The radioimmunoassay of triiodothyronine and its clinical application

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Since the identification of triiodothyronine (T3) in blood and thyroid tissue by Gross and Pitt Rivers in 1952, relatively little information had accrued until the latter part of the past decade concerning the role of this hormone in normal physiology and that of the thyroid gland. The major difficulty in obtaining this knowledge was the lack of simple, reliable and specific methods for quantitation of T3 in blood and other biological fluids. The development of gas chromatographic (Nauman, Nauman, and Werner, 1967) and saturation analysis techniques (Sterling, Bellabarba, Newman, and Brenner, 1969) for the measurement of T3 in serum provided a new impetus in this area. In 1968 Hollander established the existence of a clinical state of hyperthyroidism in which an increase of T3 appeared to be the major pathogenic factor. This finding has subsequently been confirmed by other workers (Sterling, Refetoff, and Selenkow, 1970; Wahner and Gorman, 1971). It is now well established that as much as 50% of the T4 secreted by the thyroid may be converted to T3 by peripheral deiodination (Brauerman, Ingar, and Sterling, 1970). It is even possible that T3 is the sole biologically active thyroid hormone, as conversion of T4 to T3 in vivo may be an obligatory step in the metabolic action of T4 at cellular level, T4 being thus relegated to the role of an inactive prohormone.

Although the saturation analysis technique introduced by Sterling has proved useful, it has not been widely adopted for clinical diagnostic use as it is complex, tedious to perform, and requires large volumes of blood for assay. More importantly, this method is subject to artefactual errors which produce inconstant overestimates of serum T3 concentration (Fisher and Dussault, 1971; Larsen, 1971a). A significant advance in T3 assay methodology was the production of specific T3 antibodies by Brown, Ekins, Ellis, and Reith (1970) and subsequently the development of a sensitive and precise radioimmunoassay for T3 in serum extracts (Brown, Ekins, Ellis, and Williams).

Principles and Problems of T3 Radioimmunoassay in Whole Serum

Early attempts to measure T3 in whole serum by radioimmunoassay were unsuccessful due largely to interference by endogenous thyroxine-binding globulin (TBG). Theoretically, it is possible to measure T3 by radioimmunoassay in the presence of TBG, if the avidity of the antiserum for T3 greatly exceeds that of TBG. In practice, however, this has proved very difficult. A novel approach to overcoming the problem of TBG interference has been the use of chemical compounds structurally similar to T3, which competitively inhibit binding of T3 to TBG. Compounds which have been employed successfully in this role are listed in table I. Thyroxine was first employed by Chopra, Solomon, and Beall (1971) to saturate the binding sites of TBG present in the test serum, thus displacing T3 bound to TBG; then serum T3, in addition to that added as tracer and as standard, is free to react with the specific T3 antibody. Although effective, T4 has not been widely used as an inhibitor because of the variable contamination of most T4 preparations with T3, the possibility of spontaneous deiodination of T4 to T3 in the incubation medium, and the

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**Fig 1** Standard curve for T₃ using a large selection of antisera.

Intrinsic cross reaction of T₄ with many T₃ antisera. Tetrachlorothyronine (TCT) is a potent competitive inhibitor of TBG and has been employed successfully in the radioimmunoassay of T₃ (Mitsuma, Gershengorn, Colucci, and Hollander, 1971); however, we have found that commercial preparations of TCT crossreact with every antiserum we have tested. It is possible that this cross reaction is due mainly to contamination with trichlorothyronine. Diphenylhydantoin will effectively inhibit T₃ binding to TBG (Lieblich and Utiger, 1972), but considerable practical problems have been encountered with its use due to the insolubility of this compound in aqueous solutions except under very alkaline conditions. Similar problems have been found using diazepam and other inhibitors which are relatively insoluble in aqueous solutions. Salicylate (Larsen, 1972) and methylolate (Hesch, Hüfner, and Von Zu Mühlen Mühlen, 1972) have also been used; these compounds show minimal or no cross reaction with most T₃ antisera and have the added advantage of inhibiting T₃ binding to thyroxine-binding prealbumin (TBPA) (Larsen, 1971b). Of the compounds we have tested so far, 8-anilino-1-naphthalene sulphonate acid (ANS) (Chopra, 1972; Mitsuma, Colucci, Shenkman, and Hollander, 1972; Chopra, Ho, and Lam; Eastman, Corcoran, Jequier, Ekins, and Williams, 1973) is the most potent inhibitor of T₃ and T₄ binding to TBG, showing no significant cross reaction with and causing no alter-

Table II Protocol for T₃ radioimmunoassay

<table>
<thead>
<tr>
<th>Details of Radioimmunoassay Method</th>
<th>Materials and Methods</th>
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<tbody>
<tr>
<td><strong>1 Reagents</strong></td>
<td>Barbitone buffer 0-05 M, pH 8-6 containing 0-05% bovine serum albumin</td>
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<tr>
<td></td>
<td>Serum treated with charcoal to remove T₃ and T₄</td>
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<tr>
<td></td>
<td>8-Anilino-1-naphthalene-sulphonic acid (ANS) 1 mg/ml</td>
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<td></td>
<td>T₃ Standards—Sequential dilutions in barbitone buffer</td>
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<tr>
<td></td>
<td>T₃ Antiserum 1/10000 final dilution in barbitone buffer</td>
</tr>
<tr>
<td><strong>2 Incubation Mixtures</strong></td>
<td>0-1 ml antiserum 1/10 000</td>
</tr>
<tr>
<td></td>
<td>0-1 ml ANS (100 µg)</td>
</tr>
<tr>
<td></td>
<td>0-1 ml T₃ standards or buffer in standards and tests respectively</td>
</tr>
<tr>
<td></td>
<td>0-05 ml unknown serum or T₁-free serum in tests and standards respectively</td>
</tr>
<tr>
<td></td>
<td>0-1 ml ¹²⁵I T₄ (30 pg)</td>
</tr>
<tr>
<td></td>
<td>Adjust final volume to 1-0 ml with barbitone buffer For each tube set up a control without antisera</td>
</tr>
<tr>
<td><strong>3 Incubation</strong></td>
<td>24 hours at 4°C</td>
</tr>
<tr>
<td><strong>4 Separation</strong></td>
<td>Charcoal</td>
</tr>
<tr>
<td><strong>5 Count bound and free fractions</strong></td>
<td>For each tube set up a control without antisera</td>
</tr>
</tbody>
</table>

T₄ in the standards to make the protein content especially TBG and TBPA, similar to that of the unknown sera. Thyroid hormone-free serum is readily prepared by repeated treatment of pooled serum with charcoal, using added ¹²⁵I T₄ to monitor the efficiency of extraction. The use of 100 µg ANS per 50 µl of serum represents a two to four-fold excess of the mass of inhibitor required to inhibit T₃ binding to TBG in most serum samples. All reagents are diluted in 0-05 M barbitone buffer pH 8-6 to inhibit T₃ binding to TBPA. With most

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*Recently* Sterling and Milch (J. clin. Endocr. Metab., 1974, 38, 866) have described inactivation of binding proteins by heat. Ed.

**Methods**

**ANTIsera**

The T₃ antiserum currently in use in our laboratory were raised in sheep against small doses of T₃-iodide conjugate to bovine serum albumin that include multiple intracutaneous sites. Antisera harvested 10 weeks after primary immunization were usable in titres of 1/30000 to 1/150000. Cross reactions with T₄ were less than 0-1%.

**Details of Radioimmunoassay Method**

The radioimmunoassay method is outlined in table II. It is essential to use serum free from T₃ and...
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antisera we have tested, incubation time can be shortened at higher temperatures as equilibrium is attained within one to two hours at 37°C. Charcoal separation of bound from free hormone, as previously described for T₄ (Ekins, Williams, and Ellis, 1969), is simple, quick, and inexpensive, and thus offers several advantages over double antibody separation systems.

A typical standard curve for T₃ is shown in figure 2. Using the programme of Ekins et al (1969) the assay has been optimized around the mean normal value of about 1.85 nmol/l (120 ng/100 ml) so that the standard curve permits measurement of serum T₃ concentrations from 0.15-6.0 nmol/l (10 to 400 ng/100 ml). Greater sensitivity can be achieved if required. Non-specific binding, ie, the percentage bound ¹²⁵I T₃ in the absence of antisemur, is approximately 10% and does not vary with T₃ concentration. It is implied that ANS produces total inhibition of T₃ binding to endogenous TBG; theoretically this may not be true, but in practice the residual proportion of T₃ bound to TBG is minute and is within the error of replicate determinations on the same serum sample. Ideally each serum sample should be run without antisemur to act as its own control and thus exclude any error due to residual binding of T₃ to TBG. Within-batch precision calculated from replicates for a representative assay, expressed as the mean serum T₃ concentration ± 2SD, is 0.59 ± 0.042, 1.52 ± 0.092, 3.21 ± 0.25, and 12.7 ± 2.2 nmol/l (38 ± 2.7, 99.0 ± 6.0, 208 ± 16.0 and 830 ± 140 ng/100 ml). The between-batch precision expressed as mean serum T₃ concentration ± 2SD for three quality control sera run in 11 assays was 1.36 ± 1.4, 2.74 ± 0.27, and 5.80 ± 0.46 nmol/l (88.5 ± 8.9, 177.5 ± 17.7 and 376 ± 30 ng/100 ml).

Results and Discussion of Physiological and Clinical Studies

EUHYROID SUBJECTS

Serum total T₃ ranged from 1.46 to 2.46 nmol/l (95 to 160 ng/100 ml) with a mean of 1.85, SD ± 0.27 nmol/l in 38 healthy euthyroid adults (figure 3). There was no significant difference between males (mean serum T₃ 1.83 ± 0.26 nmol/l) and females (mean serum T₃ 1.82 ± 0.29 nmol/l). Females who were pregnant or taking oral contraceptives displayed higher serum T₃ levels which parallel the changes in serum T₄ in this group, presumably due to increased circulating TBG levels (figure 3). Twenty-two euthyroid inpatients with no evidence of thyroid disease exhibited serum T₃ levels within the normal range. The results of serum T₃ determinations in our normal subjects are comparable with those reported by other workers (Brown et al, 1971; Mitsuma et al, 1971; Lieblich and Utiger, 1972; Larsen, 1972; Hesch et al, 1972), but are considerably lower than the levels measured by saturation analysis (Sterling et al, 1969; Wahner and Gorman, 1971) and by the radioimmunoassay method of Gharib, Ryan, Mayberry, and Hockert (1971) which does not employ TBG inhibitors to measure T₃ in whole serum. Subnormal T₃ levels not associated with any evidence of hypothyroidism have been found in patients with low TBG levels, in some patients with anorexia nervosa, and in the immediate newborn period (figure 3). It is intriguing that serum T₃ levels found in cord blood, in the presence of normal serum T₄ levels, modestly elevated levels of thyrotrophin (TSH) and elevated TBG levels, should be similar to the serum T₃ levels we have observed in patients with overt hypothyroidism (figure 4). The explanation for this phenomenon is unknown; however, it does emphasize the dissociation between maternal and fetal thyroid hormone secretion and also suggests that measurement of serum T₃ in cord blood cannot be used as a screening test for hypothyroidism in the newborn (Eastman et al, 1973).

PATIENTS WITH THYROID DISEASE

Hypothyroidism

In 32 clinically hypothyroid patients, in whom the diagnosis was confirmed by elevated serum TSH and/or the TSH response to thyrotrophin-releasing hormone (TRH), the mean serum T₃ concentration was 0.585 SD ± 0.38 nmol/l (38.1 ± 24.6 ng/100 ml). The serum T₃ level was below the lower limit of
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In general there was a good correlation between the serum T₃ level and the severity of the hypothyroidism. Serum T₃ concentration paralleled serum T₄ concentration with the exception of three patients who exhibited subnormal T₃ levels but normal serum T₄ levels. Contrary to some other reports we have not encountered any patients with unequivocally clinical hypothyroidism in whom the serum T₃ level is within the normal range. Eleven patients were classified as equivocally hypothyroid on clinical grounds. Serum T₃ levels were below the normal range in seven of these patients and within the lower part of the normal range in the other four (figure 4). Serum T₄ levels were within the normal range in eight of the 11 patients.

Acute falls in serum T₃ levels may occur in some patients in the absence of any classical signs of hypothyroidism, e.g., during the course of antithyroid drug therapy for thyrotoxicosis, or during the early postoperative period following pituitary or thyroid surgery. Subnormal T₃ values in these circumstances may represent transient changes in thyroid hormone output or may herald the onset of clinical hypothyroidism. Serial measurements of serum T₃ concentrations are helpful in assessing the efficacy of antithyroid drug therapy or the completeness of surgery.

**Hyperthyroidism**

In 28 untreated patients with well defined hyperthyroidism confirmed by an elevated serum T₄ and a raised free thyroxine index the serum T₃ concentration ranged from 3·0 to 16·9 nmol/l (198 to 1100 ng/100 ml) (figure 4). Four patients with clinical evidence of hyperthyroidism, but with normal serum total T₄ and free T₄ levels had serum T₃ levels ranging from 3·1 to 6·9 nmol/l (200 to 450 ng/100 ml) and a diagnosis of T₃-toxicosis was made in each of these patients according to the criteria of Hollander and Shenkman (1972). Two of the four patients had recurrent thyrotoxicosis having been already treated with antithyroid drugs for conventional thyrotoxicosis with elevated serum T₄ levels. This finding suggests that T₃-toxicosis may simply be a variant of conventional thyrotoxicosis but may be commoner in patients who have undergone previous treatment for thyrotoxicosis. The incidence of T₃-toxicosis in untreated hyperthyroid patients in this community is unknown and further experience is required before any definitive estimate can be arrived at.

**Miscellaneous thyroid disorders**

Serum T₃ levels were within the normal range in a small series of clinically euthyroid patients with multinodular goitre, untreated endocrine exophy
thalamos, treated Graves' disease, and solitary nodules proven by thyroid scintiscans (figure 5).

**SERUM T₃ IN PATIENTS ON THYROXINE REPLACEMENT THERAPY**

In patients on thyroxine replacement therapy serum T₄ estimations are not very helpful in assessing the optimal dose of T₄ for an individual patient. The daily production rate of T₄ in healthy euthyroid adults is in the vicinity of 80 to 100 µg per day (Nicoloff, Low, Dussault, and Fisher, 1972) yet most hypothyroid patients require oral doses of T₄ in excess of this amount to maintain a state of euthyroidism. Serum T₄ levels in these patients are commonly within the upper part of the normal range or modestly elevated.

Serum T₃ and T₄ concentrations were measured in 37 patients on L-thyroxine replacement therapy. Each patient was clinically euthyroid and had been on a stable dose of L-thyroxine for at least one month before investigation. The T₄ replacement dose varied from 100 µg to 400 µg per day. Serum T₃ and T₄ levels are shown in figure 6. Serum T₄ levels were raised in 18 out of 37 patients. The elevated T₄ levels were observed predominantly in patients taking 200 µg or more of thyroxine per day. By contrast, serum T₃ levels were elevated above the normal range in only three patients. Increases in serum T₃ were modest and less than the increases found in the patients with thyrotoxicosis. It is apparent that serum T₃, presumably derived from peripheral monodeiodination of T₄, more accurately reflects the metabolic status of the individual patient than does serum T₄. Although the factors responsible for this control system are poorly understood, the consistency of the T₄/T₃ ratios in the T₄-treated patients (mean ratio 83/1), at a higher level than those in the untreated euthyroid group (mean 70/1), suggests that conversion of T₄ to T₃ is dependent upon available T₄. Because the treated hypothyroid patients lack T₃ secreted directly from the thyroid, be it a partial or total lack depending upon the severity of the hypothyroidism, then it is reasonable to assume that more exogenous T₄ is required by these patients to maintain normal T₃ levels than is secreted by euthyroid subjects. This could explain the common finding of elevated serum T₄ levels¹ and higher T₄/T₃ ratios in the thyroxine-treated patients. The great variability in serum T₄ levels between patients taking the same dose of T₄ probably reflects individual variation in intestinal absorption

¹Physicians who treat hypothyroidism with just enough thyroxine to suppress the raised TSH level have recently reported that this produces normal serum T₃ (and T₄) levels, and that the patients become clinically euthyroid—see Evered et al, Brit. med. J., 1973, 3, 131, and Stock et al, New England J. Med., 1974, 290, 529. Ed.
of orally administered T₄. Further studies are in progress to assess T₄ absorption and T₄ to T₃ conversion in treated hypothyroid patients.

**Clinical Utility of Serum T₃ Determinations**

At the present time the serum T₄ concentration, interpreted in conjunction with an estimate of the degree of saturation of serum thyroid hormone-binding proteins, is generally considered to be the most specific index of thyroid function currently available. The application of radioimmunoassay to the thyroid hormones has now rendered the measurement of T₃ in serum or urine (Chan, Besser, Landon, and Ekins, 1972) a relatively simple procedure suitable for use as a diagnostic tool in the investigation of patients with thyroid disease. Although the concentration of T₃ in serum, like that of T₄, varies with changes in circulating TBG levels, nevertheless it has proved to be a precise and reliable method for the detection of thyroid dysfunction. This applies in particular to the diagnosis of hyperthyroidism. The clinical utility of serum T₃ determinations is summarized in table III. Present evidence suggests

<table>
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<tr>
<th>Table III</th>
<th>Clinical utility of serum T₃ determination</th>
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<tr>
<td>1 Diagnosis of thyrotoxicosis</td>
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<tr>
<td>2 Diagnosis of hypothyroidism</td>
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<tr>
<td>3 Assessment of acute changes in thyroid hormone secretion (a) during antithyroid drug therapy; (b) after thyroidectomy; (c) after hypophysectomy</td>
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<tr>
<td>4 Assessment of T₃ replacement therapy, especially in elderly patients with ischaemic heart disease and in young children</td>
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<td>5 Assessment of thyroid gland autonomy eg, after TRH stimulation</td>
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<td>6 Assessment of thyroid gland reserve, eg, after TSH stimulation</td>
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that the measurement of serum T₃ is a valuable adjunct to the measurement of serum T₄ and may eventually replace the latter as a more direct and precise index of thyroidal status in the diagnosis and management of patients with thyroid disease.

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**References**


