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Research Article

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Triiodothyronine (T_3)[1] was recognized to be a biologically active secretory product of the thyroid gland over a decade ago (1). Recent studies have indicated that it is formed extrathyroidally as well (2, 3). Nevertheless, relatively little information concerning the role of T_3 secretion in different thyroid disorders has been accumulated until very recently. Methods for the measurement of T_3 which require its extraction from plasma, and often its separation from thyroxine as well, have been described by several investigators (4–11). These methods have proven useful, but they are relatively complicated, the number of samples that can be assayed is limited, and they may be affected by in vitro deiodination of thyroxine. More recently the radioimmunoassay technique has been applied to the measurement of T_3. Several preliminary reports have appeared describing the preparation of antibody to triiodothyronine by immunization of animals with T_3-protein conjugates and its use for the measurement of T_3 in serum (12–15). The present report describes the development of a radioimmunoassay for the measurement of T_3, studies of the specificity of the anti-T_3 serum, and some initial studies which indicate that the method is applicable to the measurement of T_3 in unextracted serum.

METHODS

Materials. L-Triiodothyronine (T_3) was obtained from Sigma Chemical Co., St. Louis, Mo. L-T_3-^{125}I and L-thyroxine-^{125}I with specific activities of 50 mCi/mg or greater were obtained from Abbott Laboratories, Chemical Marketing Div., North Chicago, Ill. Various thyroid analogues were obtained from Sigma or provided through the courtesy of Dr. Leonard Ginger of Baxter Laboratories, Inc., Morton Grove, Ill. Bovine serum albumin (BSA) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, and human serum albumin (HSA) from Armour Pharmaceutical Co., Chicago, Ill. and Hyland Laboratories, Los Angeles, Calif.

Preparation of T_3-bovine serum albumin conjugates. The immunogen used was prepared by coupling T_3 to BSA with a water-soluble carbodiimide. In the three conjugate preparations made, 40 mg of T_3 was dissolved in 2 ml 0.1 N NaOH. To this was added 20 mg BSA in 3 ml water and then 200 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl. The pH of the reaction was adjusted to 9.0 with 0.1 N HCl. The mixture was stirred at 5°C for 24 hr and subsequently dialyzed for 96 hr against water and finally for 24 hr against 0.15 M NaCl. Conjugates of BSA alone or BSA and L-thyroxine (T_4) were prepared in a similar manner.

The final product of these reactions was analyzed for protein by the method of Lowry, Roseborough, Farr, and

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; HSA, human serum albumin; PBI, protein bound iodine; T_3, triiodothyronine; T_4, thyroxine; TBG, thyroxine binding globulin; TSH, thyrotropin.

TABLE I
Characteristics of T_3-Albumin Conjugates

	Protein	PBI*		T_3 Immunoassay	T_3/albumin
	mg/ml	*μg/mg*	*μg T_3/mg*	*μg T_3/mg*	*moles/mole*
T_3-BSA (1)	4.3	18.2	31.2	21.2	1.82
T_3-BSA (3)	4.8	34.4	59.0	76.1	6.60
T_3-BSA (3)	4.5	—‡	—	39.4	3.38
T_3-BSA (4)	5.3	—	—	78.8	6.78
BSA	5.0	<0.02	<0.04	<0.013	<0.002
T_4-BSA	5.2	—	—	0.29	0.021

* Measured by the autoanalyzer method.
‡ The symbol (—) means not measured.

Randall (16), for protein bound iodide (PBI) by the autoanalyzer technique and ultimately in the T_3 radioimmunoassay. The results of these analyses (Table I) indicate that significant conjugation of T_3 to albumin had occurred, the calculated T_3 to BSA molar ratios of the T_3-BSA conjugates ranging from 1.82–6.78 : 1. The reason for this variability is not known. Since good agreement was found between the T_3 content of the preparations calculated from the PBI data and from the immunoassay results (Table I), it was concluded that little deiodination of T_3 had occurred during the preparation of the conjugates.

Preparation and detection of anti-T_3 antibody. Rabbits were immunized by foot pad injection of 0.4–0.8 mg T_3-BSA conjugate emulsified in 0.75–1.0 ml complete Freund's adjuvant at monthly intervals. Sera were obtained before each booster injection and stored at −5°C. Sera obtained after the first injection of conjugate were found to contain some anti-T_3 antibody but those obtained after three to four injections had a much greater quantity of antibody and were used exclusively in the studies to be described. Initially, four animals were immunized and most of the studies herein reported were done using serum obtained following the third injection of one of these animals. Subsequently, additional animals have been immunized and additional anti-sera of similar potency and affinity obtained. Micro agar gel diffusion studies showed a single precipitin line when anti-T_3 sera were reacted with T_3-BSA and BSA treated with carbodiimide and a line of identity was observed when the antigens were placed in adjacent wells. No precipitin lines were seen between wells containing unmodified BSA and anti-T_3 serum.

Antibody to T_3 was detected by reaction of 0.04–0.08 ng T_3-^{125}I with varying quantities of anti-T_3 antibody for 24–48 hr and then separation of free and antibody bound T_3-^{125}I by addition of goat anti-rabbit IgG serum. With potent anti-T_3 sera as little as 0.5 μl bound 90% of the T_3-^{125}I added. The binding capacity of the most potent antiserum obtained, estimated by addition of 0.04–4.2 ng quantities of T_3-^{125}I to antiserum diluted such that 40–60% of the radioactivity of 0.04 ng of T_3-^{125}I was bound, was 600 ng T_3/ml. Less than 2.5% of the radioactivity added was precipitated when T_3-^{125}I was reacted with normal rabbit serum.

Radioimmunoassay of T_3. For T_3 assay duplicate tubes containing the following reagents were prepared: (*a*) 100 μl of unlabeled T_3 diluted in 4% HSA, 0.05 M phosphate, pH 8.0, or the serum to be assayed. Serum samples were always tested in at least two doses, usually 50 and 100 μl. When serum volumes of less than 100 μl were used, albumin-phosphate buffer was added to bring the volume to 100 μl. The concentrated human albumin solution was used to approximately equalize the protein concentration in each tube. (*b*) 50 μl T_3-^{125}I (0.04–0.08 ng, approximately 3000–6000 dpm) in 0.25% BSA, 0.05 M phosphate pH 8.0, containing 1.0 mg/ml diphenylhydantoin. To ensure solubility of the diphenylhydantoin, this solution was adjusted to pH 10.2–10.4 with NaOH. The pH of the complete reaction mixture was 8.2–8.3. (*c*) 50 μl anti-T_3 serum diluted so that 40–60% of the added T_3-^{125}I was precipitated in the absence of unlabeled T_3. The buffer used for the anti-serum dilution was 0.25% BSA, 0.05 M phosphate, pH 8.0. containing 0.05 M EDTA. With the anti-T_3 serum used in most of these studies, this dilution was 1 : 200.

After 48 hr at 5°C, goat anti-rabbit IgG serum was added in sufficient quantity to precipitate all the rabbit IgG present. The presence of human IgG (in serum) or addition of purified human IgG to solutions of T_3 in buffer did not require the addition of larger quantities of anti-rabbit IgG serum. Subsequent to an additional 18–24 hr at 5°C, the tubes were centrifuged at 1000 *g* and the radioactivity in the precipitate determined. Shorter periods of incubation of the T_3-^{125}I and anti-T_3 serum resulted in reduced binding of T_3-^{125}I to antibody. The proportion of radioactivity precipitated in duplicate tubes rarely varied by greater than 2%. When anti-T_3 antibody and/or anti-rabbit IgG were omitted from the reaction mixture, no more than 1.5% of the total radioactivity was precipitated.

Fractionation studies. Several fractionation procedures were employed in the course of these studies. For the purpose of determining the T_3 content of various T_4 preparations, 10–20 μg quantities of T_4 were chromatographed on 1 × 50 cm columns of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 0.01 N NaOH as described by Mougey and Mason (17). With the use of T_3-^{125}I and T_4-^{125}I, it was found that T_3 and T_4 were separated satisfactorily by this procedure, T_3 emerging from the column well before T_4. When unlabeled T_4 was applied to these columns, fractions corresponding to the T_3 peak and the T_4 peak, as determined by radioactivity calibration in preceding runs, were pooled, neutralized with 0.1 N acetic acid, and lyophilized. The lyophilized fractions were reconstituted in 1–2 ml for T_3 immunoassay and T_4 assay by the competitive protein binding procedure (18). In several instances, one-half of the T_4 fraction from such a chromatographic separation was rerun on the Sephadex G-25 in the same manner.

Three methods for the extraction of thyroid hormones from serum were used. One employed chromatography of serum on the cation exchange resin Dowex AG 50 W-X2 (H+ form) (Dow Chemical Co., Midland, Mich.) as described by Sterling, Bellabarba, Newman, and Brenner (8). The fraction containing both the unabsorbed serum and 0.15 M ammonium acetate, pH 8.5, which was passed through the column immediately after the serum, was lyophilized and subsequently reconstituted to the initial serum volume with 0.01 M phosphate, 0.15 M NaCl, pH 7.5. The T_3 was eluted with 7.4 N NH_4OH. This fraction was evaporated to dryness at 70°C and reconstituted to the initial serum volume with 4% HSA, 0.05 M phosphate, pH 8.0.

Thyroid hormones were also extracted from serum by ethanol precipitation. 1 volume of serum and 2 volumes of 95% ethanol were mixed, centrifuged at 1000 *g*, and samples of the resulting supernatant dried at 40°C under an air stream. In several experiments, the initial ethanol precipi-

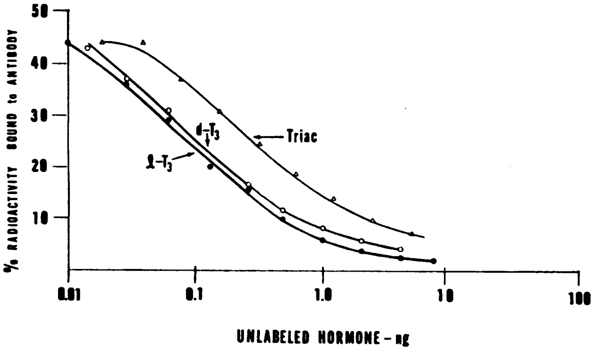

FIGURE 1 The effect of increasing quantities of unlabeled L-T_3 and T_3 analogues on the binding of T_3-^{125}I to anti-T_3 serum. Triac, triiodothyroacetic acid.

tates were recovered for immunoassay by suspension in water, dialysis, and lyophilization. The dried supernatants were suspended in a volume of 4% HSA, 0.05 M phosphate, pH 8.0, equal to the initial serum content of the dried extract. They were then centrifuged and a sample of the resulting supernatant tested in the T_3 immunoassay. The centrifugation step was included because the resuspended dried ethanol extracts contained varying quantities of particulate material. This particulate material was found to bind irreversibly, in the absence of anti-T_3 or second antibody, a variable quantity of T_3-^{125}I (4.5–24.7%) when incubated with it and the reaction mixture sedimented at the usual centrifugation speed (1000 *g*). This was not reduced by centrifugation of the initial serum-ethanol mixture at 10,000 *g*. When T_3-^{125}I was added to serum before the ethanol precipitation, 95.4 ±3.1% (SD) of the added radioactivity could be recovered after resuspension of the dried supernatants. Of this radioactivity, however, 18.6–37.2% (mean 26.0, n=9) sedimented at 1000 *g*.

T_3 was also extracted from serum to which diphenylhydantoin, in a final concentration of 500 μg/ml, had been added by treatment three times with Tetrasorb resin sponges (Abbott Laboratories, North Chicago, Ill.).

Clinical material. Serum was obtained from patients hospitalized on the Medical Service or the Clinical Research Center at the Hospital of the University of Pennsylvania or the Philadelphia Veterans Administration Hospital and from patients who were attending the Endocrine Clinic of the University Hospital. The hyperthyroid patients studied all had typical clinical and laboratory manifestations of hyperthyroidism. Each patient considered to be hypothyroid had signs and symptoms compatible with this diagnosis and, in addition, a low serum T_4 concentration, as determined by the competitive protein binding method (18). Of the 45 hypothyroid patients studied, 42 had elevated serum immunoreactive TSH concentrations (19), ranging from 10.8 to 2350 μU/ml. The remaining three patients all had serum T_4 concentrations below 2.4 μg/100 ml and undetectable or barely detectable TSH levels. After separation, serum samples were stored at − 5°C.

RESULTS

Antibody specificity studies. Results of studies showing the ability of unlabeled T_3 and various analogues of it to inhibit the binding of T_3-^{125}I to antibody are shown in Figs. 1 and 2. Addition of both T_3 and various thyroid analogues resulted in progressive reduction in the proportion of T_3-^{125}I bound to antibody. The dose response lines produced by T_3 and the compounds studied were similar in all instances. Significant inhibition of T_3-^{125}I binding to antibody occurred with as little as 0.05 ng unlabeled T_3. Multiple preparations of T_3 were tested and no significant differences among them were observed. It is apparent from Fig. 1 that the two triiodoanalogues shown were nearly as effective as L-T_3 in inhibiting the binding of T_3-I^{125} to antibody, the most potent being D-T_3. In contrast, the tetraiodo- analogues tested were effective only when much larger quantities were added (Fig. 2). Table II shows the potency, on a weight basis, of all of the various compounds tested compared to that of unlabeled T_3. Each was tested in multiple doses in at least two assays. As indicated in the table, several L-thyroxine preparations were tested. All inhibited the binding of T_3-^{125}I to antibody when added in large quantities but their potency varied considerably. Contamination of the T_4 preparations with T_3 was known in some instances, but said to be absent by the manufacturer in others. The preparations were indistinguishable when tested in the competitive displacement assay for T_4. T_3 and T_4 were tested in reaction

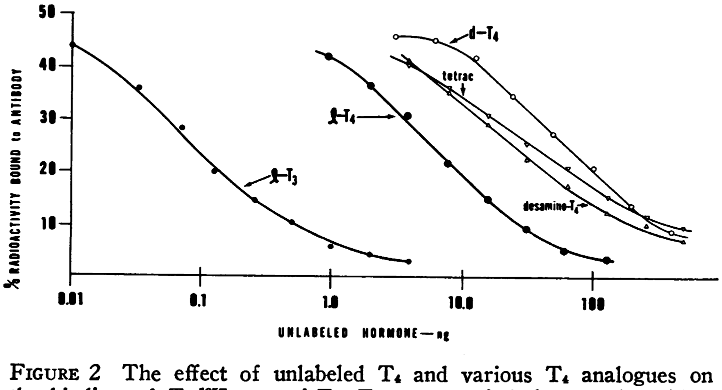

FIGURE 2 The effect of unlabeled T_4 and various T_4 analogues on the binding of T_3-^{125}I to anti-T_3. Tetrac, tetraiodothyroacetic acid.

Table II

Thyronine Derivative Cross-Reactivity in T_3 Immunoassay

Compound	Cross-reactivity
	%
L-triiodothyronine	100.0
D-triiodothyronine*	81.8
D-triiodothyronine‡	89.5
Triiodothyroacetic acid*	31.5
Triiodothyropropionic acid*	52.7
L-thyroxine*	0.14
L-thyroxine‡	0.45
L-thyroxine*	0.52
L-thyroxine‡	0.62
L-thyroxine*	1.34
D-thyroxine‡	0.10
D-thyroxine*	0.16
Tetraiodothyroacetic acid‡	0.16
Tetraiodothyroacetic acid*	0.26
Desaminothyroxine*	0.36
Monoiodotyrosine*	<0.0001
Diiodotyrosine*	<0.0001
L-3,5-diiodothyronine‡	0.094
DL-thyronine‡	<0.0012

* Obtained from Sigma Chemical Co., St. Louis, Mo.
‡ Baxter Laboratories, Inc., Morton Grove, Ill.

mixtures employing other buffers ranging in pH from 5.2 to 9.2 with similar results.

Results of T_3 immunoassay and T_4 analysis of the fractions obtained by Sephadex chromatography of several T_4 preparations are shown in Table III. Similar analyses of the T_4 preparations subjected to chromatography are also shown. In experiment 1, the T_4 fraction from the column contained the equivalent of 0.17% T_3 (1.7 ng T_3/1 μg T_4). The T_4 fraction in the second experiment contained 0.10% T_3 (1 ng T_3/1 μg T_4). In experiment 3, the T_4 fraction after the initial chromatography contained 0.03% T_3. Repurification of this T_4 fraction further lowered the T_3 content to 0.01% (0.1 ng T_3/1 μg T_4). These results indicate that the reactivity of T_4 with anti-T_3 serum can be largely attributed to T_3 contamination. The very slight T_3 immunoreactivity of the twice purified T_4 could be due to the presence of small amounts of T_3 generated from T_4 during the fractionation procedure or might represent very slight intrinsic reactivity of T_4 with anti-T_3 serum.

The reactivity of T_3-BSA and other conjugates in the immunoassay was also examined. Both T_3-BSA and T_4-BSA conjugates inhibited the binding of T_3-^{125}I to antibody, though the activity of the T_4-BSA conjugate was minimal (Table I). The dose response pattern of the conjugate inhibition was similar to that of T_3 alone. The carbodiimide treated albumin did not inhibit T_3-^{125}I binding to antibody in doses up to 0.5 mg. Both human and bovine albumin preparations also slightly inhibited the reaction when tested in doses of 5–30 mg.

Assay of T_3 in serum. Initial attempts to determine the T_3 concentration in serum in incubation mixtures not containing diphenylhydantoin yielded values of 300 ng/100 ml or higher in most instances and these values bore little relation to the quantity expected considering the clinical status of the patient. Furthermore, with many samples little dose response effect was evident when varying quantities of serum were tested. Since in all instances the amount of T_3-^{125}I bound to antibody when serum was added was low, these results suggested that T_3-^{125}I was reacting with the thyroxine binding globulin (TBG) in serum and thus was not available for binding to anti-T_3 antibody. The addition of diphenylhydantoin, a known competitive inhibitor of the binding of thyroxine to TBG (20–23), was found to prevent this. Addition of diphenylhydantoin in albumin solution in doses up to 200 μg/tube did not inhibit the binding of T_3-^{125}I to antibody or alter the sensitivity of the system to unlabeled T_3. When added in increasing quantities to different serum samples, the proportion of T_3-^{125}I bound to antibody gradually increased until a plateau was reached (Fig. 3). These findings indicate that these quantities of diphenylhydantoin prevent binding of T_3-^{125}I to TBG and allow reaction of the endogenous serum T_3 with anti-T_3 serum. In other experiments the effect of diphenylhydantoin in doses up to 200 μg/tube was studied. The results were similar to those observed when 25 or 50 μg/tube was used. On the basis of these results a dose of 50

Table III

T_3 and T_4 in Fractions after Gel Filtration of T_4

	T_3	T_4	T_3/T_4
	ng	*μg*	*ng/μg*
Experiment 1			
T_4	80.0	15.00	6.0
"T_3" fraction	60.8	0.19	320.0
"T_4" fraction	16.3	9.85	1.7
Experiment 2			
T_4	28.8	16.7	1.7
"T_3" fraction	20.5	0.64	33.8
"T_4" fraction	12.6	12.60	1.0
Experiment 3			
First column			
T_4	57.4	20.0	2.9
"T_3" fraction	25.2	0.15	164.0
"T_4" fraction	3.0	9.0	0.3
Second column			
"T_3" fraction	0.7	0.09	0.8
"T_4" fraction	0.8	7.6	0.1

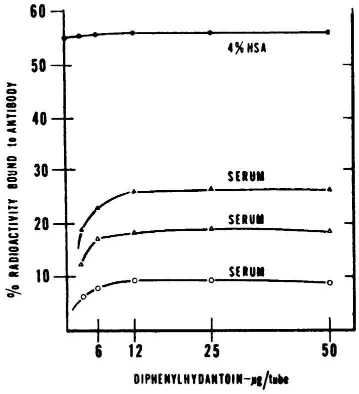

FIGURE 3 The effect of addition of increasing quantities of diphenylhydantoin to several different serum samples and 4% HSA on the binding of T_3-^{125}I to anti-T_3 serum.

µg/tube was chosen for regular use. Under these conditions, the pattern of inhibition of binding of T_3-^{125}I to antibody produced by varying doses of serum was similar to that produced by unlabeled T_3 (Fig. 4). Hypothyroid serum treated with resin sponges as outlined above did not inhibit the reaction in the presence of diphenylhydantoin whereas significant inhibition of T_3-^{125}I binding to anti-T_3 occurred in the absence of diphenylhydantoin.

Effect of added T_4 and T_3 on serum T_3 concentrations. Although the studies with the T_3 analogues showed that the anti-T_3 serum was highly specific and suggested that most of the T_4 cross-reactivity observed was due to T_3 contamination, the effect of addition of T_4 to serum on the immunoassayable T_3 concentration was studied. The T_4 preparation available which had the least cross-reactivity in the T_3 immunoassay was used (Table II). The results are shown in Table IV. For these studies, multiple serum samples of widely varying endogenous T_3 and T_4 concentrations were used. When T_4 in a concentration of 25 µg/100 ml was added, the mean serum T_3 concentration was 102 ±4.2% (SD) of the unmodified serum result. At added T_4 concentrations of 50 and 100 µg/100 ml, the mean serum T_3 values were 107.9 ±11.3% and 121.3 ±16.9% of the unmodified serum results, respectively. Thus, it is clear that T_4 in serum in any quantity likely to be encountered clinically would not artifactually elevate the serum T_3 level as measured in this system.

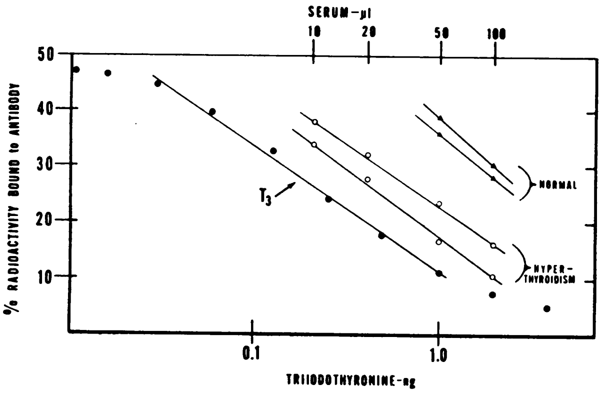

FIGURE 4 Standard assay curve and pattern of inhibition of T_3-^{125}I binding to antibody produced by different doses of several serum samples.

TABLE IV
Effect of Added T_4 on Serum T_3 Concentrations

T_4 added	No.	Effect of T_4
µg/100 ml		*% unmodified serum result* ±SD
25.0	11	102.0 ±4.2
50.0	21	107.9 ±11.3
100.0	12	121.3 ±16.9

In Table V are shown the results of serum assays after the addition of unlabeled T_3. Serum from normal, hypothyroid, hyperthyroid, and estrogen-treated subjects was enriched with T_3 in concentrations ranging from 31.2 to 500 ng/100 ml (usually in quantities ranging from 0.015 to 0.250 ng/50 µl). In all instances the mean recovery was greater than 90%. In one experiment, T_3 was reacted with serum for 24 hr at 5°C before assay. Recovery values similar to those described above were found.

Reproducibility. The quantity of T_3 found to produce a 50% reduction in the quantity of radioactivity bound to anti-T_3 antibody in 21 assays was 0.276 ±0.037 ng (SD). Both intra- and interassay variability was examined repeatedly with samples of widely varying T_3 concentration. The mean coefficient of variation (SD/mean × 100) for 15 samples, each of which was assayed in two doses twice in the same assay, was 6.0 ±4.9% (SD). The mean coefficient of variation for 50 samples assayed in two doses from two to five times in different assays was 7.9 ±4.8% (SD). No differences in reproducibility were found in assays of serum containing low, normal, or high T_3 concentrations.

TABLE V
Recovery of T_3 Added to Serum

T_3 added	No.	Recovery
ng/100 ml		% ±SD
31.2	6	95.7 ±4.1
62.5	12	92.5 ±6.9
125.0	16	95.8 ±12.2
250.0	15	96.8 ±7.4
500.0	14	102.5 ±9.2

TABLE VI
Serum T_3 Concentrations

Clinical status		T_3*	T_4*
		ng/100 ml	*μg/100 ml*
Normal	79	145 ±25	8.5 ±1.6
Hyperthyroidism	36	429 ±146	20.7 ±4.5
Hypothyroidism	45	99 ±24	2.0 ±1.2
Pregnancy or estrogen treatment	20	186 ±32	11.2 ±2.1

* Mean ±SD.

Results of serum assays. The results of measurements of serum T_3 concentrations are shown in Table VI. Since the largest quantity of serum used was 100 μl and usually 0.06 ng unlabeled T_3 produced significant inhibition of T_3-^{125}I binding to antibody, the minimum detectable T_3 concentration was 60 ng/100 ml. In normal adult subjects serum T_3 concentrations ranged from 102 to 215 ng/100 ml. The mean (±SD) in the normal sera was 145 ±25. No differences were found in serum T_3 results in males and females. In 10 normal subjects, T_3 concentrations in serum collected on different days differed by an average of 20 ng/100 ml. In hyperthyroid patients serum T_3 concentrations ranged from 205 to 793 ng/100 ml, all values but one being higher than the highest of the normal serum T_3 results. Serum T_3 concentrations ranged from < 60 to 136 ng/100 ml in the patients with hypothyroidism; in three of these T_3 levels < 60 ng/100 ml were found. In the three patients with secondary hypothyroidism, serum T_3 levels were 68, 70, and 115 ng/100 ml. The values were within two standard deviations of the normal mean in 52% of the hypothyroid patients. In pregnant women and patients receiving estrogen-containing medications, the mean serum T_3 concentration was 186 ng/100 ml.

Effects of thyroid hormones and TSH on serum T_3 concentrations. Serum T_3 concentrations in hypothyroid patients receiving various forms of replacement therapy are shown in Table VII. The T_3-treated subjects had received therapy in the dosage shown for at least 1 wk; in all others the duration of therapy at the appropriate dose was 1 month before serum sampling. In the patients receiving 25 μg T_3 daily, serum T_3 levels ranged from 90 to 370 ng/100 ml. All of these patients had elevated TSH levels. Those receiving larger doses of T_3 had, with one exception, T_3 levels greater than 200 ng/100 ml and most had TSH levels within the normal range. The range of values found in all of the T_3-treated patients varied widely; this may be due to the fact that blood sampling was not done at any fixed interval after the last previous dose of medication (see below). In the patients receiving 200 μg T_4 daily, serum T_3 concentrations were usually within the normal range. Patients receiving 300 μg T_4 daily had T_3 concentrations ranging from 159 to 260 ng/100 ml. The mean value in this group, 206 ng/100 ml, differed significantly from the normal mean ($P < 0.01$). All of these patients were considered clinically euthyroid. In only one of the T_4-treated patients was a pretreatment serum T_3 value available. This clinically hypothyroid woman initially had a serum T_3 level of 122 ng/100 ml, a serum T_4 of 1.0 μg/100 ml and a serum TSH of 255 μU/ml. The serum T_3 concentration was 225 ng/100 ml after 1 month and 159 ng/100 ml after 2 months of therapy with T_4 300 μg/day in this patient.

Serial T_3 measurements were made after a single oral dose of 100 μg T_3 in five normal subjects. The results of these studies are shown in Fig. 5. In each subject, the serum T_3 concentration increased promptly, reached a peak 2–4 hr after T_3 administration, and then declined. The serum T_3 was slightly higher than control in each subject 24 hr after the T_3 administration. T_3 in a dose of 200 μg was given to two subjects after a similar protocol. The peak T_3 values observed were 800 ng/

TABLE VII
Serum T_3 and T_4 Concentrations in Thyroid Hormone Treated Patients

	No.	T_3*	T_4*
		ng/100 ml	*μg/100 ml*
Normal	79	145 ±25	8.5 ±1.6
Thyroid treatment			
25 μg T_3/day	10	268 ±95	3.3 ±1.2
50 μg T_3/day	8	371 ±169	2.2 ±0.7
75 μg T_3/day	3	488 ±208	3.1 ±1.5
200 μg T_4/day	16	169 ±32	12.8 ±3.1
300 μg T_4/day	13	206 ±30	15.7 ±3.6

* Mean ±SD.

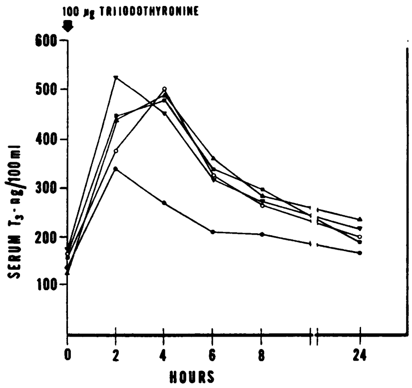

FIGURE 5 Serum T_3 concentrations in five normal subjects after the oral administration of 100 μg T_3.

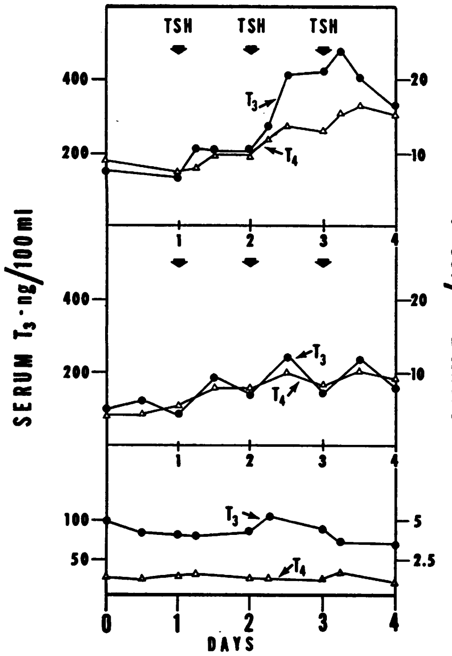

FIGURE 6 Serum T_3 and T_4 concentrations in three patients after the repeated intramuscular administration of 10 IU bovine TSH. The patients shown in the upper two panels had "hot" nodules of the thyroid (without hyperthyroidism); the third patient had primary hypothyroidism.

100 ml 8 hr after T_3 in one subject and 1,100 ng/100 ml at 6 hr in the other.

Results of serum T_3 and T_4 assays in three patients before and after the intramuscular administration of bovine TSH are shown in Fig. 6. Two of the patients had single functioning thyroid nodules and were euthyroid; the TSH was given to determine the functional capacity of the adjacent thyroid tissue. Post-TSH thyroid scans showed increased ^{131}I uptake in the extranodular thyroid tissue in both patients. In each of these patients, serum T_3 and T_4 concentrations increased subsequent to TSH administration. It is apparent that the serum T_3 concentrations increased to higher levels and returned toward control more quickly than did the T_4 concentrations. In the third subject, who had primary hypothyroidism and an elevated TSH level, the serum T_3 concentration did not change.

Studies on the stability and extraction of T_3 in serum. Storage of serum at 5°C for 7 days did not lead to significant reduction in the measured T_3 concentration. The T_3 concentration of 10 serum samples so treated averaged 91.7% (range 83.1–112.8%) of that found in paired samples stored at − 5°C.

Analysis of the fractions prepared by column chromatography on Dowex AG 50 W-X2 (Dow Chemical Co., Midland, Mich.) revealed the following results. In the nine instances in which serum was fractionated by this technique, the initial fraction containing unabsorbed protein and ammonium acetate contained no detectable T_3 (< 60 ng/100 ml). The NH_4OH eluates of these sera contained 30.2–85.3% (mean 61.2%) of the T_3 concentration found in simultaneously assayed unextracted serum. Recovery of added T_3 (100 ng/100 ml) averaged 61.0% in two experiments.

The ethanol extraction studies yielded similar results. The reconstituted ethanol precipitates did not inhibit the binding of T_3-^{125}I to anti-T_3 serum. The ethanol supernatants contained from 36.0 to 79.5% (mean 65.5%, n = 30) of the T_3 concentration found in the unextracted serum samples. Recovery of T_3 (50 and 100 ng/100 ml) added to serum in 13 instances averaged 75.6 ±11.2% (SD). These results corroborate those described previously indicating substantial loss (26.0%) of T_3-^{125}I added to serum before extraction by binding to particulate material in the ethanol supernatants and indicate that endogenous T_3 is lost in a similar manner.

It is clear that extraction of T_3 from serum by these two methods effectively removed all of the serum material capable of inhibiting the binding of T_3-^{125}I to antibody. Furthermore, the proportion of added T_3 recovered was generally similar to the proportion of T_3 found in the serum extracts compared to that found in extracted serum samples.

DISCUSSION

The studies described herein clearly demonstrate that highly specific anti-T_3 serum of suitable affinity for the detection of subnanogram quantities of unlabeled T_3 can be prepared readily. It seems clear from the data presented that the specificity of the antibody is directed primarily to the phenolic ring constituents of the thyronine molecule. Of the compounds studied, all having substantial reactivity with the antibody were 3′,3,5-triiodinated analogues. This finding was not unsuspected since it is likely that the linkage of T_3 to BSA was accomplished by formation of peptide bonds between the alanine side chain of T_3 and BSA (24). Stereospecificity was minimal since D-T_3 reacted with the anti-T_3 serum almost as well as did L-T_3. The nature of the slight reactivity of the tetraiodinated derivatives with the anti-T_3 antibody bears special comment. The gel filtration data presented suggest that, at least in the case of T_4, most of the reactivity of the T_4 preparations studied was due to contamination with T_3 and even the slight reactivity (0.01%) of the twice purified T_4 could still reflect T_3 contamination. It seems reasonable to conclude that much of the reactivity of other tetraiododerivatives tested was due to contamination with their triiodo-analogues as well.

The preparation of anti-T_3 antibody of high specificity has been reported by other workers (12–15). Using a similar coupling procedure, Gharib, Mayberry, and Ryan have prepared an anti-T_3 serum with characteristics much like those of the antisera described in this study (13). Antisera of high, though varying, specificity have been obtained also after immunization with T_3-polylysine conjugates (12) and thyroglobulin (15).

The data presented strongly suggest that this radioimmunoassay reliably measures T_3 in serum. This was achieved only with the use of diphenylhydantoin, which serves both to inhibit binding of T_3-^{125}I to serum TBG and to displace endogenous T_3 from TBG. If the latter was not the case both low serum T_3 concentrations and poor recovery of added T_3 would be expected. Neither was found. While there is controversy concerning the degree of in vivo binding of T_3 to TBG (25–28), binding of T_3 to TBG in vitro is readily demonstrable though the relative affinity of T_3 for TBG varies from $\frac{1}{3}$ to $\frac{1}{30}$ of that of T_4 in different systems (20, 23, 29). Diphenylhydantoin is a weaker competitive inhibitor of the T_4-TBG reaction than is T_3, but it would be expected to inhibit the binding of T_3 to TBG and displace T_3 from TBG more effectively than it would alter T_4-TBG interaction. The studies presented suggest that the binding of both T_3-^{125}I and unlabeled T_3 to TBG was completely inhibited by diphenylhydantoin. Whether significant quantities of endogenous T_4 were displaced from TBG by the quantity of diphenylhydantoin used in this system is not known. However, since added T_4 did not alter measured serum T_3 values, they should not be affected by endogenous T_4 displaced from TBG even if such displacement did occur. The fact that diphenylhydantoin in buffer solution did not inhibit the T_3-^{125}I–anti-T_3 reaction, whereas it does alter T_3 (and T_4) binding to TBG, provides further evidence that the binding sites for these two reactions differ substantially.

The possibility that the presence of other T_3 binding proteins in serum might influence these results also bears consideration. Such a phenomenon might influence T_3 assay results in several ways. In the first place, T_3-^{125}I could become bound and thus unavailable for binding to anti-T_3 antibody. This would result in overestimation of serum T_3 levels. Endogenously bound T_3 or T_3 displaced from TBG by diphenylhydantoin could bind to this protein(s) and thus the serum T_3 level would be underestimated. T_3 does not bind to thyroxine binding prealbumin (23, 30, 31). T_3 binding to albumin is weak and easily interrupted by a variety of materials (23, 31–34). The high affinity of the antibody for T_3 would appear to render this possibility unlikely.

The validity of the assay is also supported by the finding that the recovery of T_3 added in a wide range of concentrations and to sera of varying endogenous T_3 and T_4 concentrations was excellent. Furthermore, all of the immunoreactive material present in serum could be removed by procedures known to extract much or all of the endogenous T_3. Finally, when T_3 was added to serum which was subsequently extracted by two methods, the recovery of T_3 added in vitro approximated the proportion of endogenous T_3 found in serum extracts compared to that in unextracted serum. All of these observations strongly suggest that all of the endogenous T_3 in serum is made available to the antibody in the course of the reaction.

The serum T_3 concentration results found with this technique differ somewhat from those previously reported. In general, procedures employing column and paper chromatography coupled with displacement analysis have yielded substantially higher values. For example, Sterling and coworkers found a mean serum T_3 concentration of 220 ng/100 ml (8), and a mean normal T_3 value of 243 ng/100 ml was recently reported by Wahner and Gorman using a similar technique (11). Larsen, using a modification of this procedure, found a mean normal T_3 concentration of 180 ng/100 ml (9). Mean normal T_3 concentrations of 300 ng/100 ml or more were reported by other investigators using a variety of techniques (6, 7, 10). Little data is available as yet concerning radioimmunoassay measurements of serum T_3, but preliminary reports of values both greater and similar to those described here have recently appeared (14, 15, 35). Inadequate separation of T_3 and T_4 (9, 36), in vitro deiodination of T_4 (9, 36) or formation of T_4 esters during extraction (37) are likely explanations for the finding of higher T_3 values in the assays employing various types of chromatography. Possible sources of error in the radioimmunoassay measurements were discussed in preceding paragraphs.

As noted by others, elevated serum T_3 concentrations were found in almost all hyperthyroid patients studied. Only one such patient in this group had a serum T_3 concentration within the normal range. In hypothyroid patients, on the other hand, a substantial number had serum T_3 concentrations within the normal range although the mean values for the two groups differed significantly ($P < 0.001$). All of these patients had some symptoms and signs compatible with the diagnosis of hypothyroidism and low serum T_4 and low or elevated TSH concentrations. Similar findings have been reported by Wahner and Gorman (11). Thus a near normal T_3 concentration alone is not sufficient to sustain the euthyroid state or prevent increased TSH secretion. The normality of the T_3 concentrations in some of these patients could in part be explained by the ability of high TSH levels to preferentially stimulate thyroidal release of T_3 (38). Greater increments in T_3 than in T_4 concentrations were also observed after exogenous TSH administration in two patients in this study. In addition, when there is T_4 deficiency undoubtedly relatively more T_3 is bound to TBG than is normally the case (34). This could also explain why patients treated with sufficient T_3 to restore TSH levels to normal almost always had elevated serum T_3 concentrations.

Rapid changes in serum T_3 concentrations were observed when serial measurements were made after single oral doses of 100 μg T_3. The similarity of the peak T_3 levels suggest that fractional absorption varied little among the subjects. The wide variation in serum T_3 levels found in the T_3-treated patients (Table VII) can thus be explained by the fact that in those subjects blood sampling was not carried out at a fixed time after the previous dose of T_3. Rapid, and virtually complete, absorption and disappearance of isotopically labeled T_3 has been reported by Hays (39). In the studies described herein, the mean peak increment in serum T_3 levels was 299 ng/100 ml. Assuming the volume of distribution of T_3 to be 40 liters (40, 41), this increment would reflect the absorption of 119 μg T_3, a value in reasonable agreement with the 100 μg that was in fact administered. These results thus validate, by quite different methods, the previously published estimates of the degree of absorption and volume of distribution of T_3.

In hypothyroid patients receiving replacement therapy with thyroxine, normal or moderately elevated T_3 concentrations were found. The latter occurred largely in the patients receiving 300 μg T_4 daily and the elevations observed were modest. Considerably higher T_3 levels in patients receiving this dose of T_4 were reported by Braverman, Ingbar, and Sterling (2), but the T_3 assay employed (8) yields higher values probably because of in vitro T_4 deiodination and inadequate separation of T_3 and T_4 (9, 36). While values before and during such treatment have been reported for only one patient in the present study, there seems little doubt that these results confirm those of Braverman and coworkers indicating there is peripheral conversion of exogenously administered T_4 to T_3 in human subjects.

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REFERENCES

1. Gross, J., and R. Pitt-Rivers. 1952. The identification of 3,5,3'-triiodothyronine in human plasma. *Lancet.* **1**: 439.
2. Braverman, L. E., S. H. Ingbar, and K. Sterling. 1970. Conversion of thyroxine (T_4) to triiodothyronine (T_3) in athyreotic human subjects. *J. Clin. Invest.* **49**: 855.
3. Sterling, K., M. A. Brenner, and E. S. Newman. 1970. Conversion of thyroxine to triiodothyronine in normal human subjects. *Science (Washington).* **169**: 1099.
4. Pind, K. 1957. Paper chromatographic determination of thyroid hormone (3,5,3'-triiodothyronine) in serum without radio-iodine. *Acta Endocrinol.* **26**: 263.
5. Maclagen, N. F., C. H. Bowden, and J. H. Wilkinson. 1957. The metabolism of thyroid hormones. II. Detection of thyroxine and triiodothyronine in human plasma. *Biochem. J.* **67**: 5.
6. Nauman, J. A., A. Nauman, and S. C. Werner. 1967. Total and free triiodothyronine in human serum. *J. Clin. Invest.* **46**: 1346.
7. Hollander, C. S. 1968. On the nature of the circulating thyroid hormone: clinical studies of triiodothyronine and thyroxine in serum using gas chromatographic methods. *Trans. Ass. Amer. Physicians Philadelphia.* **81**: 76.
8. Sterling, K., D. Bellabarba, E. S. Newman, and M. A. Brenner. 1969. Determination of triiodothyronine concentration in human serum. *J. Clin. Invest.* **48**: 1150.
9. Larsen, P. R. 1971. Technical aspects of the estimation of triiodothyronine in human serum: evidence of conversion of thyroxine to triiodothyronine during assay. *Metab. (Clin. Exp.)* **20**: 609.
10. Hagen, G. A., L. I. Diuguid, B. Kliman, and J. B. Stanbury. 1970. Double isotope derivative assay of serum triiodothyronine (T_3). *Clin. Res.* **18**: 602. (Abstr.)
11. Wahner, H., and C. A. Gorman. 1971. Interpretation of serum triiodothyronine levels measured by the Sterling technique. *N. Engl. J. Med.* **284**: 225.
12. Brown, B. L., R. P. Elkins, S. M. Ellis, and W. S. Reith. 1970. Specific antibodies to triiodothyronine hormone. *Nature (London).* **226**: 359.
13. Gharib, H., W. E. Mayberry, and R. J. Ryan. 1970. Radioimmunoassay for triiodothyronine: a preliminary report. *J. Clin. Endocrinol. Metab.* **31**: 709.
14. Gharib, H., W. E. Mayberry, and R. J. Ryan. 1970. Determination of triiodothyronine concentration in human serum by radioimmunoassay. *J. Lab. Clin. Med.* **76**: 863. (Abstr.)
15. Chopra, I. J., J. C. Nelson, D. H. Solomon, and G. H. Beall. 1971. Production of antibodies specifically binding triiodothyronine and thyroxine. *J. Clin. Endocrinol. Metab.* **32**: 299.
16. Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
17. Mougey, E. H., and J. W. Mason. 1963. Separation of some iodoamino acids and iodide by gel filtration. *Anal. Biochem.* **6**: 223.
18. Murphy, B. E. P. 1965. The determination of thyroxine by competitive protein-binding analysis employing an anion-exchange resin and radiothyroxine. *J. Lab. Clin. Med.* **66**: 161.
19. Odell, W. D., J. F. Wilber, and R. D. Utiger. 1967. Studies of thyrotropin physiology by means of radioimmunoassay. *Recent Progr. Hormone Res.* **23**: 47.
20. Murphy, B. E. P. 1969. Protein binding and the assay of non-antigenic hormones. *Recent Progr. Hormone Res.* **25**: 563.
21. Wolff, J., M. E. Standaert, and J. E. Rall. 1961. Thyroxine displacement from serum proteins and depression of serum protein-bound iodine by certain drugs. *J. Clin. Invest.* **40**: 1373.
22. Oppenheimer, J. H., and R. R. Tavernetti. 1962. Displacement of thyroxine from human thyroxine-binding

globulin by analogues of hydantoin. *J. Clin. Invest.* **41**: 2213.
23. Robbins, J., and J. E. Rall. 1967. The iodine-containing hormones. *In* Hormones in Blood. C. H. Grey and A. L. Bacharach, editors. Academic Press, Inc., New York. 2nd edition. **1**: 383.
24. Likhite, V., and A. Sehon. 1967. Protein-protein conjugation. *In* Methods in Immunology of Immunochemistry. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. **1**: 150.
25. Zaninovich, A. A., H. Farach, C. Ezrin, and R. Volpe. 1966. Lack of significant binding of L-triiodothyronine by thyroxine binding globulin in vivo demonstrated by acute disappearance of ^{131}I-labeled triiodothyronine. *J. Clin. Invest.* **45**: 1290.
26. Zaninovich, A., R. Volpe, and C. Ezrin. 1969. Effects of variations of thyroxine-binding globulin capacity on the disappearance of triiodothyronine from the plasma. *J. Clin. Endocrinol. Metab.* **29**: 1601.
27. Musa, B. U., R. S. Kumar, and J. T. Dowling. 1969. Role of thyroxine-binding globulin in the early distribution of thyroxine and triiodothyronine. *J. Clin. Endocrinol. Metab.* **29**: 667.
28. Woeber, K. A., E. Hecker, and S. H. Ingbar. 1970. The effects of an acute load of thyroxine on the transport and peripheral metabolism of triiodothyronine in man. *J. Clin. Invest.* **49**: 650.
29. Hao, Y., and M. Tabachnick. 1971. Thyroxine-protein interactions. VII. Effect of thyroxine analogs on the binding of ^{125}I-thyroxine to highly purified human thyroxine-binding globulin. *Endocrinology.* **88**: 81.
30. Braverman, L., and S. H. Ingbar. 1965. Binding of 3,5, 3′-triiodothyronine in human serum during agar gel electrophoresis at pH 7.4. *Endocrinology.* **76**: 547.
31. Britton, A., B. R. Webster, C. Ezrin, and R. Volpe. 1965. The association of I^{131}-labelled thyroxine and triiodothyronine with serum proteins after starch gel electrophoresis. *Can. J. Biochem.* **43**: 1477.
32. Tabachnick, M. 1964. Thyroid-protein interactions. I. Binding of thyroxine to human serum albumin and modified albumins. *J. Biol. Chem.* **239**: 1242.
33. Sterling, K. 1964. Molecular structure of thyroxine in relation to its binding by human serum albumin. *J. Clin. Invest.* **43**: 1721.
34. Mitchell, M. L., A. H. Bradford, and S. Collins. 1964. Differences in the interaction of triiodothyronine-^{131}I with serum proteins in vitro. *J. Clin. Endocrinol. Metab.* **24**: 867.
35. Mitsuma, T., N. Nihei, M. C. Gershengorn, and C. S. Hollander. 1971. Serum triiodothyronine in man: measurements by radioimmunoassay and gas chromatography. *Clin. Res.* **19**: 377. (Abstr.)
36. Fisher, D. A., and J. H. Dussault. 1971. Contribution of methodological artifacts to the measurement of T_3 concentration in serum. *J. Clin. Endocrinol. Metab.* **32**: 675.
37. Bellabarba, D., and K. Sterling. 1969. Formation of esters of thyroxine and triiodothyronine during alcoholic extraction. *J. Clin. Endocrinol. Metab.* **29**: 1510.
38. Greer, M. A., Y. Grimm, and H. Studer. 1968. Qualitative changes in the secretion of thyroid hormones induced by iodine deficiency. *Endocrinology.* **83**: 1193.
39. Hays, M. T. 1970. Absorption of triiodothyronine in man. *J. Clin. Endocrinol. Metab.* **30**: 675.
40. Surks, M. I., and J. H. Oppenheimer. 1969. Formation of iodoprotein during the peripheral metabolism of 3,5, 3′-triiodo-L-thyronine in the euthyroid man and rat. *J. Clin. Invest.* **48**: 685.
41. Woeber, K. A., R. J. Sobel, S. H. Ingbar, and K. Sterling. 1970. The peripheral metabolism of triiodothyronine in normal subjects and patients with hyperthyroidism. *J. Clin. Invest.* **49**: 643.