Radioimmunoassay for Measurement of Triiodothyronine in Human Serum

INDER J. CHOPRA, DAVID H. SOLOMON, and GILDON N. BEALL

From the Department of Medicine, Harbor General Hospital, Torrance, California 90509, and the University of California Los Angeles School of Medicine, Los Angeles, California 90024

ABSTRACT A convenient, specific, precise, and reproducible radioimmunoassay system for measurement of triiodothyronine (T_3) in human serum has been developed. The procedure compares the ability of standards and unknowns to compete with radioactive T_3 for binding sites on a T_3-binding antiserum produced in rabbits by immunization with human thyroglobulin. The assay is set up in the presence of 250 ng thyroxine (T_4) in all tubes, to mobilize T_3 from its binding with the thyronine-binding globulin (TBG), and athyreotic sheep serum in standards to correct for the TBG in the unknowns. The method regularly detected 0.4 ng T_3, which would correspond to a T_3 concentration of 100 ng/100 ml when 400 μl of serum is analyzed. The mean recovery of unlabeled T_3 added to normal serum pools was 106%. Serial dilution of hyperthyroid sera containing high concentrations of T_3 with athyreotic sheep serum yielded expected values.

The serum T_3 concentration in 80% of 31 euthyroid normal subjects was less than 100 ng/100 ml (range $<$ 100–170 ng/100 ml); it was greater than 170 ng/100 ml in 89% of 27 sera of hyperthyroid patients with untreated Graves' disease (range $<$ 100–1300, mean 519 in 25 sera with detectable T_3). The concentration of serum T_3 fell, frequently to undetectable levels, during treatment of hyperthyroid patients with antithyroid drugs. The serum T_3 concentration in four hypothyroid patients was less than 100 ng/100 ml.

INTRODUCTION

Triiodothyronine (T_3),[1] because of its greater potency in comparison with thyroxine (T_4) (1, 2), may be expected to contribute significantly to the over-all metabolic effects of thyroid hormones. In fact, it has been suggested by some that T_4 may exert its effects at the tissue level predominantly via conversion to T_3 (3, 4). A recent report of the occurrence of hyperthyroidism with elevated serum T_3 and normal serum T_4 (5) further emphasizes the importance of this hormone and the need for accurate methods for its measurement on a large scale.

To date, there is no general agreement regarding serum concentration of this hormone. Thus, the mean values of serum T_3 in euthyroid individuals have ranged from 450 ng/100 ml (6) to 330 (7) and 220 (8) to 120 ng/100 ml (9). Most widely used among the methods available for measurement of T_3 in serum are those which involve, sequentially, extraction of thyronines, separation of T_3 from T_4, and measurement of T_3 by a competitive binding method employing thyroxine-binding globulin (TBG). Separation of T_3 from T_4 involves two possible complications: (*a*) inadequate separation with contamination of T_3 by some T_4 (10, 11), and (*b*) artifactual deiodination of T_4 with resultant formation of T_3 (11, 12); both of these phenomena would give falsely high estimates of T_3.

We have recently reported production of T_3 antibodies by immunization of rabbits with human thyroglobulin (Tg) (13); these antibodies have led to development of a radioimmunoassay (RIA) method, herein described, for measurement of T_3 in whole serum. Several experiments designed to test the validity of the method, and the results obtained by it in human sera are also presented. This method does not require prior extraction or separation of thyronines and involves use of a relatively specific T_3 binder, thereby obviating some disadvantages of earlier methods. The method has the additional advantage of allowing a large number of samples to be tested per week by a single technician, which might make wide clinical application feasible.

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[1] *Abbreviations used in this paper:* HSA, human serum albumin; PBS, phosphate-buffered saline; RIA, radioimmunoassay; T_3, triiodothyronine; T_4, thyroxine; TBG, thyronine-binding globulin; TBPA, thyroxine-binding prealbumin; TETRAC, tetraiodothyroacetic acid; Tg, thyroglobulin; TRIAC, triiodothyroacetic acid.

METHODS

Introductory comment. The basic approach to measurement of T_3 by RIA was similar to that described earlier (13). The following modifications in the method were made for adapting it to measurement of the hormone in serum. (*a*) Addition of an equal excess of cold T_4 to all standards and unknown sera for three purposes: (*i*) to displace T_3 bound to TBG and to make it available for reaction with T_3 antibody and thus measurable by RIA; (*ii*) to minimize binding of radioactive and nonradioactive T_3 to TBG; (*iii*) to equalize the amount of a cross-reacting ligand, i.e., T_4. The concentration of T_4 was adjusted to 250 ng/400 μl of test serum after taking into consideration the amount already present in serum as measured by the method of Murphy (14). This amount of T_4 corresponded to a T_4 concentration of 25 μg/100 ml in 1 ml of the reaction mixture containing 400 μl serum or to 62.5 μg/100 ml if the concentration of T_4 was referred to whole serum.

(*b*) Addition of a source of TBG to the standards in order to make them comparable with the unknown serum specimens. This is essential since TBG binds T_3 and, therefore, competes with T_3-binding antibody. Any labeled T_3 bound to TBG would not be precipitated by antiserum against rabbit gamma globulin ("second antibody") and thus would imitate the effect of additional unlabeled T_3. This modification was made by adding to the standards serum of a hypothyroid sheep obtained 21 days after total surgical thyroidectomy. Sheep serum was chosen as a source of TBG because it is most comparable with human serum among commonly available laboratory animal species (15).

Reagents. T_3-binding antiserum. The serum used was obtained from a rabbit (No. 15) immunized for 12 wk with normal human Tg, as previously described (13). 100 μl of a 1:40 dilution was added in a total reaction mixture of 1.0 ml, yielding a final dilution of 1:400. In this dilution it bound 75–80% of a tracer amount of radioactive T_3, when T_4 or TBG was not present. However, the binding of the tracer ranged between 25 and 34% when 400 μl of sheep serum and 250 ng of T_4 were present in 1 ml of reaction mixture.

Hypothyroid sheep serum. Total T_4 in the sheep serum was 4.7 μg/100 ml before surgical total thyroidectomy, and it had dropped to < 1.0 μg/100 ml at the time of bleeding 3 wk postoperatively. Serum T_3 at this time, tested on four different occasions by chemical method (16), was less than 25 ng/100 ml.[2] T_4-binding capacity of the TBG of the hypothyroid sheep serum was 32 μg/100 ml, measured by the method of Inada and Sterling (17).

Radioiodinated (^{125}I or ^{131}I) T_3 and T_4 (SA 50–70 μCi/μg) in 50% propylene glycol were obtained from Abbott Laboratories, North Chicago, Ill.

Reagent grade Na-L-T_4 and Na-L-T_3 were obtained from Mann Research Labs., New York. The thyronines were weighed, dissolved in 0.1 M NaOH, and diluted to the desired concentration in a solution made up of 100 parts 0.01 M NaOH, 50 parts propylene glycol, and 3 parts normal rabbit serum. The contribution of sodium and/or water in salts of T_3 or T_4 was considered in making dilutions. The same solutions were used up to 10 days without evident deterioration during storage in the dark at 4°C. The Na-L-T_4 was tested for contamination with T_3 using a T_3-binding antiserum, prepared by immunization of rabbits with a conjugate of T_3 with human serum albumin (HSA) by Gharib, Mayberry, and Ryan (18), and supplied to us by the courtesy of Dr. W. E. Mayberry. Hereafter this is referred to as anti-T_3-HSA. The immunoassay of reagent T_4 revealed no more than 0.15% cross-reaction with T_3. Thus, contamination of T_4 with T_3 must be even less since it is likely that there is some degree of true cross-reaction of T_4 with T_3-binding sites on anti-T_3-HSA.

Radioimmunoassay procedure. In 10×75 mm disposable glass culture tubes (catalogue No. 7810; Scientific Products, Evanston, Ill.), the various reagents were added in the following order to yield a final volume of 1 ml: (*a*) 0.1 M, EDTA pH 7.5: 100 μl. (*b*) 0.15 M sodium chloride, 0.01 M sodium phosphate buffer, pH 7.5, containing 0.1% sodium azide and 2% normal rabbit serum, phosphate-buffered saline (PBS): volume to adjust to 1 ml. (*c*) Nonradioactive T_4, 250 ng (50 μl of a solution containing 5 μg/ml) in the standards, and an amount required for a final T_4 content of 250 ng in the unknowns (variable volumes of solutions containing 5 μg/ml and/or 1 μg/ml). (*d*) Nonradioactive T_3 for standard curve. Three dilutions of T_3, i.e. 0.001 μg/ml, 0.01 μg/ml, and 0.1 μg/ml, were employed to place from 0.05 ng to 20 ng T_3 in tubes for a 10–14 point standard curve. (*e*) 400 μl sheep serum was added to the tubes for the standard curve, and 400 μl of the unknowns was added to appropriate tubes. Standard curve and unknowns were assayed at least in duplicate. The temperature of incubation in this and all subsequent steps was 4°C. (*f*) 100 μl of 1:40 dilution of T_3-binding antiserum. (*g*) After incubation overnight, 6000–7500 cpm of T_3-^{125}I ($\sim$0.15–0.25 ng T_3) in 100 μl of PBS was pipetted into all tubes, and incubation was continued for an additional period of 24 hr. Selection of 24 hr as the time of this incubation was based on pilot experiments, which indicated that maximal binding of radioactive T_3 to antibody in this system had occurred by this time and, in fact, by 16 hr. (*h*) 75–100 μl of a previously titered goat anti-rabbit γ-globulin was added, and the tubes were reincubated for 20–24 hr. They were then centrifuged at 2000 rpm for 30 min, the supernatant was aspirated, and radioactivity in the precipitates was determined by 10-min counts in a Nuclear-Chicago Autogamma counter. Each assay additionally included two tubes without T_3-binding rabbit antiserum; the counts precipitated in these tubes, which ranged from 2 to 3% of total counts added, were taken to be nonspecifically bound or trapped in the final precipitate and were subtracted from the counts in all tubes. In each assay run there were also at least two tubes which contained all reagents outlined above except nonradioactive T_3. The counts precipitated in these tubes were arbitrarily assigned a value of 100%, and the counts in the other standards and unknowns were expressed as a per cent of the counts in the zero-T_3 tubes. A standard curve was plotted as shown in Fig. 1. The T_3 content in 400 μl of unknown serum was determined from the standard curve.

Specificity of T_3 measurements by RIA was assessed by studying the displacement of T_3-^{125}I from T_3-binding antibody by two or more doses of a variety of thyroid analogues. In the case of compounds which appeared to cross-react to any significant degree, a full dose-response curve was then studied.

D-T_3, D-T_4, L-thyronine, 3,5-L-diiodothyronine, tetraiodothyroacetic acid (TETRAC), triiodothyroacetic acid (TRIAC), tetraiodothyropropionic acid, diiodothyropropionic acid, monoiodotyrosine, and diiodotyrosine were purchased from Sigma Chemical Co., St. Louis, Mo. 3,5,3′-Triiodothyropropionic acid and 3-monoiodothyronine were provided by

[2] Measurements made through courtesy of Doctors D. A. Fisher and J. H. Dussault.

courtesy of Warner-Lambert Research Institute, Morris Plains, N. J., research affiliate of Warner-Chilcott Laboratories.

Sources of sera. 31 euthyroid sera were tested. 20 were from normal, healthy laboratory workers, and 11 were from patients with nonthyroid diseases.

Sera were obtained from 24 untreated hyperthyroid patients with Graves' disease and 4 patients with idiopathic hypothyroidism. The diagnosis was verified by clinical examination, serum total T_4, and thyroid uptake of radioiodine. In addition, sera were obtained from sequential blood samples from a patient who had ingested 90 tablets of thyroid USP (3 grains each) in a suicidal attempt.

Sources of serum with elevated serum TBG concentration were three patients with carcinoma of the prostate receiving stilbesterol, 10–15 mg/day, one *postpartum* woman receiving stilbesterol, 5 mg/day, and three normal women receiving contraceptive pills.

Assessment of validity of method. The validity of the entire method and particularly the suitability of sheep serum as a source of TBG in the standard tubes to match the human TBG in unknown sera was assessed by testing (*a*) the recovery of varying amounts of nonradioactive T_3 added to 400 μl of various pools of human serum, and (*b*) the T_3 concentration in several dilutions of T_3-rich sera from hyperthyroid patients using sheep serum as the diluent. Other attempts to validate the results of T_3 concentration in human sera as measured by the proposed RIA included the following: (*a*) Measurement of T_3 in thyronine concentrates of sera. Thyronines were extracted from 8 to 10 ml sera by passing through a column of anion exchange resin (Bio-Rad AG X_2) chloride form, 200–400 mesh as described by Dussault, Lam, and Fisher (16). Approximately 25,000 cpm of T_3-^{131}I was added for later recovery calculations. All of the eluate with 50% acetic acid was collected rather than discarding the first 4 ml (which contains predominantly T_4) as described by Dussault et al. (16). The eluates were dried in a water bath at 40°C under a stream of nitrogen. At this point the recovery of T_3-^{131}I averaged 69% (range, 61.5–78%). The dried extract was reconstituted in 0.9 ml of methanol–2 N NH_4OH (99:1). 400 μl of the extract was transferred in duplicate to 10 × 75 mm assay tubes, and counted with a gamma counter to determine the proportion of starting sample represented by this extract and the amount of T_3 represented by radioactive T_3. Assuming T_4 was extracted with the same efficiency as T_3 and measuring the T_4 concentration in the starting serum

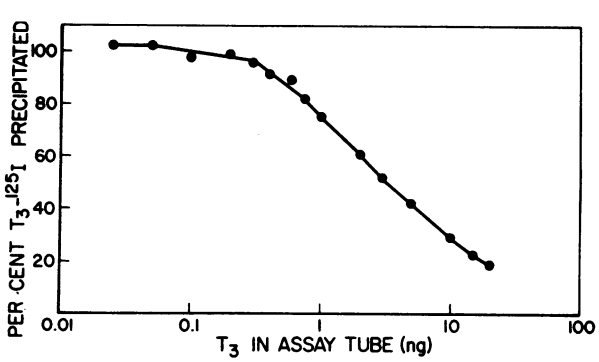

FIGURE 1 Dose-response curve. Inhibition of binding of T_3-^{125}I by unlabeled T_3 is shown on a semilogarithmic plot. Each point is a mean of triplicates. 400 μl athyreotic sheep serum and 250 ng T_4 were added as described in the text.

TABLE I

*Relative Cross-Reactivity with T_3-Binding Antibody of Thyroid Hormone Derivatives and Some Other Compounds of Interest**

Compound	Relative reactivity (arbitrary value if L-T_3 = 100)
D-T_3	100
TRIAC	18.0
Triiodothyropropionic acid	12.0
L-T_4	1.3
D-T_4	0.5
TETRAC	0.7
Tetraiodothyroproprionic acid	<0.3
3,5-L-Diiodothyronine	6.5
3,5-Diiodothyropropionic acid	<0.3
3-Monoiodothyronine	<0.5
L-Thyronine	0.3
Diiodotyrosine	<0.1
Monoiodotyrosine	<0.1
Dilantin	<0.1
Mercury (Mercurhydrin)	<0.1
Potassium iodide	<0.1

* All cross-reaction studies in this Table were performed using the assay system described in Methods, including the addition of 250 ng T_4 to each tube.

by the method of Murphy (14), the amount of T_4 in this extract was also estimated. Cold T_4 in 70% alcohol was then added to the extract to adjust the total T_4 in the tube to 500 ng. For the standard curve, tubes containing 500 ng T_4 and varying amounts of T_3 were prepared. All tubes were dried at 40°C under a stream of nitrogen. To all tubes 100 μl EDTA, 200 μl PBS, 500 μl 5% HSA, 100 μl 1:20 dilution of T_3-binding antiserum, and 100 μl T_3-^{125}I were then added and incubated as described. The radioactivity bound to rabbit T_3-binding antibody was separated by precipitation with a goat antiserum against rabbit γ-globulin. The standard curve was plotted as described above. (*b*) Measurement of T_3 in human sera by using anti-T_3-HSA (*vide supra*) in the RIA procedure described above.

Assessment of sensitivity. The threshold was defined as the smallest amount of nonradioactive T_3 in the presence of which the radioactivity bound to antibody was significantly lower ($P < 0.05$) than in the tubes with no nonradioactive T_3. It was determined in standard curves run in triplicate or quadruplicate.

RESULTS

Standard curve. Fig. 1 shows a typical standard curve obtained in the presence of 400 μl sheep serum and 250 ng T_4.[3] The index of precision (λ) was 0.07.

[3] With T_4 addition of 500 and 1000 ng per 400 μl sheep serum, the standard curves were shifted progressively to the right thereby resulting in fall in sensitivity. The detection threshold for T_3 was approximately 0.75 ng for 500 and approximately 1.0 ng for 1000 ng T_4.

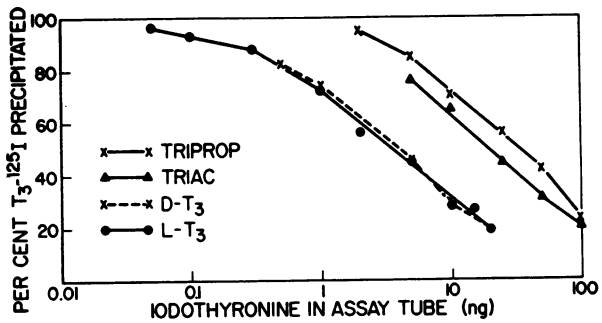

FIGURE 2 Displacement curves of D-T_3, TRIAC, and triiodothyropropionic acid (TRIPROP) compared with L-T_3. Increasing amounts of thyroid hormone analogues were added to the reaction mixture and treated as described in the text.

The threshold was 0.4 ng in this assay and varied between 0.3 and 0.4 ng in other runs, corresponding to a T_3 concentration of 75 and 100 ng/100 ml, respectively.

Specificity. The relative avidity of various thyroid analogues for the T_3-binding sites in the rabbit antiserum is presented in Table I. The dose-response curve with D-T_3 was almost superimposible on that of L-T_3, whereas that of TRIAC and triiodothyropropionic acid was to the right of and essentially parallel to the dose-response curve obtained with L-T_3 (Fig. 2). Other thyroid analogues reacted minimally in the system. Some other compounds which might be of interest in connection with the assay of T_3 were also studied. Dilantin, mercury, and iodide had no discernible effect in concentrations up to 10 mg, 2 mg, and 4 g per 100 ml, respectively. Human Tg had no effect with additions of up to 1000 ng/assay tube. An addition of 10,000 ng of Tg caused displacement of T_3-^{125}I from antibody equivalent to that by 1.4 ng T_3.

Serum T_3 concentration in health and disease. The range of serum T_3 concentration in various sera is presented in Fig. 3. The majority of the normal sera, 25 of 31, contained T_3 in a concentration less than the detection threshold of the method, i.e., 100 ng/100 ml; among the six sera in which T_3 was detectable, the values were 100 in two, 110 in another two, and 150 and 170 ng/100 ml in the remaining two individuals. Among the 27 sera from hyperthyroid patients, T_3 was detected in 25, with a range from 160 to 1300 ng/100 ml (mean, 519).

T_4 and T_3 concentrations in serial bleedings of 10 Graves' disease patients during treatment with antithyroid drugs are presented in Table II. In all patients serum T_3 decreased toward or to normal levels during therapy. In some, the fall of T_4 to normal levels was more rapid, whereas in others T_3 fell more rapidly. T_4 and T_3 concentrations during relapse of hyperthyroidism in three patients are also presented in this Table. Hyperthyroidism had recurred within 4 wk of the termination of a 1 yr course of antithyroid drug therapy in all three of these patients. In two of these patients in whom serum T_3 concentration before treatment was measured, it was much higher at the time of relapse than before treatment, whereas the converse was true with regard to serum T_4 concentrations.

Fig. 4 presents the curve describing disappearance of T_3 from serum in the patient who had allegedly ingested 17.3 g of desiccated thyroid. Approximately 30 hr after the overdose, when the patient was first seen, serum T_3 was 950 ng/100 ml. It was 500 ng/100 ml at 24 hr and 275 ng/100 ml at 40 hr after admission to the hospital (half-life approximately 24 hr). The corresponding values of the serum T_4 concentration were 45.2, 41.2, and 36.0 μg/100 ml, respectively (half-life approximately 135 hr). Despite serum T_3 and T_4 concentrations which were both markedly elevated on admission, the patient's only clinical sign of hyperthyroidism was mild tachycardia.

Serum T_3 concentration and other pertinent data in patients taking estrogen are depicted in Table III. Only two of the seven individuals tested had detectable T_3 in serum, with values of 167 and 275 ng/100 ml.

Precision of T_3 measurement in human serum was estimated by examining the coefficient of correlation (r) between duplicates. For 19 unknowns, with serum T_3 concentration ranging from 125 to 1300 ng/100 ml, coefficient of correlation was 0.9859, indicating that 97% of the variance was attributable to the substance measured,

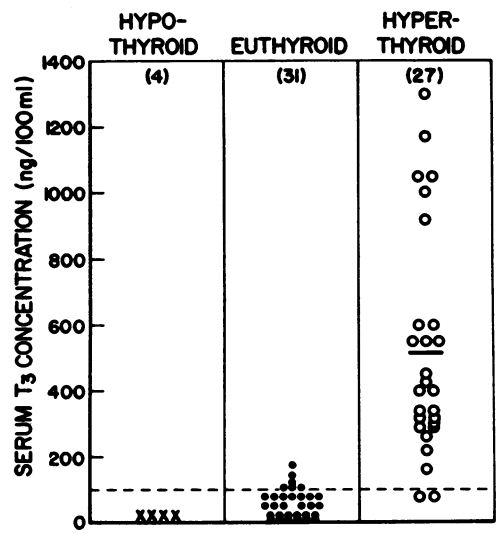

FIGURE 3 T_3 concentrations in human sera. The broken horizontal line represents the usual detection threshold (100 ng/100 ml). The solid horizontal line represents the mean serum T_3 concentration in 25 hyperthyroid patients in whom it could be detected.

TABLE II

Effect of Antithyroid Drug Therapy on T_4 and T_3 Concentrations in Sera of Patients with Active Graves' Disease

Patient		Before treatment	During antithyroid drug therapy*			Relapse after withdrawal of treatment
D. G.	T_4‡	27.3	4.1	4.0	2.8	
	T_3§	1300	275	<100	150	
			(1.5)	(4.5)	(7.0)	
C. A.	T_4	25.4	14.4			
	T_3	550	<100			
			(3.5)			
C. L. A.	T_4	23.8	4.8	3.0	10.8	17.8
	T_3	<100	<100	<100	<100	1025
			(3.5)	(5.0)	(13.0)	
C. J.	T_4	24.9	13.8	4.8		
	T_3	350	307	<100		
			(2.0)	(5.0)		
O. G.	T_4	22.0	18.4	13.4	9.0	
	T_3	450	324	<100	250	
			(1.5)	(6.0)	(8.0)	
M. G.	T_4	18.0	6.0	3.9	8.6	
	T_3	300	215	130	100	
			(1.5)	(4.5)	(6.0)	
R. J.	T_4	24.4	15.6	12.3		
	T_3	925	200	<100		
			(1.2)	(3.0)		
V. C.	T_4	22.0	5.8	3.8	7.0	15.8
	T_3	—	<100	110	110	550
			(1.5)	(4.5)	(9.5)	
T. F.	T_4	18.3	6.8			
	T_3	287	<100			
			(1.5)			
A. B.	T_4	30.0	3.8	7.2		19.6
	T_3	200	<100	<100		425
			(3.0)	(12.0)		

* Antithyroid drug used was methimazole in the case of C. J. and A. B. and propylthiouracil in the others. Numbers in parenthesis indicate the approximate period of treatment in months.
‡ Micrograms/100 milliliters.
§ Nanograms/100 milliliters.

i.e., T_3. The mean (±SEM) value for per cent departure of duplicates from their mean in the same assay was 4.84 ±0.78.

To examine reproducibility, a comparison was made of the mean T_3 concentrations of 9 sera run in duplicate in different assays. The coefficient of correlation was 0.9572, indicating that 9% of the total variance was due to methodologic variation of which approximately two-thirds is attributable to day-to-day factors and one-third to within assay factors (see above). The mean (±SEM) value for per cent departure of duplicates from their mean in different assays was 6.67 ±1.30.

Validation of the method. To examine the applicability of sheep serum in standards, the recovery of cold T_3 from five different pools of normal sera was examined. In 15 experiments where 0.5–10 ng of T_3 was added to 400 μl normal human serum the mean (±SEM) recovery of T_3 was 106.5 (±4.4)%.

When serial dilutions were made by adding sheep serum to serum of hyperthyroid patients, the results varied randomly around expected values (Table IV).

To check by chemical means the results obtained in normal subjects, sera of four normal laboratory workers, which appeared to contain less than 100 ng/100 ml of T_3 as measured by RIA, were extracted to concentrate T_3. Accounting for recovery, an extract representing about 3 ml of serum was analyzed for T_3 concentration using the T_3-binding antiserum. T_3 content in these extracts could now be read in the midportion of the standard curve. The T_3 concentration in these sera was 97,

TABLE III

Serum T_3 Concentration in Individuals Taking Estrogen

Patient	Condition for which estrogen administered	T_4-binding capacity of TBG	Serum T_4 concentration	Serum T_3 concentration
		μg/100 ml	*μg/100 ml*	*ng/100 ml*
P. S.	Carcinoma prostate	32.5	12.8	275
J. D.	Carcinoma prostate	37.0	12.6	167
J. D.	Carcinoma prostate	63.0	13.6	<200*
S. D.	Contraception	—	12.6	<133‡
D. M.	Contraception	38.0	12.2	<133‡
B. T.	Contraception	—	11.0	<133‡
M. P.	Lactation suppression	30.5	9.9	<100

* 200 μl sample tested.
‡ 300 μl sample tested.

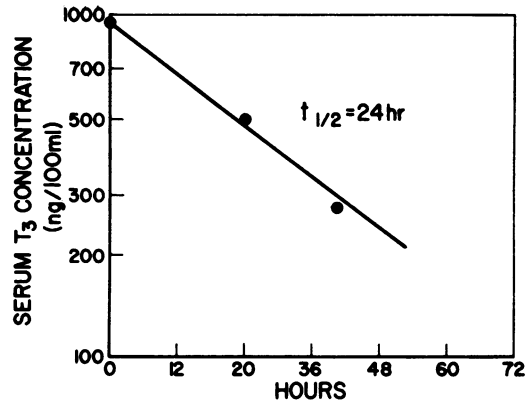

FIGURE 4 Disappearance of T_3 from serum of a patient who had allegedly ingested 17.3 g of desiccated thyroid. Serum T_3 concentration is plotted on the *y*-axis on a logarithmic scale and time in hours on *x*-axis on an arithmatic scale. Zero hr represents the time when the patient was first seen, approximately 30 hr after overdose.

88, 103, and 75 ng/100 ml, indicating that the RIA had not failed to detect any considerable amount of T_3.

To further check our results, we employed a T_3-binding antiserum (anti-T_3-HSA) which had some 700 times higher avidity for T_3 than for T_4. During preliminary experiments, described in more detail below, it became clear that excess T_4 was also necessary with this antiserum, just as with ours, in order to mobilize T_3 from TBG. The results obtained with the two antisera are shown in Table V. There was a reasonably good agreement between the two antibodies in estimating T_3 concentrations in both normal and hyperthyroid sera.

Critical importance of adding an adequate source of TBG to the standards. The need to include a comparable source of T_3-binding protein(s) in the standards to correct for those present in the unknown sera became obvious by study of the effect of hypothyroid sheep serum on the binding of radioactive T_3 to the antibody. When varying amounts of hypothyroid sheep serum were incubated with a fixed amount of antibody and T_3-^{125}I with *no* nonradioactive T_3 or T_4 added, a displacement curve was obtained as shown in Fig. 5. It was essentially parallel to the displacement curve obtained with varying amounts of nonradioactive T_3. The same was true with anti-T_3-HSA. With this antiserum, 100 μl of sheep serum caused displacement of radioactive T_3 from antibody equivalent to that with 0.90 ng of nonradioactive T_3. As the sheep serum actually contained minimal T_3 (less than 25 ng/100 ml), this pronounced displacement must be predominantly due to its TBG content. Similarly, 100 μl of the sera from five normal subjects seemed to be equivalent to 0.68–1.2 ng of unlabeled T_3 (mean 0.96) which would be equivalent, it should be noted, to from 680 to 1200 ng/100 ml. 100 μl of five hyperthyroid sera appeared to behave like 0.95–1.35 ng of T_3 (mean 1.12 ng). As some of the displacement of radioactive T_3 from antibody observed in the case of human sera may be expected to be due to their T_3 content, it became pertinent to know the T_3 concentration which would be measured if no T_4 were added in the regular RIA system. When this was done, a fairly satisfactory standard curve was obtained, adding T_3 freshly to sheep serum as in the usual RIA. However, in human serum, where the T_3 was already firmly bound to TBG, it apparently was not free to

TABLE IV

Effect of Dilution of Sera from Hyperthyroid Patients with Hypothyroid Sheep Serum on Estimates of T_3 Concentration

Patient	Serum dilution assayed....0	1/2	1/3	1/4	1/6	1/8	1/12	1/16
	T_3							
	ng/100 ml whole serum							
D. G.	1300	1250	—	1320	—	1440	—	1280
A. D.	1050	—	786	900	—	1040	1200	—
C. L. A.	1175	—	975	1000	960	1000	1200	
C. A.	600	600	600	700	—	—	—	—
A. A.	425	500						

react with T_3-binding antibody, since even hyperthyroid sera, previously shown to have high concentrations of T_3 in the regular RIA, now gave subthreshold readings. It was thus evident that the addition of excess T_4 is necessary in order to be able to measure T_3 in human serum and that the displacement of T_3-^{125}I from antibody by human sera in the absence of added T_4 could be, if anything, only minimally due to their T_3 content and was essentially due to TBG (not thyroxine-binding prealbumin [TBPA] as this does not bind T_3 to any significant extent [19]).

Effect of varying amounts of TBG in the presence of excess unlabeled T_4. The marked displacement effect of sheep serum on the binding of radioactive T_3 to antibody described above became much less pronounced when the same experiment was conducted in the presence of excess T_4 (250 ng). If one expressed the per cent T_3-^{125}I bound to antibody in the presence of 400 µl sheep serum and 250 ng T_4 as 100% (as has been done regularly in the RIA described), a reduction of the volume of sheep serum to 300 µl increased the binding to antibody by only 2% and a reduction to 200 µl increased it by another 4%. Conversely, an increase of sheep serum to 600 µl decreased the binding of T_3-^{125}I to antiserum by only 10%. Presumably, then, in this system at least, a variation of ±25% in TBG concentration in the human sera as compared with the sheep serum or within themselves, would not cause a major change in the estimate of T_3 concentration.

TABLE V

Comparison of Serum T_3 Concentration in Human Sera as Estimated by Radioimmunoassay Using Two Different T_3-Binding Antisera

	Patient	Serum T_3 concentration: By using rabbit antiserum against human thyroglobulin	By using rabbit antiserum against T_3-HSA conjugate*
		ng/100 ml	
Euthyroid	H. J.	115	<100
	R.	100	145
	A. E.	110	140
	J. K.	170	150
	R. G.	<100	125
Hyperthyroid	C. A.	600	525
	A. D.	1050	1200
	C. L. A.	1175	1400
	M. S.	325	425
	K. M.	550	500
	Mean ±SEM	740 ±160‡	810 ±203‡

* Produced by Gharib et al. (18).

‡ Difference not statistically significant.

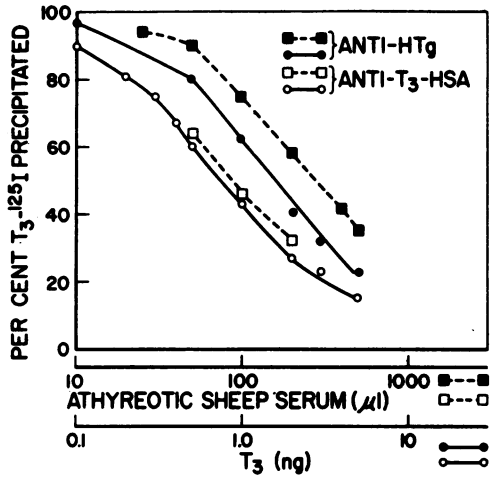

FIGURE 5 Displacement curves obtained with addition of increasing volumes of athyreotic sheep serum compared with those obtained with increasing amounts of L-T_3. Excess T_4, as routinely used, was omitted for this study. Two T_3-binding antisera have been studied. ●——● represents the inhibition curve with L-T_3 against the antiserum produced by immunization against human thyroglobulin. It was used in a dilution of 1:400 which bound 80% of tracer T_3-^{125}I. ■ - - - ■ is the curve obtained with athyreotic sheep serum using this antiserum. ○——○ represents the displacement curve with L-T_3 using anti-T_3-HSA (Gharib et al. [18]). It was used in a dilution 1:1000 which bound 84% of tracer T_3-^{125}I. □ - - - □ represents the displacement curve with athyreotic sheep serum (same as above) using anti-T_3-HSA.

Effect of inhibition of TBPA. As sheep serum contains little or no TBPA (15), TBPA in human sera may have an effect on estimates of their T_3 concentration. However, this did not appear to be the case when recovery of cold T_3 added to normal human serum pool was compared in tubes containing either PBS or 0.07 M barbital buffer, pH 8.6, the latter to inhibit TBPA (19). The serum pool appeared to contain less than 0.3 ng T_3/400 µl serum, when either of the two buffers were used. When 1.0 ng T_3 was added to parallel tubes with each buffer, a mean (±SEM) value of 1.32 ±0.02 and 1.35 ±0.05 ng T_3/400 µl, was obtained in triplicates incubated in PBS and barbital buffer, respectively. These values were not significantly different from each other.

DISCUSSION

The precision and reproducibility of the proposed method were satisfactory. The specificity of T_3-binding by the antiserum was quite acceptable; the affinity of T_3 for the antibody was much greater than that of all thyroid analogues except D-T_3. In the case of triiodothyroacetic acid and triiodothyropropionic acid, there was significant cross-reaction, approximating 18% and 12%, re-

spectively. It should be emphasized, however, that these are maximal estimates, since some contamination of these iodoamino acids by T_3 cannot be excluded and would produce indistinguishable effects. In any case, the practical significance of these cross-reactivities is difficult to assess, because little information is available regarding their presence or concentration in human serum. On the other hand, cross-reaction with T_4, even if quite limited could be significant because of the large amounts present in serum compared with T_3. Interference from variations in T_4 in test specimens was eliminated by adding a large excess of unlabeled T_4 and by keeping its amount constant in all unknowns and also in the standards. The results were checked by using another T_3-binding antiserum (anti-T_3-HSA) which cross-reacted with T_4 only very minimally. It was of interest, however, to note that addition of an excess of T_4 was also required in the case of this highly specific antiserum, to mobilize T_3 from TBG in the test specimen. Without the excess T_4, T_3 was undetectable even in hyperthyroid sera shown in the usual RIA to contain a high concentration of T_3. In addition, the excess of T_4 dampened the effects of variations in TBG.

Although 250 ng of T_4 may not be enough to saturate completely the binding sites on the amount of TBG in 400 μl of serum, the results indicate that it is adequate to mobilize at least 5 ng of T_3 (5.2 ng T_3/400 μl sample = 1300 ng/100 ml, the highest value obtained in our studies). At equilibrium, it is possible that a proportion of T_3 may be actually bound to TBG, but the same would apply to the radioactive T_3 added, both to the samples and the standards. The method reported here, like any RIA, must be viewed as depending entirely on a comparison between the standards and unknowns. It appears that essential identity was achieved by the methodologic modifications used in our method.

The sensitivity of the method was such that it should have readily detected T_3 in all normal sera if the serum T_3 concentration in such sera were above 170 ng/100 ml, as reported by other methods (6–8). However, with the values obtained being less than 100 ng/100 ml (i.e. < 0.4 ng/400 μl) in most normal subjects, sensitivity remains a major problem. It may be noted that the same T_3-binding antiserum, when used without excess T_4 and sheep serum, can easily detect 0.1 ng T_3/assay tube (13). Reduction in sensitivity due to the additions was not unique to this antibody but was also noted with anti-T_3-HSA. With this antiserum, sensitivity has been reported to be 0.05 ng/assay tube (18), but it dropped to 0.4 ng when excess T_4 and hypothyroid sheep serum were added as described above.

The T_3 concentration in euthyroid individuals reported here is lower than that found by previous methods (6–8). The mean normal value by the most commonly used method is 220 ng/100 ml (8). The possibility of an error in our method due to a considerable amount of T_3 in the sheep serum used in standard curve was excluded by chemical analysis which showed T_3 of less than 25 ng/100 ml. The true serum T_3 concentration could not be underestimated by the RIA by more than this amount. Most values reported in the literature have been obtained by methods involving chemical extraction and separation of T_3 from T_4 before final measurement by competitive protein binding. Artifacts during the processing of samples in these methods may lead to spuriously high estimates of T_3 concentration (10–12). When Benotti, Grimaldi, Pino, and Maloof (10) analyzed the T_3 isolated from paper chromatography by gas chromatography, significant contamination by T_4 ($\sim 0.5\%$) was observed. Considering the mean serum T_4 concentration in euthyroid individuals to be 6.48 μg/100 ml (20), 0.5% of T_4 could raise the estimates of T_3 concentration by about 80 ng/100 ml, since T_4 causes 2.5 times greater displacement of radioactive T_3 from TBG than an equal weight of T_3 (12). In fact, this does seem to be the case. Recently, using a binding protein 30 times more specific for T_3 than T_4, Ekins, Brown, Ellis, and Reith have reported T_3 concentrations in normal sera ranging between 70 and 160 ng/100 ml (mean 120 ng) (9). Hollander, using a gas chromatographic method, has reported earlier a mean serum T_3 concentration even higher than that obtained by other methods (6). However, later investigations from his laboratory have indicated that those values may have been spuriously elevated. Changes in the method have lowered his estimate of mean normal T_3 concentration to approximately 138 ng/100 ml, which would be more comparable with the results reported in the present study.[4]

Dussault et al. (16), using a chemical method, have carefully investigated the inadequacies of separation of thyronines as well as the contribution of T_3 derived from in vitro deiodination of T_4 to the final estimate of T_3 concentration. When they correct the T_3 concentration for methodological artifacts, the values obtained in normal subjects range from less than 25–203 ng/100 ml (mean 98 ± 48). 61% of 31 normal subjects tested had a serum T_3 concentration less than 100 ng/100 ml, in close agreement with the results reported in this paper.

The RIA method affords an adequate, but by no means perfect, separation of hyperthyroid patients from normal subjects and hypothyroid patients. 89% of hyperthyroid patients had a serum T_3 concentration above upper limit of normals (170 ng/100 ml). Similarly, diminution in serum T_3 concentrations in hyperthyroid patients during treatment indicated the usefulness of the

[4] Hollander, C. S. Personal communication.

method in following the response to therapy. However, the method does not distinguish hypothyroid patients from most euthyroid subjects.

Turnover of T_3 has been calculated to approximate 60 μg/day (21) in comparison with about 90 μg/day of T_4 (22). This estimate of T_3 turnover is based on a turnover rate of 52% per day, a volume of distribution of 43 liters and a mean serum T_3 concentration of 273 ng/100 ml (21). However, more recent work has described T_3 degradation rate as approximately 70% per day, corrected for newly formed iodoprotein, and the volume of distribution has been in the range of 26–35 liters (23).[5] Using these figures and a median normal serum T_3 concentration of less than 100 ng/100 ml, as reported here, it would seem that the normal T_3 utilization rate should be less than 25 μg a day. As T_3 is about 3–4 times as potent in its metabolic effect as T_4, even this lower estimate does not minimize the possible significance of the contribution of T_3 to total thyroid hormone economy.

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