee technique (21). All T_4 values are corrected for 80% extraction of T_4 into ethanol and are expressed as total T_4.

Serum specimens. Samples of blood were generally obtained at 7 a.m. from patients at Presbyterian-University Hospital, Oakland Veterans Administration Hospital, and Montefiore Hospital, all in Pittsburgh, Pa. Some patients were hospitalized on the Clinical Research Unit of Presbyterian-University Hospital. After clotting, the samples were centrifuged and the serum frozen at −20°C until assay. When thyroid-stimulating hormone (TSH) was administered, 10 U were given i.m. and specimens were obtained at various intervals as described.

Materials. All solutions were made using distilled, deionized water and reagents were obtained from commercial sources: "Morpho" carbodiimide (CDI) from Aldrich Chem-Chemical Co., St. Louis, Mo., crystalline BSA used in ical, "ethyl" CDI and L-thyroxine (free acid) from Sigma coupling from ICN Nutritional Biochemicals Div., Cleveland, Ohio, crystalline HSA and Na 3,5,3′ L-triiodothyronine from Mann Research Labs., Inc., New York. Statistical analyses were performed by standard methods (22).

RESULTS

Standard curve. Addition of increasing quantities of T_3 results in progressive decreases in the per cent T_3-^{125}I bound as is shown in Fig. 1 (curve A). At the antiserum concentration generally used (1/106,000), the per cent T_3-^{125}I bound in the 0 tube is 50–60%. The presence of 0.1 ng T_3 causes displacement of 55–65% of the T_3-^{125}I bound at 0 T_3 concentration. The displacement curve in the presence of salicylate and T_3-free serum is slightly lower and has a steeper initial slope than that which is obtained in the presence of buffer alone (curve B). The effect of salicylate on the standard curve can be appreciated by comparison of curve A with curve C, obtained by omitting salicylate from tubes containing human serum. Displacement of tracer T_3 from antibody in the latter circumstances is obscured by the binding of T_3-^{125}I presumably to the TBG present in human serum.

When increasing quantities of hyperthyroid serum are assayed, the displacement curve is superimposable on the standard curve in T_3-free serum. A representative example of such an experiment is shown in Fig. 2. In this figure, the per cent T_3-^{125}I bound is expressed relative to the 0 value. Thus, as little as 5 μl of this patient's serum provided significant displacement of ^{125}I from the antibody.

Cross-reactivity of the antiserum with L-thyroxine, monoiodotyrosine (MIT), and diiodotyrosine (DIT).

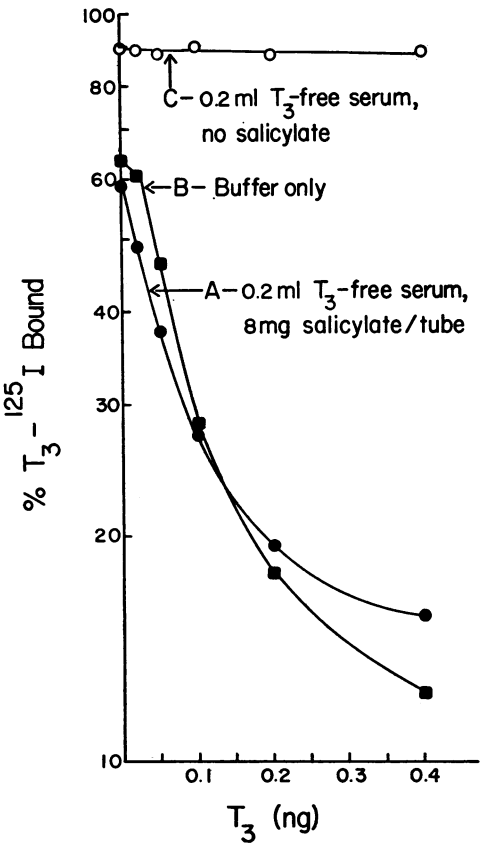

FIGURE 1 Comparison of standard curves performed under various conditions. The per cent T_3-^{125}I bound is not corrected for the per cent T_3-^{125}I bound in the absence of antibody (blank). The blank values were as follows: curve A, 8.2%; B, 3.3%; C, 87%. The standard error of the mean of the triplicate determinations is less than 2% for all points.

TABLE II

Effect of T_4 Enrichment of Human Serum on T_3 Concentrations

	Initial T_4	Final T_4	Net increase T_4	Initial T_3	Final T_3	Net increase T_3	Net increase T_3/net increase T_4
	ng/ml	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	
A	61*	1154	1093	0.41‡	1.53§	1.12	0.0010
B	43	526	483	0.20‡	0.80§	0.60	0.0012
						Mean	0.0011

* T_4 values are the mean of duplicate determinations.

‡ Calculated from the dilution of pooled serum with T_3-free serum (A = 1/1, B = 1/3).

§ T_3 values are the mean of duplicate determinations at two dilutions.

Table II shows the results of the T_3 assay in sera enriched with T_4 as described above. It would appear that in this assay system, approximately 100 ng of T_4 are required to produce displacement of T_3-^{125}I from the antibody equivalent to 0.1 ng of T_3 (approximately 50% displacement). The above calculation for cross-reactivity with T_4 assumes that there is no T_3 present as a contaminant in the T_4 preparation. The data were alternatively consistent with the interpretation that even after repeated chromatography, there is a 0.1% T_3 contamination of the T_4 preparation. From our previous studies, the latter seems more likely (5). If the T_4 preparation were free of T_3, the T_4 present in normal concentration in human serum (approximately 80 ng/ml), would result in an artifactual overestimate of only

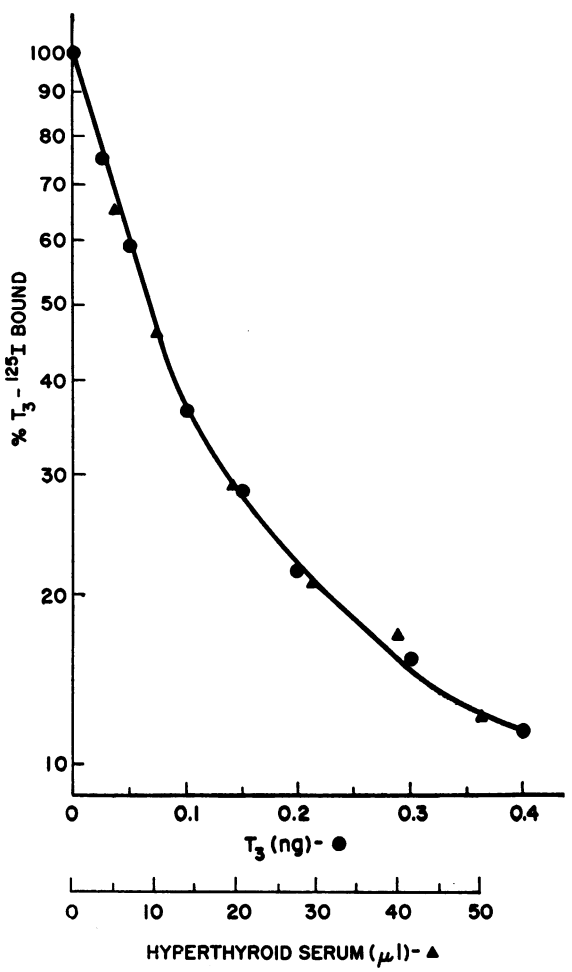

FIGURE 2 Comparison of displacement of T_3-^{125}I by T_3 and by hyperthyroid serum. Results are the mean of triplicate determinations. The standard error of the mean is less than 2% of the counts of tracer bound at 0 T_3 for all points. The estimated T_3 concentration in this hyperthyroid serum was 7.4 ng/ml and the T_4 concentration 23.2 μg/100 ml.

TABLE III

Recovery of T_3 Added to Normal Serum

	Portion	T_3 Expected	T_3 Recovered	Recovered/ expected
	μl	ng	ng	%
Pool + 4 ng/ml T_3*	100	0.48	0.48‡	100
	50	0.24	0.24	100
	25	0.12	0.13	108
	10	0.048	0.051	106
			Mean	103.5±2.1 (SE)

* Endogenous T_3 concentration of the pool was 0.81 ng/ml.
‡ Mean of duplicate determinations.

0.08 ng of T_3/ml due to cross-reaction with T_4. There is no displacement of T_3 by as much as 1000 ng of MIT or DIT.

Recovery of T_3 added to normal human serum. When normal human serum was enriched with 4 ng of T_3/ml and various portions were assayed, the mean recovery was 103% over a broad range of T_3 concentrations (Table III). Recovery of T_3 from a sample of human serum containing 2 ng/ml added T_3 was 1.98±0.14 (SD) ng/ml in 10 consecutive assays.

Reproducibility of the assay. Results of T_3 determinations in three different serum preparations of varying T_3 content are given in Table IV. Using serum of extremely low T_3 concentration (assay of 25 and 50 pg of T_3) the per cent standard deviation within the assay is 9.2. However, at higher concentrations (assay of 80 and 160 pg or 100 and 200 pg), the within-assay per cent standard deviation is substantially less: 2–4%. The between-assay variation is slightly greater, though with more experience with the assay this has been decreased to about 5%.

Further evidence of the precision of this assay is given in Table V. This table presents the results of five consecutive immunoassays. The data demonstrate that the quantities of T_3 recovered in different portions of various unknown samples is directly proportional to the volume of the unknown serum assayed. Thus, the calculated T_3 concentration is constant regardless of the quantities of serum assayed within the limit of the standard curve. This is true when both small (less than 100 pg) and large (greater than 100 pg) quantities of T_3 are measured in the greater portion. This provides assurance that the T_3-free serum used as diluent to achieve a constant serum volume of 0.2 ml does not either artifactually lower or increase the estimated T_3 concentration. In practice, we have attempted to assay sufficient quantities of serum to measure between 50 and 250 pg in the portions sampled. This allows at least two points on the standard curve above and below the unknown value.

TABLE IV

Reproducibility of the T_3 Immunoassay

Sample	T_3	% SD	N
	ng/ml		
Within assay			
A	0.25±0.023*	9.2	9‡
B	0.81±0.029	3.6	10
C	1.03±0.03	2.2	10
Between assays			
D	0.75±0.06	8.0	10

* Mean ±SD of duplicate determinations.
‡ N refers to the number of pairs.

Results of T_3 assays in euthyroid subjects, patients with hyper- and hypothyroidism, pregnant patients at delivery, and cord sera. The mean T_3 level in 38 clinically euthyroid subjects was 1.10 ng/ml ±0.25 (SD). This is indicated in Fig. 3 as a shaded area which is 1 SD above and below the mean. Hyperthyroid subjects have values which range from a low of 1.88 ng/ml in a subject with hyperthyroidism and TBG deficiency to a high of 23.8 ng/ml in a subject with a T_4 level of 50 μg/100 ml. This patient had been treated for a period of months with small doses of iodide which had obviously not controlled her hyperthyroidism. The values in hypothyroid patients were obtained before institution of treatment. The mean T_3 level in 25 hypothyroid patients was 0.39±0.21 ng/ml. Thus, there would appear to be greater overlap between this group and the normals than was observed with the hyperthyroid patients. All of these patients were spontaneously hypothyroid or had become hypothyroid after surgery.

TABLE V

Results of Determinations in Paired Portions of Different Volumes in Five Consecutive Assays

Quantity of T_3 in larger portion					
<0.100 ng			>0.100 ng		
Volumes*	Pairs	T_3 recovered	Volumes	Pairs	T_3 recovered
μl		ng	μl		ng
200	24	0.0677±0.0047‡	200	45	0.219±0.012
100		0.0332±0.0025	100		0.108±0.005
100	3	0.077 ±0.0061	100	17	0.220±0.021
50		0.039 ±0.0036	50		0.109±0.011
50	8	0.0578±0.0120			
25		0.0332±0.0075			

* The volumes refer to the quantity of the portions of unknown serum which was assayed in duplicate. Each unknown was measured in only one pair.
‡ Mean ±SE.

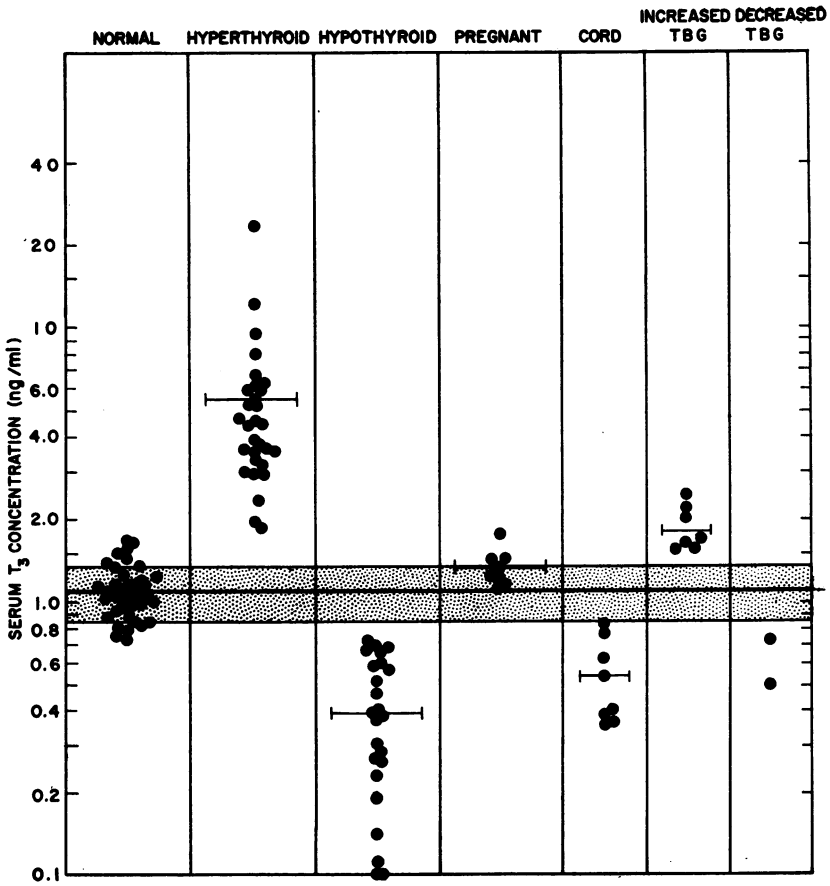

FIGURE 3 Results of T_3 determinations in various groups of patients. The heavy line and the stippled area represent the mean ±1 SD in 38 euthyroid subjects. The bars indicate the mean in various groups. T_3 levels are plotted on a log scale to allow convenient presentation of both high and low levels.

In eight pregnant patients at the time of delivery, the T_3 level was in the upper normal range (mean 1.33 ng/ml). Of great interest are the T_3 values in cord sera obtained simultaneously with the maternal sample in these patients. The mean T_3 level (0.53 ng/ml) is about 2 SD below the mean normal value. The paired cord-maternal T_3 levels are significantly different by paired t test ($P < 0.001$). The implications of this observation are discussed further below.

Serum T_3 concentrations were examined in seven euthyroid patients with increased TBG levels (mean 45 μg T_4/100 ml, range 31–65 μg T_4/100 ml). The mean serum T_3 concentration in these patients was 1.78 ±0.25 (SD) ng/ml or about 3 SD above the normal mean. Two euthyroid patients with TBG deficiency (7.9 and 10.7 μg T_4/100 ml) have been identified and both have T_3 levels in the low normal range.

Correlation of T_3 and T_4 concentrations. In Fig. 4, a plot is constructed of the T_3 and T_4 levels determined on the same specimens. In general, there is the expected good correlation between T_3 and T_4 levels in normals and hyperthyroid subjects. It would appear, however, in the hypothyroid subjects, that there is more overlap with the normals in T_3 levels than in T_4 levels. The data from the cord blood obtained at the time of delivery are the only group of results that appear to deviate from the general pattern. Here, there appears to be a distinct decrease in the serum T_3 relative to T_4 levels. Both the cord sera and the maternal samples had increased TBG binding capacities (35–50 μg T_4/100 ml).

Response of T_3 to physiologic influences. In Fig. 5, the utility of this assay in observing rapid physiologic changes in thyroidal T_3 secretion is illustrated. The data from the TSH injections in euthyroid subjects is representative of that we have seen in a series of seven such studies in which T_3 and T_4 concentrations after TSH were compared (Table VI). There is a substan-

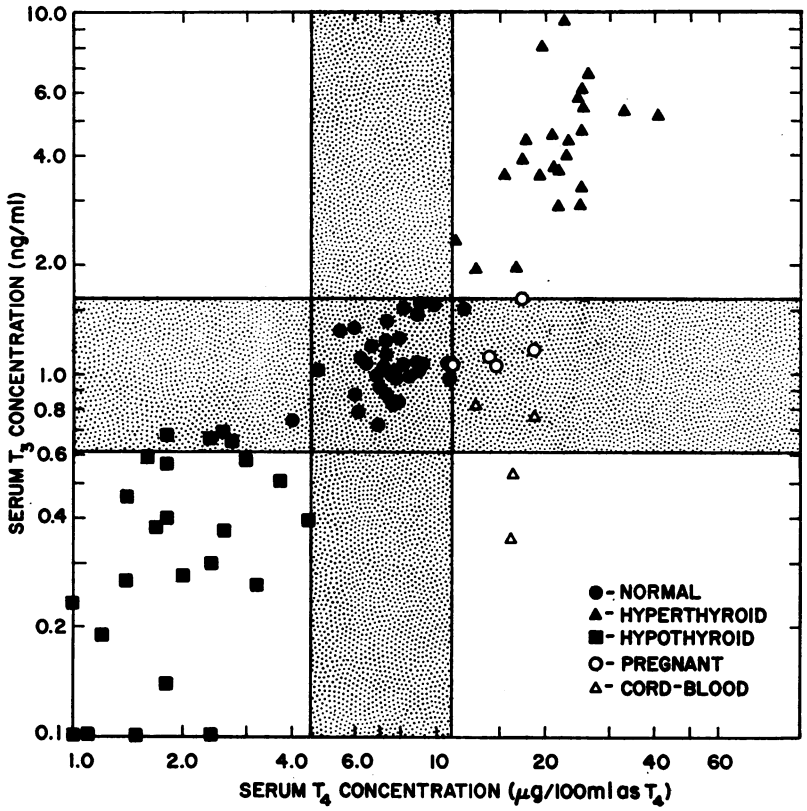

FIGURE 4 T_3 and T_4 concentrations measured in the same serum samples from normals, from patients with various thyroid diseases, from pregnant patients at the time of delivery and in cord sera. The stippled area represents the mean ±2 SD for both T_3 and T_4 levels based on the data obtained in these normal subjects. No attempt was made to quantitate precisely levels of T_3 less than 0.1 ng/ml or less than 1.0 μg of T_4/100 ml. The log scale is used to allow presentation of a broad range of values.

tial increase in T_3 concentrations after TSH which reaches a mean level of 205% of the baseline value by 8 hr. The T_4 levels have increased only 41% at this time and even by 24 hr have not increased to the degree that the T_3 levels do. It is not as yet clear from these studies when the peak T_3 levels are reached but it appears to be somewhere between 8 and 24 hr after TSH injection. In both euthyroid patients shown in Fig. 5, as well as in the others we have studied, further injections of TSH result in additional increases in T_3 and T_4 levels.

The rapid decrease in T_3 levels after either removal of the pituitary or thyroid gland is depicted in the same figure. There is a prompt decrease the day of surgery, but little if any decrease is observed over the next few days. As might be expected, T_3 levels fall significantly faster than do T_4 levels under these circumstances. The half-life for T_3 has been estimated to be approximately 1 day while that for T_4 in the euthyroid patient can be expected to be about 7 days (23). This would appear to account for the rapid decrease of T_3 in the initial 24 hr after subtotal thyroidectomy or hypophysectomy. The reasons for the failure of serum T_3 to reach undetectable levels are discussed further below. Both patients subsequently became clinically hypothyroid with decreases of T_4 and T_3 into the hypothyroid range.

DISCUSSION

Methodology. The direct immunoassay of T_3 in human serum has been technically difficult due to: (*a*) small quantities of T_3 present in the serum, (*b*) the presence in serum of relatively large amounts of a similar hormone, T_4; and (*c*) the presence of TBG, a protein which has a relatively high affinity for the hormone to be measured. The method described above appears to provide a satisfactory solution to these problems. The sensitivity of the assay as described is sufficient to measure 25 pg of T_3 and with further dilution of the antiserum as little as 12.5 pg of T_3. The latter allows quantitation of

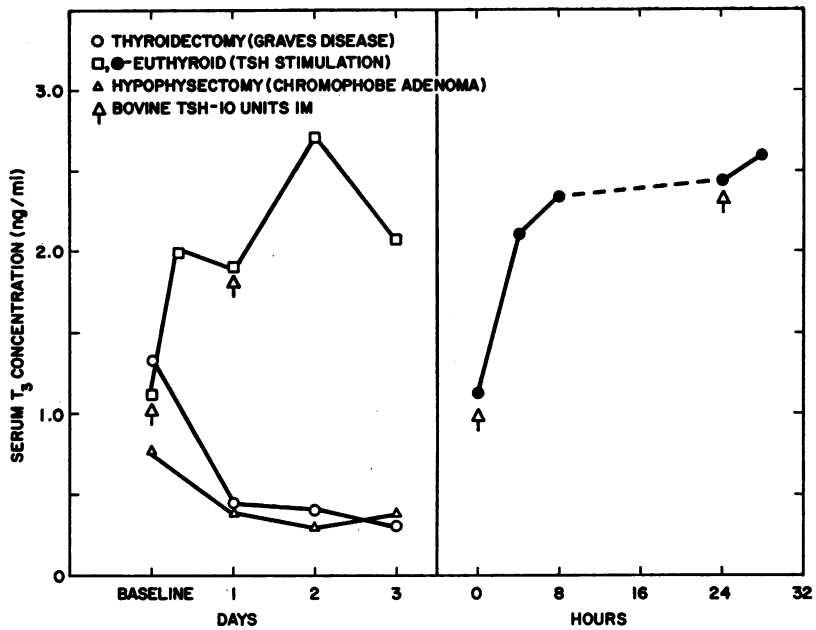

FIGURE 5 Response of T_3 to alterations in thyroid hormone secretion. The results of T_3 levels in four patients are presented. Two of these were given 10 U of TSH at the times indicated by the arrows. Thyroid secretion was inhibited in two patients, one by thyroidectomy and the other by hypophysectomy.

T_3 in whole serum containing as little as 0.06 ng/ml, levels rarely reached even in profound hypothyroidism.

It is not clear from our studies whether the high titer of antisera obtained in these rabbits is due to more than chance. The initial production of antibody to T_2 was reported by Brown, Ekins, Ellis, and Reith (9). However, these authors as well as the other laboratories in which various T_3-protein conjugates have been utilized, have not reported antiserum with usable dilutions higher than 1–4,000 (9–13). It is possible that the partial insolubility of the T_3-BSA conjugate is related to its increased efficacy, but this is pure speculation. It would appear from preliminary data, that antisera obtained from the three other animals immunized with the

TABLE VI

Effect of Exogenous TSH on Serum T_3 and T_4 Concentrations in Euthyroid Subjects

Subject	Serum T_3 concentration			Serum T_4 concentration		
		After 10 U TSH*			After 10 U TSH*	
	Basal	8 hr	24 hr	Basal	8 hr	24 hr
	ng/ml			*μg/100 ml*		
D. F.	1.14	2.00	1.93	8.8	11.9	13.1
D. L.	1.63	3.50	3.00	9.4	10.6	15.9
H. W.	1.14	2.35	2.44	6.4	9.8	11.2
E. O.	0.73	1.14	1.35	6.9	11.6	10.2
E. A.	0.93	1.75	1.58	7.6	8.0	11.5
S. B.	0.56	1.82	1.24	4.7	8.3	8.6
C. S.	1.00	2.07	2.94	7.4	11.7	17.8
Mean ±SEM	1.02±0.13	2.09±0.27	2.07±0.28	7.3±0.6	10.3±0.6	12.6±1.2
Per cent increase		105	103		41	72

* Bovine TSH given intramuscularly.

initial BSA-T_3 conjugate are of equally potent affinity, although two of the animals died before high titers were attained. This suggests that the high titer is probably not an unusual response of a single animal. In addition to the high titer and high affinity, the antiserum shows very low, if any cross-reactivity with T_4, MIT, or DIT.

The use of salicylate is a critical factor in the success of this assay as is demonstrated in Fig. 1. Its effect would appear to confirm previous evidence that salicylate inhibits the binding of T_3 to TBG (15, 16). We have shown that salicylate concentrations of approximately 10-fold greater magnitude are required to cause displacement of T_4 equivalent to that of T_3 in dilute human serum. Therefore salicylate has the theoretical advantage of displacing more T_3 from TBG than T_4 and would tend to decrease the potential for cross-reactivity of T_4 with T_3 in the assay.

This property would presumably also be shared by the compounds used by other investigators to inhibit T_3-TBG binding. Tetrachlorthyronine has been reported to be effective by Mitsuma, Gershengorn, Colucci, and Hollander and diphenylhydantoin by Lieblich and Utiger (12, 13). A preliminary study reported by Gharib et al. does not appear to consider the influence of the TBG in the serum on the T_3 assay (10). This may account for the higher normal values reported by these authors since addition of TBG could simulate addition of T_3 by competing with the antibody for tracer T_3.

Since the presence of serum (probably primarily due to its TBG content) alters the standard curve, it is necessary to include a constant amount of serum in the unknown and standards during the assay. Furthermore, since changes in serum TBG content (idiopathic or pregnancy related) cause parallel changes in the per cent T_3-^{125}I bound in the absence of antibody as described above, it is necessary to monitor the blank value for each unknown serum. With these data, the observed per cent bound can be corrected for that per cent of T_3-^{125}I that would be bound in the absence of antibody. However, as may be readily appreciated, this is a somewhat tedious procedure in that eight tubes are required for duplicate immunoassay of T_3 in two dilutions. We are currently accumulating a larger body of data over a period of intensive use of this assay to determine whether or not individual blanks are necessary when T_4 and TBG levels are approximately normal. The significance of the small changes in the blank value is dependent on the quantity of T_3 in the portion which has been assayed. If this is small and the results are high on the curve the 2–3% change is almost negligible. On the other hand, if the per cent bound is low, then the difference of 3% represents a larger fraction of the observed per cent bound and may change the T_3 value considerably. The demonstrated precision of the assay as described appears to justify the use of the simultaneous blanks in that differences of greater than 6% in normal or hyperthyroid T_3 values are statistically significant when the samples are measured in the same assay. The dextran-coated charcoal may offer some advantages over double-antibody systems in that the effect of TBG alterations alone on the distribution of T_3-^{125}I in the serum sample can be recognized and a correction applied.

The use of charcoal to obtain a T_3-free serum has not previously been described. The best justification for the use of this preparation is presented in Table V. This shows that virtually identical concentrations of T_3 are calculated from the measurements at two different dilutions of the same sera using a variety of volumes at both high and low T_3 levels. If this T_3-free serum did not behave identically with normal serum one would anticipate a systematic difference in the two values.

Observed T_3 values in various clinical states. In general, the T_3 values observed in normals, hyper- and hypothyroid subjects are comparable to those reported by other authors using a radioimmunoassay for T_3. The normal value of 1.1 is somewhat lower than that reported by Mitsuma et al. (1.4 ng/ml) as is the mean value in hypothyroid serum (0.39 ng/ml as opposed to 0.60 ng/ml [12]). The values are likewise lower than those of Lieblich and Utiger (normal 1.5 ng/ml and hypothyroid 0.99 μg/ml [13]). Our results are higher than those reported by Chopra, Solomon, and Beall (80% of normals less than 1 ng/ml) but the assay described by these authors was not sufficiently sensitive to measure T_3 concentrations less than 1 ng/ml (11). The values in hyperthyroid serum are similar to those previously reported (10–13). The T_3 concentrations are significantly lower than the levels we have previously obtained using a modification of the Sterling assay (5). In sera which we have assayed by both methods, we find the results using immunoassay to be 60–70% of those initially reported. We believe the present values to be more accurate and attribute the differences between the two to artifactual elevation of T_3 values due to T_4 to T_3 conversion and some contamination of the T_3 spot with T_4 when the Sterling method is used (5). The data in Figs. 3 and 4 suggest that there is some overlap between T_3 levels in normals and the various thyroid states, but probably no more than has been observed for T_4 levels.

The serum T_3 values obtained in pregnant patients would appear to be similar to the results observed for T_4 levels in such patients, i.e. they are in the upper portion of the normal range presumably reflecting the presence of increased TBG levels. The group in which the T_3 results deviate strikingly from the expected values is that of the cord sera. In these eight subjects the T_3 values are distinctly lower than would be expected based on T_4 values and some are clearly in the hypothyroid range.

We have obtained similar low values in both umbilical artery and vein sera suggesting that this value is truly representative of the neonatal T_3 levels. Therefore, one may speculate that this low T_3 value is causally related to the increase in TSH secretion which has been observed by several investigators in the early hours of neonatal life.

Aside from the diagnosis of various thyroid states, the immunoassay for T_3 appears to be a useful tool in other studies of thyroid function. The effects of TSH on T_3 levels are quite marked at short time intervals after administration of TSH. Thus, in the euthyroid patients studied, a mean increase of 105% in T_3 levels has been observed within 8 hr after TSH administration. Triiodothyronine levels would then appear to be a more sensitive index of TSH response than the T_4 levels which are increased only 41% at this time.

In addition, it would appear from limited experience that the T_3 level may have some predictive value in the assessment of the future thyroid function of patients subjected to pituitary or thyroid surgery. Because of the short half-life of T_3, elimination of thyroidal T_3 secretion is associated with a rapid decrease of the previously normal T_3 into the hypothyroid range. The residual T_3 in the serum of these two patients is presumably in large part derived from T_4 to T_3 conversion. While more experience is needed to determine whether or not patients with a decrease of T_3 into the hypothyroid range immediately postoperatively will invariably remain hypothyroid, there are obvious diagnostic advantages to a test sensitive to rapid changes in thyroid function.

One of the most intriguing aspects of the immunoassay of T_3 is the ability of the rabbit to generate a specific antibody against a compound which differs by only one atom (albeit a large one) from another hormone. It has been our experience that while the specificity of the antisera are not nearly so great, excellent titers of anti-T_3 antisera are also stimulated by thyroglobulin, though this experience is not universal (24). One may speculate that perhaps T_3 is the better antigen because T_4, even in thyroglobulin, is deiodinated after injection into the animal leaving only, or primarily, T_3 as the antigen.

ACKNOWLEDGMENTS

The author would like to express his appreciation to Miss Jitka Dockalova, Mrs. Darina Sipula, and Miss Regina Oswalt for their careful technical assistance.

These studies were supported by National Institute of Health Grant No. AM-14283 from the National Institute of Arthritis and Metabolic Diseases, the Health Research and Services Foundation of Pittsburg, Pa., Grant No. M-10, the Dreyfus Charitable Fund, and the General Clinical Research Center Grant No. FR-56 from the National Institutes of Health.

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