

[54] RADIOIMMUNOASSAY FOR MEASUREMENT OF THYROXINE (T₄) AND TRIIODOTHYRONINE (T₃) IN BLOOD SERUM

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[52] U.S. Cl. 424/1; 436/500; 436/539; 436/540; 436/541; 436/542; 436/804; 436/817; 436/825; 436/826; 23/230 B; 23/230.3

[58] Field of Search 424/1; 23/230 B

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[57] ABSTRACT

This invention relates to a highly accurate, rapid and simple estimation of thyroxine (T₄) directly from blood serum and also relates to the accurate measurement of triiodo-L-thyronine (T₃) directly from blood serum. More specifically, the invention relates to a rapid, specific and reliable radioimmunoassay (RIA) technique for measurement of both T₄ and T₃ in unextracted serum. The method requires very small amounts of serum, e.g., 25 microliters (μl) to measure T₄ concentration in nearly all specimens representing clinical states of eu-, hypo- and hyperthyroidism, and 250 μl to measure T₃ concentrations in specimens representing most clinical states.

11 Claims, 3 Drawing Figures

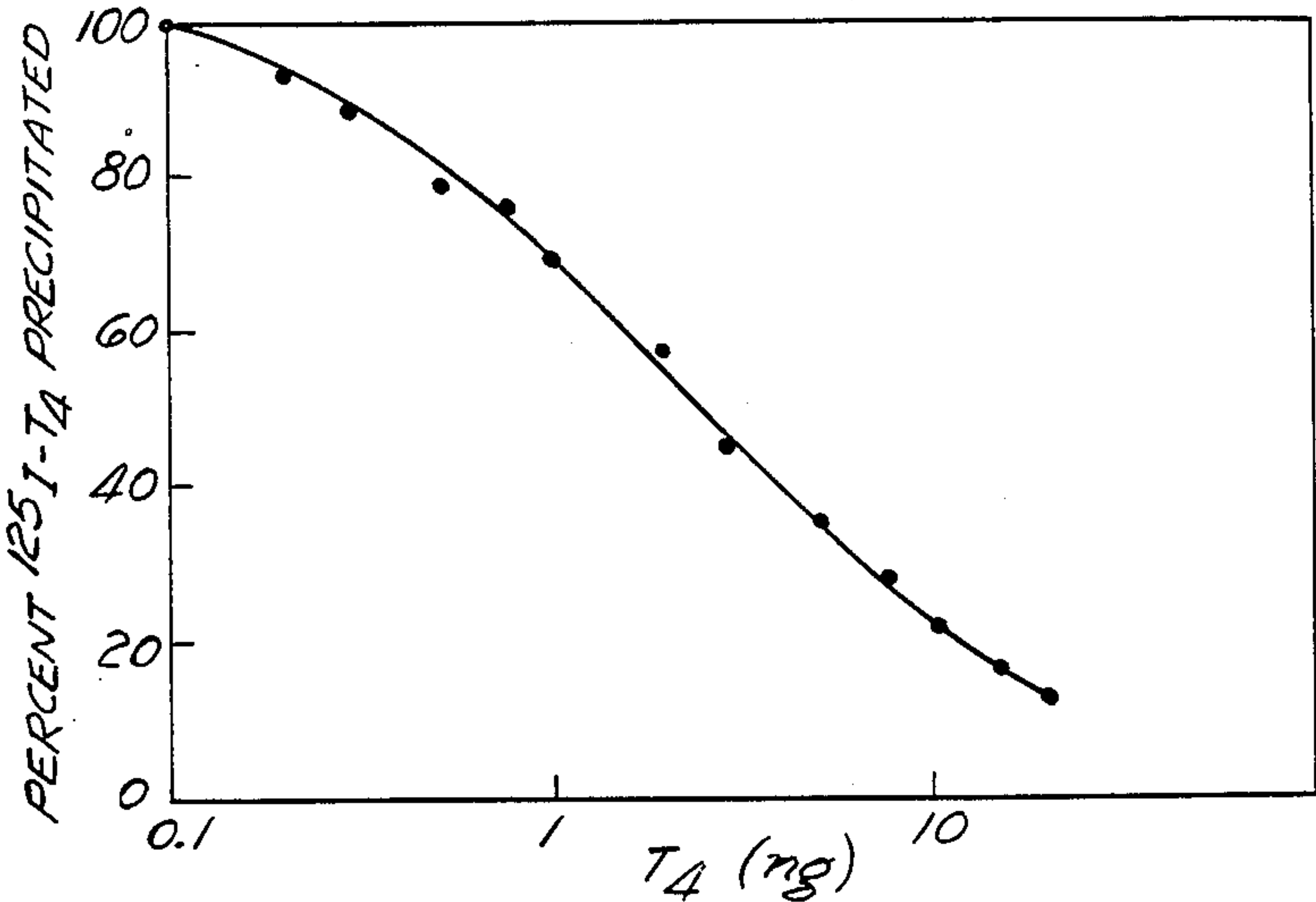


Fig. 1

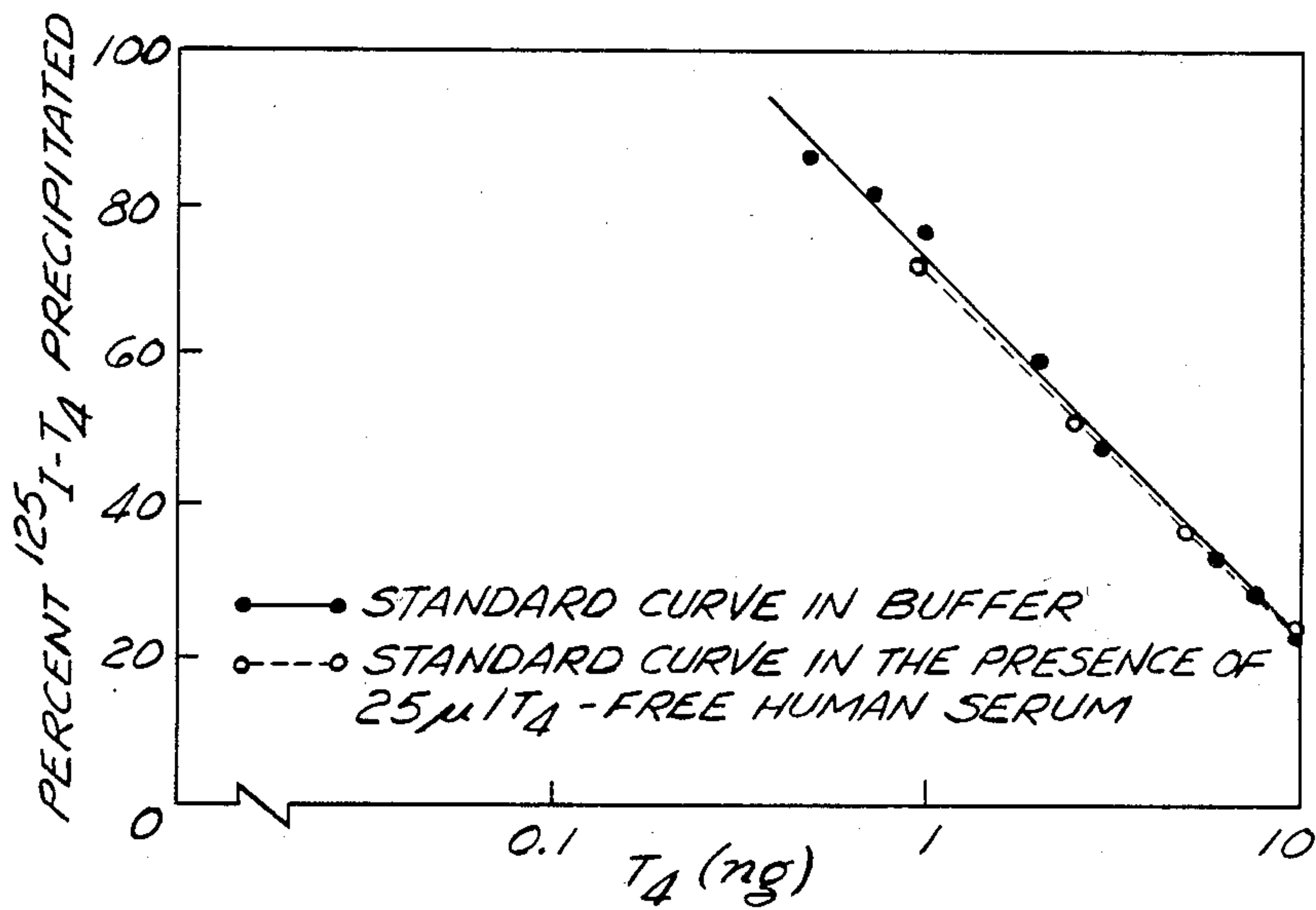
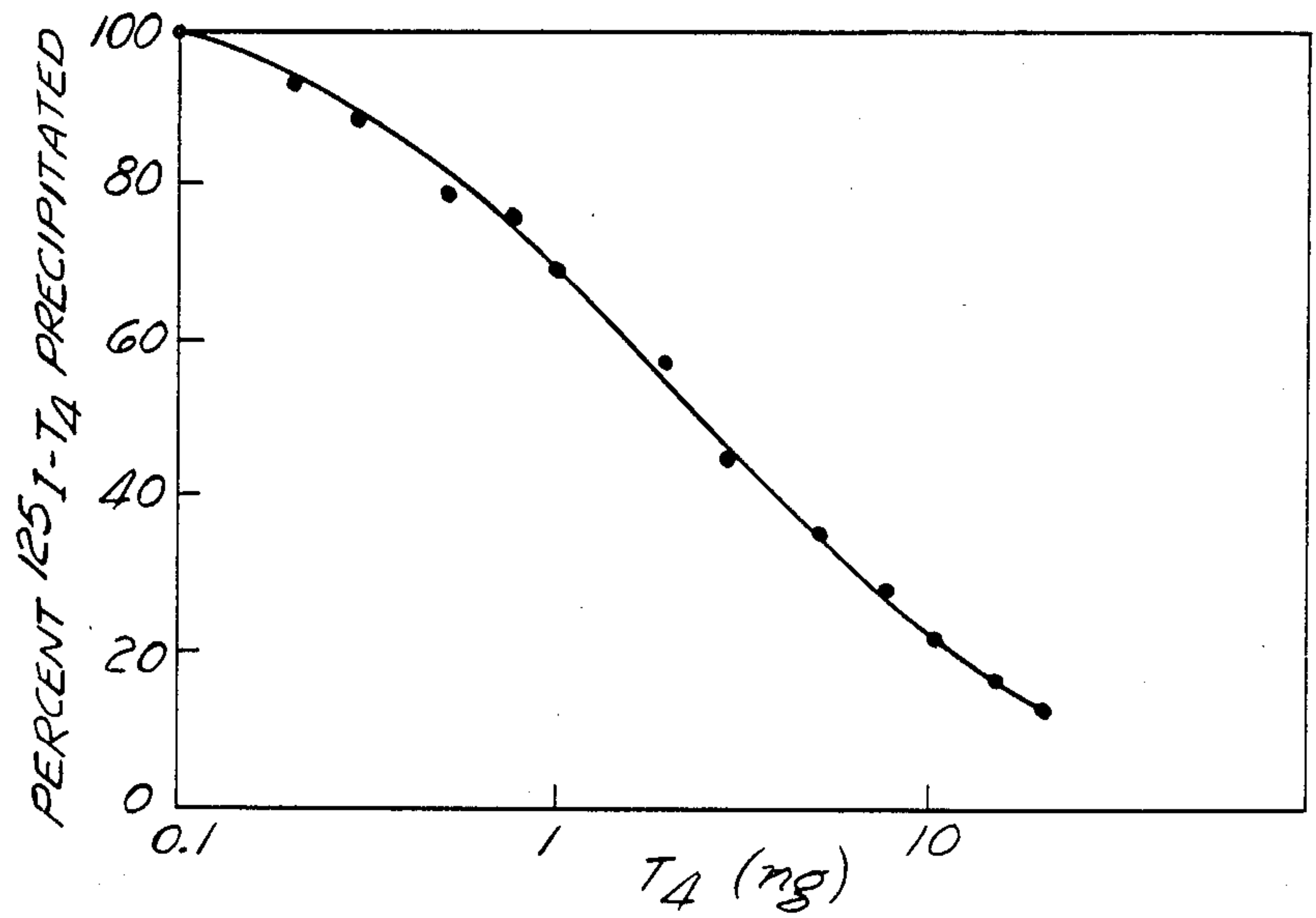
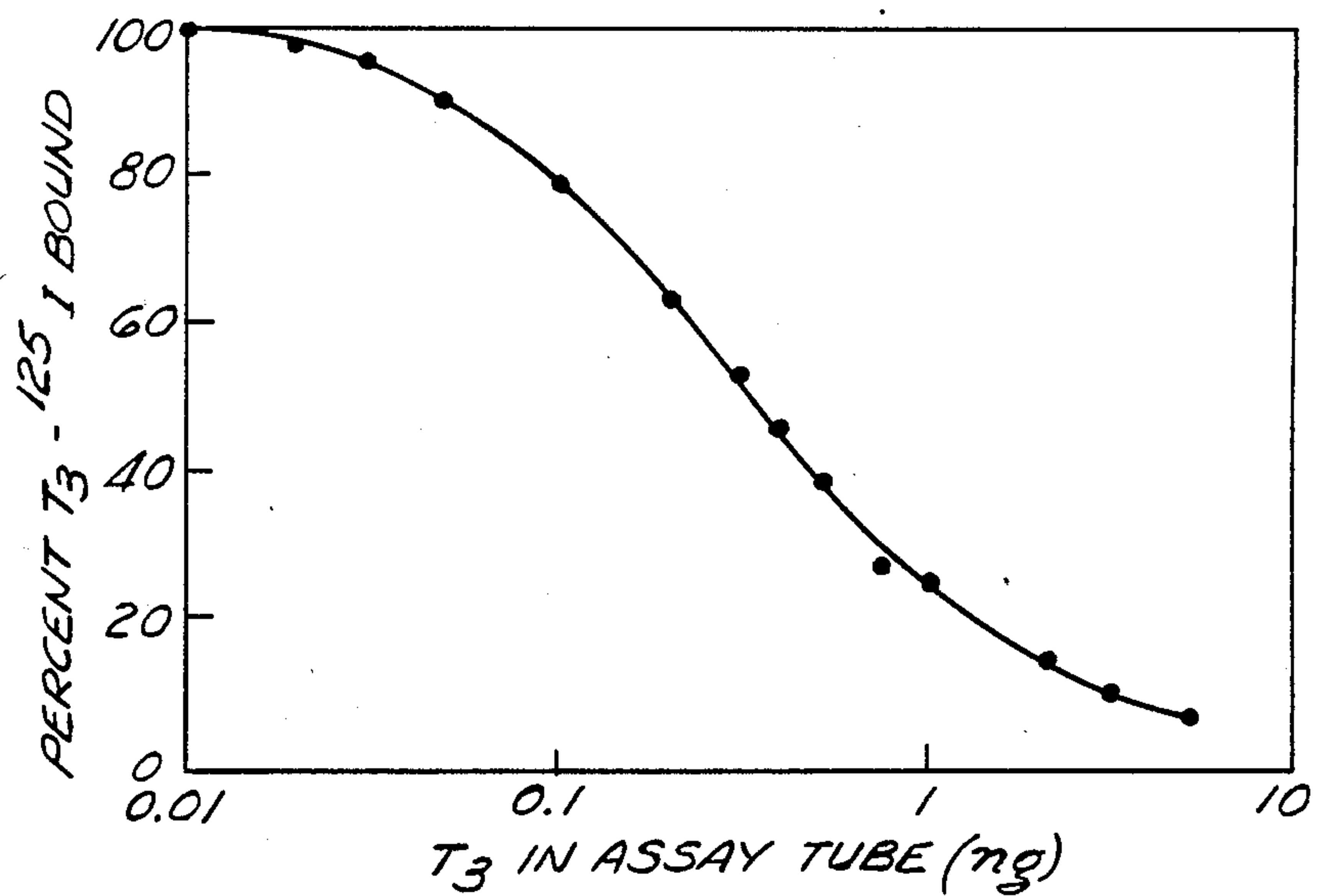


Fig. 2

Fig. 3



RADIOIMMUNOASSAY FOR MEASUREMENT OF THYROXINE (T₄) AND TRIIODOTHYRONINE (T₃) IN BLOOD SERUM

Matter enclosed in heavy brackets [] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education and Welfare.

BACKGROUND OF THE INVENTION

In a normal human serum, about 99.97% of thyroxine (T₄) and 99.7% of triiodothyronine (T₃) present, is bound to proteins such as thyroxine-binding globulin (TBG), prealbumin (TBPA) and albumin; the remaining 0.03% of T₄ and 0.3% of T₃ is present as unbound (dialyzable or free) hormones. The serum concentration of thyroid hormones, among other facts such as thyroidal secretion and degradation, is also dependent on serum concentration of TBG; other binding proteins in serum are relatively less important since their binding affinity for thyroid hormones is far less than that of TBG. The concentration of TBG is elevated in conditions such as pregnancy, estrogen treatment or a genetic abnormality; serum total T₄ (and T₃) is increased in these situations. Conversely, serum TBG concentration is decreased during treatment with androgens or due to a genetic deficiency of TBG. This is associated with subnormal concentrations of thyroid hormones. However, in either of the above-mentioned situations of altered TBG concentration, the patient remains eumetabolic and concentrations of free T₄ and (and T₃) and that of total T₄ (and T₃) corrected for TBG abnormality are within the range of normal subjects.

Until a few years ago, serum T₄ concentration was assessed indirectly by measurements of organic (protein bound or butanol extractable) iodine. However, these measurements were frequently erroneous because of iodide contamination in the laboratory or of administration of iodide containing drugs to the patients. In order to overcome these problems, a competitive-protein binding assay (CPBA) for T₄ was introduced by Murphy and Pattee (1). This procedure employs the principle of saturation analysis, and quantitates T₄, relatively specifically, by measurement of displacement of radioactive T₄ from T₄-binding sites on TBG. It allows measurements of T₄ in a range of 3-20 ug % using a butanol-ethanol extract of serum. In the Murphy-Pattee method, one must first extract thyroxine from the blood serum with alcohol or butanol-ethanol; this introduces error since the extraction procedure itself results in the extraction of varying amounts of thyroxine. The drying of the extract is also time consuming, and must be accomplished before one commences the measurement of T₄.

The Murphy-Pattee procedure thus leaves one with a dried extract containing T₄ as well as much non-thyroxines, such as lipids. To the tubes containing the dried extract, one then adds a 1/32 diluted human serum containing radioactive T₄ (T₄*). The T₄ in the dried extract competes with T₄* for binding sites on TBG. After equilibrium is reached one separates the unbound T₄ by means of an ion exchange resin and measures the amount of radioactive counts (of T₄*) remaining bound to TBG. The quantitation of T₄ is accomplished by

reading from a standard curve, prepared simultaneously with known amounts of T₄.

(1) Murphy and Pattee J. Lab. Clin. Med. 66:161 July 1965

The disadvantages of the Murphy-Pattee method are that it requires an extraction procedure; it is not sensitive enough and it is cumbersome for processing a large number of samples.

A radioimmunoassay (RIA) has been previously developed and reported, by the applicant herein in conjunction with others, which employs a highly specific antibody to T₄ for its subsequent measurement instead of utilizing TBG from human serum. In this method, a rabbit anti-thyroglobulin antiserum is employed as the T₄ binding protein. Further, in this method, the T₄ must be first extracted as in the Murphy-Pattee method but the method is more sensitive than Murphy-Pattee. This RIA method of applicant herein is reported, in detail, in *Journal of Clinical Endocrinology*, 33:865, 1971, incorporated herein by this reference. In general, the precision, reproducibility and practicality of this RIA is comparable to those of competitive protein binding assay (CPBA) using serum TBG as the T₄ binding protein.

SUMMARY OF THE INVENTION

I have found that I can achieve significantly improved measurement of T₄ and T₃ directly from unextracted serum by displacing T₄ and T₃ from serum thyroxine (or T₃) binding globulin (hereinafter generically referred to as TBG by means of a foreign compound (i.e., one not itself a thyroid hormone) which compound not only has the capability of preventing completely the binding of radioactive T₄ and radioactive T₃ (T₄* or T₃*, respectively) or T₄ and T₃ to TBG, respectively, but does not inhibit in any way the reaction of T₄ and T₃ with T₄-antibodies used as the hormone binding proteins (instead of TBG). Foreign compounds falling in this category will be termed herein, and in the claims simply as "blocking agent" or "blocking agents".

The novel approach adopted herein is believed to be broadly new and is not dependent upon the use of a particular blocking agent. Many different types of blocking agents have been proven to have the desired qualities aforementioned, e.g., 8-anilino-1-naphthalene-sulfonic acid (ANS), 3-(4-anilino-1-naphthylazo) 2,7-naphthalene disulfonic acid (ANNDS), 2,4 6-trinitro benzene sulfonic acid (TNBS), naphthalene sulfonic acid, Thimerosal, 5-5-diphenyl 2-thiohydantoin, Doxepin Hcl, Diazepam, sodium salicylate, prochlorperazine, halofenate (MK-185) Merck, Sharpe and Dohme.

Further, the inhibition of the binding of T₄ (or T₃) to thyroxine binding prealbumin (TBPA) is accomplished by the setting up of the assay in a suitably buffered medium, e.g., containing barbital ions, e.g., barbital buffer, and having a pH of between about 6.8-9.6. T₄ binding by prealbumin is also affected by the presence of compounds such as ANS, or sodium salicylate.

Thus, with respect to the measurement of T₄ by my novel RIA, the process involves only the following few steps: (1) incubation for one hour, of human serum, in the presence of radioactive T₄, T₄ antibody, and one of the blocking agents mentioned above or others in a barbital or other suitable buffer - to thereby displace T₄ bound to TBG in the serum and make it available for reaction with T₄ antibody (and thus measurable by RIA techniques) while minimizing or completely inhibiting the binding of the added radioactive T₄ to TBG. Finally, the radioactive T₄ bound to the antibody is sepa-

rated from free radioactive T₄. This may be accomplished in various ways, e.g., by use of a "second antibody", e.g., goat anti-rabbit gammaglobulin which precipitates the antibody bound radioactivity which may then be counted. A standard curve is prepared with known amounts of T₄ and unknowns are read off the curve. Using the just-described RIA, it is feasible to quantitate T₄ in a wide range (1 to 40 ug %) using only 15 to 25 ul of unextracted serum.

Measurement of T₃ in serum has been more involved and difficult than that of T₄. On an average, T₃ is present in normal serum in concentrations about 1/70 that of T₄. However, the importance of its quantitation in serum has been highlighted by recent suggestions that T₃ may be the predominant biologically active thyroid hormone and that T₄ exerts its biological effect only after prior conversion to T₃ in the body. Several instances of hyperthyroidism due to elevated serum T₃ (and normal serum T₄) have been described. A popular method for measurement of T₃ involves three steps, i.e., extraction of thyronines from serum by column chromatography, separation of T₄ from T₃ by paper chromatography and finally quantitation of T₃ by competitive protein binding assay using TBG in a manner similar to that for T₄ measurement by Murphy-Pattee. However, chromatographic separation of T₄ from T₃ may not always be complete. It may also be associated with in vitro conversion of T₄ to T₃, thereby leading to inappropriate high values. These problems have been circumvented by the use of a radioimmunoassay (RIA) method for quantitating T₃ in unextracted serum by a method similar to that above-described for T₄ assay.

Thus, improvements in accurate measurement of serum T₃ by RIA requires, according to this invention, adequate blocking of thyronine-binding globulin (TBG) in serum, by means of the same blocking agents heretofore enumerated. The approach to T₃ measurement is precisely the same as for T₄ measurement; the improvement in the T₃ RIA being due primarily to the use of blocking compounds such as ANS, which block T₃ binding sites. ANS, when used in a concentration of 1 ug/ul. test serum, displaces nearly all T₃ bound to TBG and prevents completely the binding of radioactive T₃ to TBG. ANS has negligible affinity for T₃ -binding sites on the rabbit antiserum used in this RIA (which antiserum was produced by immunization with T₃ -enriched thyroglobulin). The T₃ -antibody may be precipitated with a "second antibody" and the bound radioactive T₃ is separated from free T₃, and counted by previously known methods, to provide an accurate measurement of T₃.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an example of the standard curve relating T₄ (ng) to percent radioactive T₄ precipitated;

FIG. 2 illustrates two more standard curves indicating the substantially completely blocking of TBG by the particular blocking agent utilized, viz. ANS; and

FIG. 3 is an example of the standard curve relating to T₃ (ng) to percent radioactive T₃ precipitated

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. T₄ Assay

In general, the method requires the incubation for about one hour of human serum, in the presence of a barbitol or other suitable buffer and in the presence of T₄ antibody, T₄*, and a blocking agent present in suffi-

cient quantity to displace T₄ bound to TBG in the serum and make it available for reaction with the said T₄ antibody. One can readily assess the amount of any particular blocking agent required to meet the foregoing condition. Thus, by way of example only, the ability of ANS to displace T₄ has been demonstrated by its displacement of radioactive T₄ from TBG in a CPBA system, (1) supra. 100 ug of ANS was found to displace 18 nanograms (ng) T₄. Thus, when 100 ug of ANS is added 25 ul of serum, it will displace all T₄ up to a concentration of 72 ug per 100 ml. of serum. Even though the upper concentrations of T₄ found are well below 72 ug/100 ml, an excess of ANS, e.g., 150 ug added to 25 ul of serum, may be and is used, in the instant assay procedure. In general, then, it is well within the skill of the art, to determine the required minimal quantity of blocking agent required for the assay procedure. Further examples of the displacement ability of other blocking agents are set forth in Table I below, the first compound set forth in Table I being ANS for ready comparison.

TABLE I

BLOCKING AGENT	AMOUNT T ₄ DISPLACED FROM TBG
(100 ug utilized except where noted otherwise)	
1. ANS	18
2. ANNDS (50 ug)	>20
3. TNBS	7.1
4. αNaphthalene Sulfonic Acid	3.0
5. 5,5 diphenyl-2 thiohydantoin	6.1
6. Doxepin HCl	8.2
7. Diazepam	6.3
8. Prochlorperazine	7.2
9. Dilantin ®	5.7
10. Thimerosal (1 mg.)	8.4
11. Sodium Salicylate (10 mg.)	6.1
12. Halofenate* (MK-185)	8.4
13. Chlorpromazine HCl ((50 ug)	5.6

*Proprietary compound supplied by Merck, Sharp & Dohme

The amount of T₄* added is determined by that quantity required to give to the assay tube, in which the assay is being conducted, a measurable counting rate, after reaction with the T₄ antibody. The amount of radioactive T₄ to be added is not critical and may vary over a wide range depending upon the sensitivity of the counting equipment. One may, for example, utilize sufficient radioactive T₄ initially, to cause a counting rate of 2000-6000 CPM (counts per minute) to result from measurement of the T₄*-antibody precipitate. One may also desire to measure the free radioactive T₄, in which event, the counting rate of the free radioactive T₄ should be preferably at this range. Of course, as the counting equipment becomes more sensitive, the amount of radioactive T₄* to be added may be further reduced.

Preparation of antibodies specific to T₄ is known and will be described in some detail hereafter. The amount of T₄-antibody added, to a predetermined quantity of human serum, e.g., 25 ul is that quantity having the ability to bind substantial quantities of T₄*, e.g., from about 20-60% of radioactive T₄, in the absence of any non-radioactive T₄.

The contents of the assay tubes are buffered, preferably by barbitol ions, to a pH in the range of from about 6.8 to 9.6, with a pH of about 8.6 being preferred.

Incubation of the contents of the assay tubes, containing measured amounts of T₄*, T₄ antibody, and block-

ing agent in a measured amount of serum, takes place over a period of an hour or so at room temperature and for five minutes at 4° C. During this period of incubation, competitive reactions between T₄* and T₄, on the one hand, with T₄ antibody on the other, takes place, and near equilibrium of these competing reaction is attained.

The radioactive T₄ bound to antibody (antibody bound radioactivity) is then separated from the free radioactive T₄ by anyone of a number of methods. For example, a charcoal suspension when added to the final reaction mixture, after the end of the incubation period, adsorbs the free radioactivity. The reaction mixture, containing the charcoal, is centrifuged and the charcoal settles. One may count either the bound radioactivity in the supernatant, or the free radioactivity in the charcoal.

Alternatively, one may add a polyethylene glycol (Carbowax 6000) to the reaction mixture. Polyethylene glycol will precipitate gammaglobulins, and when added to the reaction mixture, will precipitate the antibody bound radioactivity, and may be measured as indicated above.

The method of precipitation of antibody bound radioactivity presently preferred, involves the use of a second antibody which will precipitate the antibody bound radioactivity. After a suitable period of incubation; e.g., 24 hours at 4° C., the antibody bound radioactivity may then be counted. Measurement of T₄ concentration is made by correlating the antibody found radioactivity with a standard curve previously prepared.

B. T₃ Assay

In general, the method of measuring T₃ requires the incubation for about 20 hours of human serum, in the presence of a barbital or other suitable buffer and in the presence of T₃ antibody, T₃*, and a blocking agent, present in sufficient quantity to displace all T₃ bound to TBG in the serum and make it available for reaction with said T₃ antibody. It has been demonstrated that TBG competes with T₃ antibody for radioactive T₃ as well as for non-radioactive (or stable) T₃. The concentration of blocking agent required to prevent this competition or interference from TBG is readily determined. In general, the binding of T₃ to TBG is much weaker than is T₄ to TBG and the amount of blocking agent required is less per given amount of human serum than is required for the T₄ assay. Thus, for example, 1 ug/ul serum of ANS is utilized in a T₃ assay as compared with 4-6 ug/ul ANS for a T₄ assay. The ability of ANS to displace T₃ has been demonstrated by its displacement of radioactive T₃ from TBG. 10 ug. of ANS was found to displace 11.2 nanograms (ng) T₃. Thus, when 200 ug. of ANS is added to 250 ul of serum, it may displace all T₃ up to a concentration of 88 ug T₃ per 100 ml. of serum. Even though the upper concentrations of T₃ found are well below 2.5 ug/100 ml, an excess of ANS, e.g., 250 ug is normally added to 250 ul of serum in the instant assay procedure. In general, then, it is well within the skill of the art, to determine the required minimal quantity of blocking agent required for the assay procedure. The procedure for determining the minimal quantity for ANS is set forth in Example 2 hereof. Further, examples of the displacement ability of other blocking agents are set forth in Table II below, the first compound set forth in Table II being ANS for ready comparison.

TABLE II

BLOCKING AGENT	AMOUNT T ₃ DISPLACED FROM TBG (ng.)
(10 ug utilized except where noted otherwise)	
1. ANS	11.2
2. ANNDS	10.4
3. TNBS (100 ug.)	7.0
4. αNaphthalene Sulfonic acid (100 ug)	7.0
5. 5,5 diphenyl-2 thiohydantoin	7.6
6. Doxepin HCl	10.8
7. Diazepam	7.0
8. Prochlorperazine	7.0
9. Thimerosal (100 ug.)	8.0
10. Sodium Salicylate (1 mg)	11.0
11. Halofenate* (MK-185)	5.2
12. Chlorpromazine HCl (25 ug)	6.6

*Proprietary compound supplied by Merck, Sharp & Dohme.

The amount of T₃* added is determined by that quantity required to give to the assay tube, in which the assay is being conducted, a measurable counting rate, after reaction with the T₃ antibody. The amount of radioactive T₃ to be added is not critical and may vary over a wide range depending upon the sensitivity of the counting equipment. One may, for example, utilize sufficient radioactive T₃ initially, to cause a counting rate of 2000-6000 CPM (counts per minute) to result from measurement of the T₃*-antibody precipitate. One may also measure the free radioactive T₃, in which event, the counting rate of the free radioactive T₃ should be preferably at this range. Of course, as the counting equipment becomes more sensitive, the amount of radioactive T₃ to be added may be further reduced.

Preparation of T₃ antibodies specific to T₃ is known and will be described in some detail hereafter. The amount of T₃ antibody added, to a predetermined quantity of human serum, e.g., 250 ul, is that quantity having the ability to bind from about 20-60% of radioactive T₃ added in the absence of any non-radioactive T₃.

The contents of the assay tubes are buffered, preferably by Barbital ions, to a pH in the range of from about 6.8 to 9.6, with a pH of about 8.6 being preferred.

Incubation of the contents of the assay tubes, containing measured amounts of T₃, T₃ antibody, and blocking agent in a measured amount of serum, takes place over a period of 20 hours or so, at 4° C. During this period of incubation, competitive reactions between T₃* and T₃, on the one hand, with T₃ antibody on the other, takes place, and near equilibrium of these competing reactions is attained.

The radioactive T₃ bound to antibody (antibody bound radioactivity) is then separated from the free radioactive T₃ by anyone of a number of methods. For example, a charcoal suspension when added to the final reaction mixtures, after the end of the incubation period, adsorbs the free radioactivity. The reaction mixture, containing the charcoal, is centrifuged and the charcoal settles. One may count either the bound radioactivity in the supernatant, or the free radioactivity in the charcoal.

Alternatively, one may add a polyethylene glycol (Carbowax 6000) to the reaction mixture. Polyethylene glycol will precipitate gammaglobulins, and when added to the reaction mixture, will precipitate the antibody bound radioactivity, and may be measured as indicated above.

The method of precipitation of antibody bound radioactivity presently preferred, involves the use of a sec-

ond antibody which will precipitate the antibody bound radioactivity. The antibody bound radioactivity is then counted and measurement of T_3 concentration is made by correlating the antibody bound radioactivity with a standard curve previously prepared.

C. Specific Examples

EXAMPLE 1

The following example illustrates the use of ANS as a blocking agent in the measurement of T_4 at a variety of concentrations, and illustrates, as well, the making of a standard curve, to allow accurate correlation of the unknown T_4 concentrations with the standard curve. This example serves as the basis for a paper to be shortly published in *Journal of Clinical Endocrinology*, 34: 938, 1972 under the title "A Rapid Radioimmunoassay For Measurement of Thyroxine in Unextracted Serum".

The reagents employed are:

1. T_4 -binding antiserum or T_4 antibody: The serum used was obtained from a rabbit immunized with normal human thyroglobulin (Tg), as described by Chopra et al., *J. Clinical Endocrinology* 32:299. It was used in a final dilution of 1:300; in this dilution it bound approximately 50% of a tracer amount of radioactive T_4 .
2. Radioactive (^{125}I) T_4 (SA 50–75 uc/ug) in 50% propylene glycol was obtained from Abbott Laboratories, North Chicago, Illinois.
3. ANS was obtained from K & K Laboratories, Hollywood, Calif.
4. Reagent grade Na-L- T_4 (non radioactive T_4) was obtained from Mann Research Laboratories, New York. It was weighed and dissolved in 0.1M NaOH and diluted to desired concentrations in 0.075M barbital buffer, pH 8.6, containing 2% normal rabbit serum (NRS). The NRS is employed as a carrier protein to render the antibody precipitate visible, in the final step, but is not present in sufficient quantity to interfere with the T_4 measurement.

The steps of the radioimmunoassay (RIA) procedure follows:

In 10×75 mm disposable glass tubes, the various reagents were added in the following order to yield a final volume of 0.5 ml:

1. Three hundred ul of 0.075 M barbital buffer which contained 2% NRS, 150 ug ANS and approximately 10,000 CPM (counts per minute) of ^{125}I - T_4 (~0.2 ng T_4). The stock solution of ANS (50 mg per 100 ml of buffer) was made fresh before every assay.
2. One hundred ul of various dilutions of T_4 , i.e. 0.5 ng/ml to 200 ng/ml. were employed to yield 0.05 to 20 mg T_4 for a 10 to 14 point standard curve (see FIG. 1).

In the case of the unknown, 25 ul of serum was employed, followed by 75 ul of .075 barbital buffer containing 2% NRS but without additional ANS or ^{125}I - T_4 . The standard curve and unknowns were assayed at least in duplicate.

3. To all tubes, 100 ul of a 1:60 dilution of T_4 antibody was added.

Steps 1 and 3 were conveniently and accurately performed using an automatic pipettor (Repipet 1.0 ml, Lab Industries, Berkeley, California). All tubes were briefly swirled after steps 2 and 3. The tubes were then incubated for 1 hr at room temperature and 5 min at 4C. Pilot experiments had indicated that a state of near-

equilibrium was reached during this period of incubation.

4. To precipitate ^{125}I - T_4 bound to antibody, approximately 40–50 ul of a previously titered goat antirabbit γ -globulin was added and tubes were reincubated overnight (~20 hr) at 4C. The details of separation of bound from free radioactivity, correction for nonspecific binding or trapping or ^{125}I - T_4 in the precipitate and plotting of standard curves have been described recently in a RIA for triiodothyronine (T_3). Chopra et al. *Radioimmunoassay for measurement of triiodothyronine in human serum*, *J. Clin. Invest.* 50:2033, Oct. 1971. The results are set forth below:

- FIG. 1 shows the typical standard curve obtained. The curve is essentially linear between 0.3 to 10 ng, allowing measurement of serum T_4 over the range of 1.2 to 40 ug per 100 ml when 25 ul serum was assayed. The index of precision (λ) was 0.063 in this and another standard curve.

- FIG. 2 shows a comparison of standard curves obtained in barbital buffer and in the presence of 25 ul of T_4 -free serum. The two standard curves were nearly superimposable, indicating that TBG in the serum was adequately blocked under the assay conditions used.

- Table III illustrates the results of serum T_4 as determined by the RIA described in this example and those obtained by CPBA in sera from euthyroid subjects and patients with or without thyroid functional abnormalities.

TABLE III

Source of sera		Comparison of estimates of serum T_4 concentration by this RIA and CPBA.	
		Serum T_4 ug/100 ml	
No.		CPBA	RIA
Euthyroid	40	7.51 \pm 0.31*	8.33 \pm 0.38
Hyperthyroid	40	19.9 \pm 0.83	24.6 \pm 1.39
Hypothyroid	7	3.34 \pm 0.13	3.45 \pm 0.36
Estrogen-treated	8	12.7 \pm 0.61	13.2 \pm 0.79

*Mean \pm SEM

- In euthyroid individuals the mean serum T_4 by this RIA was 10.9% higher than that by CPBA. This difference was statistically significant. It is attributable, in part, to losses in T_4 during extraction of serum in CPBA, since the recovery of radioactive T_4 in the butanol-ethanol extraction averaged 88%.

- Precision of T_4 measurements by the RIA, described herein, was assessed by comparing the duplicates within assays. The mean value for percent departure of duplicates from their mean in 85 sera was 4.32 \pm 0.38.

- Reproducibility of estimates of serum T_4 by RIA was studied by comparison of T_4 concentration in 10 sera measured in duplicate in different assays. The mean value for percent departure of duplicates from their mean was 7.1 \pm 1.22. The working time involved in setting up an assay comprised of 94 tubes was only 116 min. This includes 56 min. spent in pipetting standards and test sera which would be common to all methods.

- The RIA proposed here is adequately sensitive, precise and reproducible. The requirement of only 50 ul of sample for duplicate determinations of serum T_4 over a range of 1.2 to 40 ug per 100 ml, in one attempt, not only makes the assay useful for routine clinical purposes but also for measurement of T_4 in serum of infants and small experimental animals where sample availability may be limited. Since T_4 -binding antisera (a T_4 antibody) can be raised quite regularly when rabbits are immunized

with Tg in Freund's adjuvant, this RIA appears to be a very practical method for measurement of serum T₄. The practicality of RIA is further emphasized by the simplicity of the procedure described here as well as the short working time involved.

The specificity of the T₄-binding antiserum is also quite acceptable.

EXAMPLES 2-13

The procedure of Example I may be followed, except that the following compounds listed below may be employed instead of ANS. The amounts used will be proportional to the blocking power of the compounds as listed in Table I, taking into account solubility considerations. Thus, 2 ug ANNDS per ul of serum can be successfully employed. The results should be comparable to those of Example I.

EXAMPLE	COMPOUND
2	ANNDS
3	TNBS
4	α Napthalene Sulfonic Acid
5	5,5 diphenyl-2 thiohydantoin
6	Doxepin HCl
7	Diazepam
8	Prochlorperazine
9	Dilantin ®
10	Thimerosal
11	Sodium Salicylate
12	Halofenate* (MK-185)
13	Chlorpromazine HCl

*Proprietary compound supplied by Merck, Sharp & Dohme.

EXAMPLE 14

The following example illustrates the use of ANS as a blocking agent in the measurement of T₃ at a variety of concentrations, and illustrates, as well, the making of a standard curve, to allow accurate correlation of the unknown T₄ concentrations with the standard curve. This example serves as the basis for a paper to be shortly published in the Journal of Laboratory and Clinical Investigation under the title: An Improved Radioimmunoassay of Triiodothyronine in Serum.

The reagents employed are:

1. T₃-thyroglobulin conjugate was first prepared in order to make T₃ antibody. The procedure was as follows: L-triiodothyronine (T₃) was conjugated to human thyroglobulin (Tg) by a modification of the method of Oliver et al.,: The measurement of digitoxin in human serum by radioimmunoassay, J. Clin. Invest. 47:1035-1042, 1968. The method of preparation of human Tg was the same as described earlier. Chopra et al.: Production of antibodies specifically binding triiodothyronine and thyroxine, J. Clin. Endocr. 32:299-308, 1970. To 100 mg. of Tg in 2 ml. of phosphate buffered saline (0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.5, PBS), was added 10 mg. of Na-1-T₃ (Mann Research Laboratories, New York) dissolved in 2 ml. of dimethyl formamide and 20 mg. of 1-cyclo-hexyl-3 (2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (Morpho-CDI, Aldrich Chemical Co., Inc., Milwaukee, Wis.). The solution was kept at room temperature in the dark with occasional stirring for 18 hours. The reaction mixture was then dialyzed against 3 changes of normal saline, each time using 4 l for 24 hours at 4° C. The conjugate was stored frozen (-10° C.). Pilot experi-

ments using radioactive T₃ indicated that 60% of the T₃ was incorporated into the conjugate.

2. T₃-binding antiserum was then prepared. The serum used in RIA was obtained from a New Zealand rabbit immunized with 8 injections of the aforescribed Tg-T₃ conjugate, 1 to 2 mg each, emulsified in 1 ml. of complete Freund's adjuvant, at 2-3 week intervals. One hundred ul of a 1:400 dilution of antiserum was used in a final reaction mixture of 1 ml. At this final dilution of 1:4000 it bound 33% of a tracer amount (0.1 ng) of radioactive T₃.
3. In order to make the standard curve (FIG. 3) hypothyroid sheep serum was employed. This sheep serum was obtained from an adult sheep 6 week after a surgical total thyroidectomy. Total T₄ in this sheep serum, as measured by a sensitive RIA for T₄, was less than 1.0 ug per 100 ml. T₄-binding capacity of the thyroxine-binding globulin (TBG) of sheep serum was 17.5 ug per 100 ml.
4. Radioiodinated (¹²⁵I) T₃ (SA 90-100 uci/ug was obtained from Industrial Nuclear Co., Inc., St. Louis, Mo.
5. Reagent grade Na-1-T₃ (non-radioactive) was obtained from Mann Research Labs., New York. It was dissolved and diluted to a concentration of 100 ug/ml in .01M NaOH containing 20% propylene glycol. Dilutions for use in standard curve, i.e., 0.1 ng/ml to 100 ng/ml T₃, were made in 0.075M barbitol buffer, pH 8.6, containing 1% normal rabbit serum and 0.1% sodium azide. Hereafter, this diluent is referred to as barbitol buffer.
6. ANS was obtained from K&K Laboratories, Hollywood, Calif. The steps of the radioimmunoassay procedure follow—In 10×75 mm disposable glass culture tubes, the various reagents were added in the following order: (a) Barbitol buffer, volume to adjust to a final volume of 1 ml; (b) ANS, 250 ug (100 ul of a solution containing 2.5 mg/ml); (c) 250 ul of hypothyroid sheep serum in the standards and an equal volume of test serum in all other tubes; (d) various volumes of four dilutions, e.g., 0.1 ng/ml, 1.0 ng/ml, 10.0 ng/ml and 100 ng/ml, of non-radioactive T₃ to provide 10 pg to 10 ng T₃ in tubes for a standard curve (FIG. 3); (e) 100 ul of 1:400 dilution of T₃-binding rabbit serum; (f) approximately 7000 cpm of T₃-¹²⁵I (~0.1 to 0.15 ng T₃) in 100 ul of barbitol buffer.

After a brief mixing, the tubes were incubated at 4° C. for 24 hours. To precipitate T₃-¹²⁵I bound to rabbit anti-T₃, 75 ul of a previously filtered goat anti-rabbit γ -globulin was added, and the tubes reincubated at 40° C. for 20-24 hour. The details of subsequent separation of bound from free radioactivity, correction for nonspecific binding or trapping of T₃-¹²⁵I in the precipitate and plotting of the standard curve have been described previously. Chopra et al.: Radioimmunoassay for measurement of triiodothyronine in human serum, J. Clin. Invest. 50:2033-2041, 1971.

The results are set forth below:

FIG. 3 shows a typical standard curve obtained in the presence of 250 ul sheep serum and 250 ug ANS. The threshold was 50 pg in this assay and varied between 30 and 50 pg in other assays, corresponding to a T₃ concentration of 12 and 20 ng/100 ml, respectively.

Adequacy of the amount of ANS employed in RIA—It has been previously demonstrated that T₃-binding proteins in serum, such as TBG, interfere in

RIA of T₃ by competing with T₃-binding antibody for radioactive (and stable) T₃. The concentration of ANS required to prevent this interference was determined by adding 25 to 500 ug ANS to tubes containing the typical reaction mixture but no stable T₃; the proportion of T₃-¹²⁵I bound to T₃-binding antibody was compared to that bound to antibody in the absence of sheep serum and ANS. Radioactivity bound to antibody in the presence of 200 ug of ANS was only slightly less (95-98%) than that bound to antibody in plain buffer, indicating thereby an almost complete neutralization of the inhibiting effect of sheet serum TBG. An excess of ANS, i.e., 250 ug. was employed in the final RIA procedure.

Serum T₃ concentration in health and disease—Table IV presents the data on serum T₃ concentration in 148 subjects of whom 96 were healthy and euthyroid, 30 hyperthyroid, 12 hypothyroid and 10 euthyroid with elevated serum TBG either due to estrogen treatment or to a genetic abnormality.

TABLE IV

Serum T ₃ concentration in health and disease.		
Source of Sera	No.	Serum T ₃ (ng/100 ml)
Euthyroid	96	112.8 ± 3.3 (45-216)*
Hyperthyroid	30	490.7 ± 42.3 (176-1120)
Hypothyroid	12	40.1 ± 7.6 (<12-104)
Euthyroid with elevated serum TBG	10	157.6 ± 31.2 (50-353)

*Mean ± S.E.M. (range)

In the euthyroid subjects, serum T₃ varied from 45 to 216 ng per 100 ml with mean ± S.E.M. of 112.8 ± 3.29 ng per 100 ml. The serum T₃ concentration of 30 hyperthyroid patients was 490.7 ± 42.3 ng/100 ml. In 12 hypothyroid subjects, serum T₃ ranged from 12 to 104 ng/100 ml (mean, 40.1 ± 7.65); serum TSH as measured by an RIA² in eight of these patients ranged between 62.5 and 280 uu/ml (normal range 1 to 10 uu/ml). In 10 sera from euthyroid subjects with high serum TBG, T₃ was 157.6 ± 31.2 ng/100 ml; serum T₄ in these sera, measured by the method of Murphy et al: (The determination of thyroxine by competitive protein binding analysis employing an anion exchange resin and radiothyroxine, J. Lab. Clin. Med. 66:161-167, 1965) was 14.8 ± 1.42 ug per 100 ml, range 10-22.5 (normal 4-11). The maximal T₄-binding capacity of TBG as measured in serum of 7 of these subjects by the method of Inada and Sterling (Inada, M. and Sterling, K.: Thyroxine transport in thyrotoxicosis and hypothyroidism, J. Clin. Invest. 46:1422-1450, 1967,) ranged between 40.7 and 56.3 ug per 100 ml (mean 46.9).

² Gross, J., Pitt-Rivers, R. and Trotter, W. R.: Effect of 3:5:3'-L-triiodo-thyronine in myxedema, Lancet 1:1044-1045, 1952.

This procedure is sensitive enough to allow reliable measurements of serum T₃ not only in hyperthyroid patients but also in euthyroid and hypothyroid subjects. While this may be attributed in part to the use of a T₃-binding antiserum which usually allows detection of 0.03 ng of T₃ in comparison to 0.1 ng T₃ detected by the antiserum used previously known, the major improvement in sensitivity is a result of the use of nonthyroid hormone blocking agent, e.g. ANS. Also, ANS has some advantages over compounds, such as Dilantin and tetrachlorthyronine including better solubility at pH of RIA, lower cost and ready availability.

A mean serum T₃ concentration of 113 ng per 100 ml in euthyroid subjects obtained by the use of the present method is comparable to 120, 110 and 105 ng per 100 ml observed by some other investigators, using another RIA of T₃. However, it is believed that the difference in

the mean normal serum T₃ of 138 ng per 100 ml reported by these investigators, (Mitsuma et al.,: Radioimmunoassay of triiodothyronine in unextracted human serum. J. Clin. Endocr. 33:364-367, 1971) and 113 ng per 100 ml obtained by this procedure is indicative of random sample variation.

It is concluded that measurement of serum T₃ by RIA affords an adequate separation of hyperthyroid and hypothyroid patients from normal subjects.

EXAMPLES 15-26

The procedure of Example 14 would be followed except that the below listed compounds will be employed instead of 250 ug ANS, respectively. The amounts used will be proportional to the blocking power of the compounds as listed in Table II, taking into account solubility considerations. Thus, 1 ug ANNDS per ul of serum can be successfully employed. The results would be comparable to those obtained in Example 14.

EXAMPLE	COMPOUND
15	ANS
16	ANNDS
17	TNBS
18	αNaphthalene Sulfonic acid
19	5,5 diphenyl-2 thiohydantoin
20	Doxepin HCl
21	Diazepam
22	Prochlorperazine
23	Thimerosal
24	Sodium Salicylate
25	Halofenate* (MK-185)
26	Chlorpromazine HCl

*Proprietary compound supplied by Merck, Sharp & Dohme.

While various modifications of the invention have been herein described, various modifications of this invention will become apparent to those skilled in the art, and the scope of the invention is to be determined by the claims which follow.

I claim:

1. A method of measurement of the concentration of a particular thyroid hormone selected from the group consisting of thyroxine (T₄) and triodo-L-thyronine (T₃) in unextracted human serum, which comprises:

- A: adding to a measured quantity of unextracted human serum,
- a. a blocking agent selected from the group consisting 8-anilino-1-naphthalene sulfonic acid and 3-(4-anilino-1-naphthylazo) 2,7 naphthalene disulfonic acid, and present in an amount sufficient to displace essentially all of said particular thyroid hormone to be measured from thyroxine-binding globulin (TGB),
 - b. buffering ions to buffer said serum to a pH of between about 6.8 to about 9.6,
 - c. radioactive thyroid hormone, of the type to be measured, in an amount which will give a measurable counting rate of either antibody bound or free radioactivity after reaction equilibrium has been reached as set forth in Step B below; and
 - d. an antibody in sufficient quantity to bind a significant quantity of said radioactive thyroid hormone in the absence of any of the particular nonradioactive thyroid hormone to be measured,

B: allowing reaction of both particular thyroid hormone, to be measured, and said radioactive thyroid

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hormone, with said antibody to proceed substantially to equilibrium to thereby produce antibody bound radioactive thyroid hormone;

C: separating said antibody bound radioactive thyroid hormone, to be measured from said free radioactive thyroid hormone;

D: measuring the quantity of radioactive thyroid hormone selected from antibody bound radioactive thyroid hormone and free radioactive thyroid hormone;

E: preparing a standard curve with known amounts of the particular thyroid hormone to be measured, and

F: correlating the quantity of radioactive thyroid hormone measured with a known amount of said particular thyroid hormone read from said standard curve.

2. The method of claim 1 wherein said thyroid hormone to be measured is thyroxine, said blocking agent is 8-anilino-1-naphthalene disulfonic acid, said blocking agent being added to said human serum in quantities of at least about 4 ug/ul serum.

3. The method of claim 1 wherein said buffering ions are barbital ions, and said pH of said serum is about 8.6.

4. The method of claim 1 wherein said radioactive thyroid hormone is initially present in the order of 10,000 counts per minutes.

5. The method of claim 1 wherein said antibody bound radioactive thyroid hormone after reaction, gives a counting rate of from about 2000-6000 counts per minute.

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6. The method of claim 1 wherein said antibody bound radioactive thyroid hormone, to be measured, is separated from said free radioactive thyroid hormone by precipitation of said antibody bound radioactive thyroid hormone with a second antibody.

7. The method of claim 1 wherein said antibody bound radioactive thyroid hormone, to be measured, is separated from said free radioactive thyroid hormone by precipitation of said antibody bound radioactive thyroid hormone with gamma globulin.

8. The method of claim 1 wherein said antibody bound radioactive thyroid hormone, to be measured, is separated from said free radioactive thyroid hormone by precipitation of said antibody bound radioactive thyroid hormone with charcoal suspension.

9. The method of claim 1 wherein said antibody bound radioactive thyroid hormone, to be measured, is separated from said free radioactive thyroid hormone by precipitation of said antibody bound radioactive thyroid hormone with polyethylene glycol.

10. The method of claim 1 wherein said thyroid hormone to be measured is T₄, said blocking agent is 2,7 naphthalene disulfonic acid, said blocking agent being added to said human serum in quantities of at least about 2 ug/ul serum.

11. The method of claim 1 wherein said thyroid hormone to be measured is T₃, said blocking agent is 2,7 naphthalene disulfonic acid and said blocking agent is added to said human serum in quantities of at least about 1 ug/ul serum.

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**UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION**

PATENT NO. : RE.32,098
DATED : March 25, 1986
INVENTOR(S) :INDER J. CHOPRA

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 13, line 20, "disulfonic" should read:
sulfonic;

Column 14, line 22, prior to "2,7" insert:
3-(4 anilino-1-naphthylazo)

line 27, prior to "2,7" insert:
3-(4 anilino-1-naphthylazo)

Signed and Sealed this

Twelfth Day of August 1986

[SEAL]

Attest:

DONALD J. QUIGG

Attesting Officer

Commissioner of Patents and Trademarks