Use of 8-Anilino-1-naphthalenesulfonic Acid in Radioimmunoassay of Triiodothyronine

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Abstract □ The rationale for the use and effects of 8-anilino-1-naphthalenesulfonic acid in the radioimmunoassay of triiodothyronine is discussed. Historical background for the use of fluorescent probes, such as 8-anilino-1-naphthalenesulfonic acid, to study protein sites is outlined. By using 8-anilino-1-naphthalenesulfonic acid to block the binding of triiodothyronine to thyroxine binding globulin, it is possible to design a sensitive and precise radioimmunoassay for measurement of triiodothyronine in unextracted serum samples. Recovery of triiodothyronine added to serum was excellent, and hyperthyroid serum assayed at various dilutions produced a curve that was virtually identical with the standard curve. Standard curves are prepared either in triiodo-/tetraiodothyronine-free serum or human serum albumin to maintain the same milieu between standards and serum samples. Triiodothyronine values by radioimmunoassay ranged from 80 to 250 ng/100 ml in 243 euthyroid subjects. In patients with thyroid disease, the serum triiodothyronine concentrations were as follows: hypothyroid (n = 27), <36–145 ng/100 ml; and hyperthyroid (n = 42), 273– >1000 ng/100 ml. Twenty-two normal pregnant women had triiodothyronine serum concentrations ranging from 174 to 410 ng/100 ml. To assess a patient's thyroid status properly, serum triiodothyronine measurements are required. The triiodothyronine assay appears to be a sensitive index, particularly in the evaluation of thyroid hyperfunction.

Keyphrases □ 8-Anilino-1-naphthalenesulfonic acid—used as probe for radioimmunoassay of triiodothyronine, relevance to evaluation of thyroid hyperfunction □ Triiodothyronine—use and effects of 8-anilino-1-naphthalenesulfonic acid, radioimmunoassay □ Thyroid hyperfunction—triiodothyronine radioimmunoassay using 8-anilino-1-napththalenesulfonic acid as fluorescent probe □ Radioimmunoassay—8-anilino-1-naphthalenesulfonic acid as fluorescent probe in analysis of triiodothyronine □ Fluorescent probes—8-anilino-1-naphthalenesulfonic acid, radioimmunoassay of triiodothyronine

In a previous paper (1), it was demonstrated that highly specific antibodies to triiodothyronine can be produced by coupling it to human serum albumin and that the antiserum was suitable for the detection of picogram quantities of triiodothyronine from unextracted serum. Additional clinical experience with the radioimmunoassay of triiodothyronine has demonstrated quite clearly that serum proteins, particularly thyroxine binding globulin, compete with the antibody for binding of both labeled and unlabeled triiodothyronine. This interference has significant effects on the assay and, of course, on the triiodothyronine value reported for the patient.

Thyroxine, tetrachlorothyronine, diphenylhydantoin, sodium salicylate, and 8-anilino-1-naphthalenesulfonic acid are some of the compounds used by various investigators to block the binding of triiodothyronine to thyroxine binding globulin (2–6).

Additional problems may be caused by displacement of the radioactive label from the antibody in the presence of serum, even though it is free of the antigen in question. Therefore, most radioimmunoassays include antigen-free serum or plasma in the preparation of the standard curve. Mitsuma *et al.* (3) and Larsen (5) observed displacement of labeled triiodothyronine by triiodothyronine-free human serum. It has been suggested that the dextran-coated charcoal method of separating antibody bound ^{125}I-triiodothyronine from free ^{125}I-triiodothyronine has the advantage over double-antibody systems in that the effect of thyroxine binding globulin on the distribution of ^{125}I-triiodothyronine in the serum sample can be recognized and a correction applied (5). The comparative merits of these two systems in the radioimmunoassay of triiodothyronine are presently being evaluated in this laboratory.

This paper examines: (*a*) the rationale for the use of 8-anilino-1-naphthalenesulfonic acid in the triiodothyronine assay; (*b*) effects of adding 8-anilino-1-naphthalenesulfonic acid to the assay; and (*c*) any possible differences that may result from preparation of the standard curve in triiodo-/tetraiodothyronine-free serum, plain phosphate-buffered saline buffer, or human serum albumin.

EXPERIMENTAL

Materials—The following were used: L-triiodothyronine[1], charcoal[2], ^{125}I-triiodothyronine[3] with specific activity of 350–544 mCi/mg, and the probe, 8-anilino-1-naphthalenesulfonic acid[4]. Antiserum to triiodothyronine was produced in rabbits as described previously (1).

Preparation of Triiodo-/tetraiodothyronine-Free Serum—To maintain the same milieu between standards and serum sample and to eliminate any differences that may occur between standard curves prepared in buffer and in serum, a serum with a very low triiodo-/tetraiodothyronine content was prepared as follows. Ten grams of charcoal[2] was added to 100 ml of normal human serum and incubated at 4° for 24 hr. The serum was then centrifuged for three 1-hr periods at 15,000×g. The procedure removed over 95% of the triiodo- and tetraiodothyronine (comparing values before and after charcoal addition by radioimmunoassay and competitive protein binding assay) and did not affect thyroxine binding globulin binding capacity as shown by the triiodothyronine resin uptake test.

Determination of Quantity of 8-Anilino-1-naphthalenesulfonic Acid Required to Prevent Thyroxine Binding Globulin Interference—Various concentrations of 8-anilino-1-naphthalenesulfonic acid were incubated with ^{125}I-triiodothyronine, triiodothyronine antiserum, and serum samples from euthyroid, hyperthyroid, hypothyroid, and pregnant patients. In other experiments, an excess of the probe (700 μg/0.1 ml of serum) and no probe were added to the assay system to determine effects on the triiodothyronine values of a group of euthyroid patients. The object of using 8-anilino-1-naphthalenesulfonic acid in the assay of triiodothyronine is twofold: (*a*) to displace triiodothyronine bound to thyroxine binding globulin in the patient's serum so that the triiodothyronine can be recognized by the triiodothyronine antibody, and (*b*) to prevent binding of ^{125}I-triiodothyronine

[1] Sigma Chemical Co.
[2] Norit A, Sigma Chemical Co.
[3] Abbott Laboratories.
[4] Eastman Kodak Co.

to thyroxine binding globulin so that all of the ^{125}I-triiodothyronine is available for binding to the triiodothyronine antibody.

Patient Dilution Curve—Hyperthyroid serum was assayed at various dilutions from 0.010 to 0.100 ml. Triiodo-/tetraiodothyronine-free serum or human serum albumin (4.5%) was used to maintain a consistent serum sample size of 0.100 ml.

Triiodothyronine Radioimmunoassay Procedure—Reagents were added to disposable 10 × 75-mm polystyrene tubes as follows:

Preincubation—

1. Delivery of the triiodothyronine standard solution was such that the concentration ranged from 10 to 1000 pg for a 10-point standard curve or 0.1 ml of the serum to be assayed. Each point on the standard curve was assayed in triplicate.
2. Triiodo-/tetraiodothyronine-free serum (0.1 ml) or 4.5% human serum albumin was added to the 0% control tubes, the 100% control tubes, and tubes containing the standard curve.
3. A 1:5000 dilution (0.1 ml) of triiodothyronine antibody containing 1% normal rabbit serum and 0.05 *M* disodium edetate in phosphate-buffered saline buffer (pH 7.4) was added to all tubes except the 0% control tubes.
4. Phosphate-buffered saline buffer (pH 7.4) was added sufficient to adjust the final total volume to 1.0 ml. The tubes were vortexed and incubated at 4° for 24 hr.

Incubation 1—

5. Phosphate-buffered saline buffer (0.1 ml) containing 50 pg of ^{125}I-triiodothyronine (350–544 mCi/mg), 60 μg of 8-anilino-1-naphthalenesulfonic acid, and 0.25% human serum albumin was added to all tubes. The tubes were vortexed again and incubated at 4° for an additional 48 hr.

Incubation 2—

6. Precipitation of ^{125}I-triiodothyronine bound to antibody was accomplished by adding 0.1 ml of a previously titered goat anti-rabbit γ-globulin to all tubes and incubating at 4° for 24 hr.

Separation and Calculations—The details of separation of antibody-bound ^{125}I-triiodothyronine and free ^{125}I-triiodothyronine by centrifugation, correction for nonspecific binding, and calculation of results were described previously (1).

Serum Specimens—Serum was obtained from euthyroid healthy volunteers, normal pregnant women, and hyper- and hypothyroid patients[5]. The blood was allowed to clot, and the serum was separated by centrifugation and frozen at −20° unless assayed within 2 days.

RESULTS

Probe Concentration—Concentrations ranging from 70 to 1400 μg of the probe were added to serums containing different quantities of thyroxine binding globulin. Maximum percent binding of ^{125}I-triiodothyronine to triiodothyronine antiserum occurred when the probe concentration was approximately 70 μg/0.1 ml of serum in all samples (Fig. 1). Further dilution of the probe in a range of 3.75–150 μg/0.1 ml of serum showed maximum percent binding occurring between 37.5 and 75 μg. As a result, 60 μg/0.1 ml of serum was chosen for routine use in all triiodothyronine assays. The effects of no probe and an excess concentration of probe on the triiodothyronine assay were studied by assaying 18 euthyroid serum samples. Triiodothyronine serum concentrations in euthyroid serum samples containing no probe ranged from 330 to 540 ng % (437 ± 13.1, mean ± *SE*). In samples containing 60 μg of probe, the triiodothyronine concentration was 150–245 ng % (189 ± 5.9, mean ± *SE*). The samples containing 700 μg of probe contained 153–267 ng % triiodothyronine (196 ± 7.1, mean ± *SE*).

Standard Curves—Standard curves prepared in 4.5% human serum albumin or in plain phosphate-buffered saline buffer were virtually superimposable, while the curve prepared in triiodo-/tetraiodothyronine-free serum was shifted slightly to the right of that in plain phosphate-buffered saline buffer in the area of concentration of 10–200 pg triiodothyronine. Figure 2 shows a comparison in the displacement of ^{125}I-triiodothyronine by increasing quantities of triiodothyronine and a hyperthyroid serum. Results of diluting the serum with either triiodo-/tetraiodothyronine-free serum or 4.5% human serum albumin to maintain a consistent serum sample of 0.1 ml are shown in Table I. Displacement of 50% of ^{125}I-triiodothyronine from the antibody was produced by a mean triiodothyronine concentration of 118.6 ± 2.2 (*SE*) pg in 10 assays.

[5] Followed or treated at the Wilford Hall USAF Medical Center.

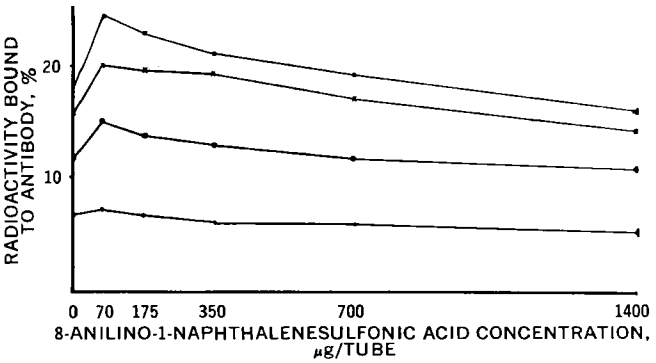

Figure 1—*Effect of addition of increasing quantities of 8-anilino-1-naphthalenesulfonic acid to different serum samples on the binding of ^{125}I-triiodothyronine to triiodothyronine antiserum. Key: ■, serum from hypothyroid subject; ×, serum from euthyroid subject; ○, serum from pregnant subject; and ●, serum from hyperthyroid subject.*

Recovery of Triiodothyronine Added to Serum—Serum from euthyroid, hypothyroid, and pregnant subjects was enriched with triiodothyronine in concentrations ranging from 50 to 500 ng/100 ml. The recovery of added triiodothyronine (Table II) averaged 105.3 ± 2.7% (mean ± *SE*).

Reproducibility of Triiodothyronine Assay—Within-assay variability was evaluated by duplicate determinations of serum from a euthyroid subject assayed as four pairs and was found to be 210 ± 1.3 (*SE*) ng %. The percent standard deviation within the assay was 1.9%. Between-assay variability was evaluated by duplicate measurements of euthyroid and hyperthyroid serums in the same and in 10 different assays by four technicians. The euthyroid serum triiodothyronine concentration was 160 ± 3.5 (*SE*) ng %, and the percent standard deviation was 6.9. The hyperthyroid serum was assayed as 283 ± 5.0 (*SE*) ng %, and the percent standard deviation was 7.3%.

Clinical Results—Triiodothyronine levels were determined in euthyroid subjects, hyper- and hypothyroid patients, and a group of normal pregnant patients (Table III). The quantity of serum assayed was 0.1 ml, and 27–52 pg of unlabeled triiodothyronine [36 ± 2.2 (*SE*) pg for 10 assays] produced meaningful inhibition (20%) of ^{125}I-triiodothyronine binding to antibody; therefore, the minimum detectable triiodothyronine concentration was generally 36 ng/100 ml. The 243 euthyroid subjects had tetraiodothyronine concentrations of 4.5–11.5 μg/100 ml [7.7 ± 1.5 (*SD*) μg %, uncorrected for extraction, extraction efficiency = 83.2%] as determined by the Murphy (7) procedure. The triiodothyronine resin

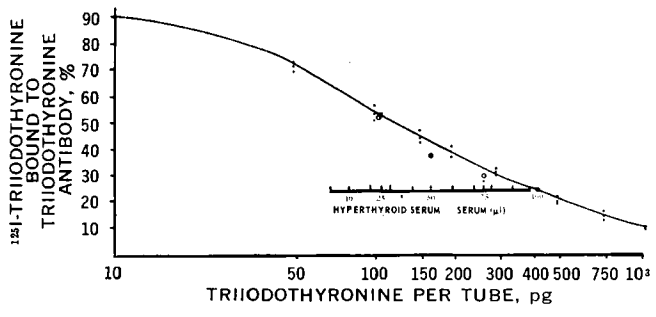

Figure 2—*Dose-response curve prepared in 0.1 ml of 4.5% human serum albumin, showing inhibition of binding of ^{125}I-triiodothyronine by unlabeled triiodothyronine and a hyperthyroid serum. Serum is matched to curve at the 0.1-ml dilution and shows reasonable fit at various dilutions along the standard curve. Key: ×, serum diluted to 0.1 ml with 4.5% human serum albumin; and ○, serum diluted to 0.1 ml with triiodo-/tetraiodothyronine-free serum.*

Table I—Triiodothyronine Concentrations in Dilutions of Serum from a Hyperthyroid Patient

Amount of Serum Assayed, ml	Triiodothyronine[a]	Triiodothyronine
0.1	410[b]	410[c]
0.075	335	305
0.050	205	205
0.025	103	105
0.010	44	54

[a] Triiodothyronine in ng/100 ml for all dilutions. [b] Serum diluted to 0.1 ml with 4.5% human serum albumin. [c] Serum diluted to 0.1 ml with triiodo-/tetraiodothyronine-free serum.

uptake values for this group ranged from 24.1 to 36.4% [30.4 ± 2.7 (*SD*) %]. In hyperthyroid patients, serum triiodothyronine concentrations ranged from 273 to >1000 ng/100 ml, all values being greater than the highest of the euthyroid group. There was some overlap between the hypothyroid and euthyroid subjects. Six patients in the hypothyroid group had triiodothyronine concentrations in the euthyroid range of 80–250 ng/100 ml. Normal pregnant women from 19.5 to 40 weeks of gestation had serum triiodothyronine concentrations of 170–410 ng/100 ml.

DISCUSSION

A major improvement in the radioimmunoassay of serum triiodothyronine has been accomplished by the use of 8-anilino-1-naphthalenesulfonic acid, which is a fluorescent dye with the general formula $C_6H_5NHC_{10}H_6SO_3H$. The use of dyestuffs to characterize human serum was introduced over 50 years ago when the ability of serum and its fractions to bind dyes was demonstrated (8, 9). Bennhold (10) proposed the transporting function of human serum albumin through the similar use of a variety of dyes.

Many studies from 1935 to 1955 elaborated on the use of dyestuffs for serum protein characterization. The practical importance of the phenomenon of protein absorption of dyes was demonstrated, thus establishing a foundation for research in this area (11, 12). In the early 1950's, Weber (13) and Lawrence (14) developed and utilized the method of measurement of the polarization of light emitted by excited fluorescent molecules in characterizing the binding properties of human serum albumin. They reported later on the significance of particular fluorescing dyes of the naphthalene and acridine series which possessed anilino or certain substituted anilino groups. They demonstrated that only in the presence of organic molecules such as those in serum would these dyes fluoresce in aqueous solvents (15).

By 1968, the development of the use of fluorescent probes had significantly advanced. Stryer (16) showed 8-anilino-1-naphthalenesulfonic acid to be an excellent probe in characterizing the binding of heme to apomyoglobin and apohemoglobin and utilized its ability to displace heme from the binding sites on each.

Table II—Recovery of Triiodothyronine Added to Serum

Patient Serum	Initial Serum Triiodothyronine[a]	Triiodothyronine Added	Triiodothyronine		Recovery, %
			Expected	Recovered	
Pregnant subject	255	50	305	300[b]	98.4
		100	355	345	97.2
		200	455	490	107.7
		500	755	920	121.9
Hypothyroid subject	62	50	112	112	100
		100	162	158	97.5
		200	262	260	99.2
		500	562	610	108.5
Euthyroid subject	159	50	209	205	98.1
		100	259	265	102.3
		200	359	390	108.6
		500	659	820	124.4
	Average (mean ± *SE*):				105.3 ± 2.7

[a] All triiodothyronine serum concentrations in ng/100 ml. [b] Mean of duplicate determinations.

Table III—Serum Triiodothyronine Concentrations

Clinical Status	Number	Range, ng/100 ml	Mean Range, ng/100 ml
Euthyroid	243	80–250	183 ± 41 (mean ± *SD*)
Hyperthyroidism	42	273–>1000	—
Hypothyroidism	27	<36–145	—
Normal pregnancy	22	174–410	275 ± 12.4 (mean ± *SE*)

McClure and Edelman (17–19) elaborated on this by using different fluorescent probes with several serum proteins and studying the binding characteristics of each.

More recently, Green *et al.* (20) used 8-anilino-1-naphthalenesulfonic acid to characterize the binding site of thyroxine binding globulin, demonstrating that 8-anilino-1-naphthalenesulfonic acid competes with thyroxine for the same binding site. In the field of radioimmunoassay, Chopra utilized this property of 8-anilino-1-naphthalenesulfonic acid to prevent protein interference in the radioimmunoassay of triiodothyronine (6) and thyroxine (21).

It has been established that thyroxine binding globulin is a glycoprotein consisting of a linear series of peptide bonds with amino acid and carbohydrate side chains (19, 22, 23). Due to environmental interaction with solvents, with other solutes, and within itself, thyroxine binding globulin takes on a folded and twisted configuration (19). With the use of fluorescent probes, thyroxine binding globulin has demonstrated only one binding site for triiodothyronine and tetraiodothyronine. This has been characterized as being in a severe fold of a hydrophobic nature interspersed with amino acid residues that possibly can ionize in the presence of strong electronegative groups to become positively charged (14–17, 19, 20, 22–27).

The bond also exhibits properties of hydrogen bonding with triiodothyronine and tetraiodothyronine, due perhaps to the presence of the imidazole ring of histidine, the nitrogens of which have a certain affinity for hydrogen (25, 27, 28). Thus, thyroxine binding globulin apparently binds to small molecules exhibiting a hydrophobic nature and capable of hydrogen bonding and/or possessing high electronegativity. Additional studies using analogs of tetraiodothyronine have shown that molecules possessing hydroxy groups, amino side chains, a preponderance of iodine on a phenyl ring structure (distal to the amino acid group), and large, bulky naphthoxy or naphthalene groups have a definite and greater affinity for thyroxine binding globulin (26, 27). An examination of the chemical structure of triiodothyronine and 8-anilino-1-naphthalenesulfonic acid shows that both of these small molecules meet these criteria and may, therefore, be expected to exhibit a certain affinity for thyroxine binding globulin.

The hydrophobic nature of the diphenyl ether structure of triiodothyronine or the naphthalene group of the probe satisfies one basic requirement of binding to thyroxine binding globulin (14–17, 26, 27). (The bulky naphthalene structure of the probe satisfies another requirement of strong affinity for thyroxine binding globulin. This suggests that the binding site of thyroxine binding globulin is better occupied by a molecule of tighter fit such as that exhibited by tetraiodothyronine analogs with naphthoxy or naphthalene groups.) The triiodothyronine molecule possesses iodine on the outer phenolic ring, and the iodine's electronegative character is particularly important in the distal conformation. This conformation shows not only tighter binding but more hormonal activity (29, 30). This further suggests that an induced positive ionic residue is in close proximity to the electronegative iodine of the distal conformation rather than the proximal triiodothyronine conformation due to the relative bond strengths (27).

Similarly, the sulfonate group of the probe possesses high electronegativity and would seem to induce electrostatic attractions with amino acid residues that easily assume a positive ionic behavior (14, 25). The hydrogen bonding proposed between the hydroxyl group of triiodothyronine and the thyroxine binding globulin binding site is also important. The presence of this bond would attest to a definite affinity of thyroxine binding globulin for triiodothyronine as proposed (25–28). The relative impor-

tance of each characteristic in the affinity of thyroxine binding globulin for triiodothyronine and 8-anilino-1-naphthalenesulfonic acid infers a difference in binding ability.

Comparisons of the relative affinity of tetraiodothyronine and triiodothyronine for thyroxine binding globulin have been made. When assuming that levothyroxine has a relative affinity of 100, distal triiodothyronine has an affinity of 3.3 and proximal triiodothyronine has an affinity of 0.8 (27). The association constants of tetraiodothyronine and the probe for thyroxine binding globulin have been reported as follows: K_a tetraiodothyronine = 2.35 ± 0.43 × 10^{10} m^{-1} at 23°, and K_{assoc} 8-anilino-1-naphthalenesulfonic acid = 2.09 ± 0.16 × 10^6 m^{-1} at 37° (20). Scatchard (31) reported the K_{assoc} 8-anilino-1-naphthalenesulfonic acid for thyroxine binding globulin as 4.19 ± 0.16 × 10^6 m^{-1} at 23°. Thus, when triiodothyronine and the probe compete for thyroxine binding globulin binding sites, there is indeed a competitive equilibrium reaction.

It can be stated, therefore, that thyroxine binding globulin has a decreasing affinity for tetraiodothyronine, triiodothyronine, and the probe, respectively, and that when the probe is present in sufficient concentrations, it displaces almost all of the tetraiodothyronine and triiodothyronine from thyroxine binding globulin. The quantity remaining bound to thyroxine binding globulin would be inconsequential.

If endogenous triiodothyronine was not displaced from thyroxine binding globulin, unmeasurable serum triiodothyronine concentrations would be expected. This is not the case. Recovery of triiodothyronine added to serum is also excellent, thus suggesting strongly the validity of the radioimmunoassay method. When 500 ng % of triiodothyronine was added to the serums from pregnant and euthyroid subjects, the percent recovery was much greater than expected. This was primarily due to the flattening out of the curve in the assay system at levels >500 ng/100 ml. When hypothyroid serum is used, percent recovery is excellent all along the standard curve. Excess concentrations of the probe appear to have no significant effects on the antigen–antibody reaction, as shown by the lack of significant differences (paired t-test, p >0.10) in mean serum triiodothyronine concentrations of 18 euthyroid subjects in assays containing 60 and 700 μg 8-anilino-1-naphthalenesulfonic acid/assay tube. This would also suggest that even with probe concentrations in excess of those required to displace triiodothyronine and tetraiodothyronine from thyroxine binding globulin since tetraiodothyronine has a greater affinity for thyroxine binding globulin than either triiodothyronine or the probe (thus requiring more 8-anilino-1-naphthalenesulfonic acid for displacement from thyroxine binding globulin), tetraiodothyronine does not alter the measured triiodothyronine value because there is little difference between the means of the 60 and 700 μg 8-anilino-1-naphthalenesulfonic acid–triiodothyronine values. This would be expected since the triiodothyronine antibody was previously shown to lack significant cross reaction with tetraiodothyronine (1).

Significant differences of triiodothyronine values did not occur between standard curves prepared in 4.5% human serum albumin or triiodo-/tetraiodothyronine-free serum. Since the standard curve containing the triiodo-/tetraiodothyronine-free serum was not displaced to the left of that prepared in buffer, it may be assumed that the triiodo-/tetraiodothyronine-free serum is, in fact, essentially free of triiodothyronine. The slight displacement of the serum standard curve to the right of the buffer curve may be due to nonspecific interactions of serum proteins on the equilibrium reaction. Tubes containing triiodothyronine antibody, ^{125}I-triiodothyronine, and either triiodo-/tetraiodothyronine-free serum or human serum albumin (100% tubes) have a lower percent binding than those containing only buffer. This suggests that triiodothyronine may be binding to certain serum proteins or, again, that there is a nonspecific inhibition by serum protein on the antigen–antibody equilibrium. Serum triiodothyronine concentrations are significantly higher when the standard curve is prepared in plain buffer, due to the higher percent binding of ^{125}I-triiodothyronine to the antibody when serum proteins are not present.

With the use of 8-anilino-1-naphthalenesulfonic acid as a thyroxine binding globulin blocking agent and either 4.5% human serum albumin or triiodo-/tetraiodothyronine-free serum in the preparation of the standard curve, the radioimmunoassay procedure is an accurate measure of the concentration of triiodothyronine in serum. The excellent recovery of exogenous triiodothyronine added to various serum samples, the virtually identical curve of different dilutions of serum from hyperthyroid subjects with that of the standard curve, and the satisfactory within- and between-assay variability all validate the radioimmunoassay procedure.

The triiodothyronine serum concentration in euthyroid subjects of 183 ± 41 (*SD*) ng % reported here is in reasonable agreement with the mean range of values of 102–218 ng/100 ml determined by other radioimmunoassay procedures (32, 33). Differences in triiodothyronine serum concentrations determined by different radioimmunoassay systems may be due to: (*a*) specific factors within the assay such as sensitivity, specificity, and nonspecific serum effects; (*b*) geographic location and dietary iodine; and (*c*) sample size.

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