The radioimmunoassay of triiodothyronine and its clinical application

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Since the identification of triiodothyronine (T_3) 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 T_3 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 T_3 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 T_3 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 T_4 secreted by the thyroid may be converted to T_3 by peripheral deiodination (Brauerman, Ingbar, and Sterling, 1970). It is even possible that T_3 is the sole biologically active thyroid hormone, as conversion of T_4 to T_3 *in vivo* may be an obligatory step in the metabolic action of T_4 at cellular level, T_4 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 T_3 concentration (Fisher and Dussault, 1971; Larsen, 1971a). A significant advance in T_3 assay methodology was the production of specific T_3 antibodies by Brown, Ekins, Ellis, and Reith (1970) and subsequently the development of a sensitive and precise radioimmunoassay for T_3 in serum extracts (Brown, Ekins, Ellis, and Williams).

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Principles and Problems of T_3 Radioimmunoassay in Whole Serum

Early attempts to measure T_3 in whole serum by radioimmunoassay were unsuccessful due largely to interference by endogenous thyroxine-binding globulin (TBG). Theoretically, it is possible to measure T_3 by radioimmunoassay in the presence of TBG, if the avidity of the antiserum for T_3 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 T_3, which competitively inhibit binding of T_3 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 T_3 bound to TBG; then serum T_3, in addition to that added as tracer and as standard, is free to react with the specific T_3 antibody. Although effective, T_4 has not been widely used as an inhibitor because of the variable contamination of most T_4 preparations with T_3, the possibility of spontaneous deiodination of T_4 to T_3 in the incubation medium, and the

Compound	*Authors*
Thyroxine	Chopra *et al* (1971)
Tetrachlorthyronine	Mitsuma *et al* (1971)
Salicylate	Larsen (1972)
Merthiolate	
Diphenyl hydantoin	Hesch *et al* (1972)
Diazepam	
8-Anilino-1-naphthalene sulphonic acid	Chopra (1972); Mitsuma *et al* (1972); Chopra *et al* (1972); Eastman *et al* (1973)

Table I *Inhibitors of T_3-TBG binding useful in T_3 radioimmunoassay*

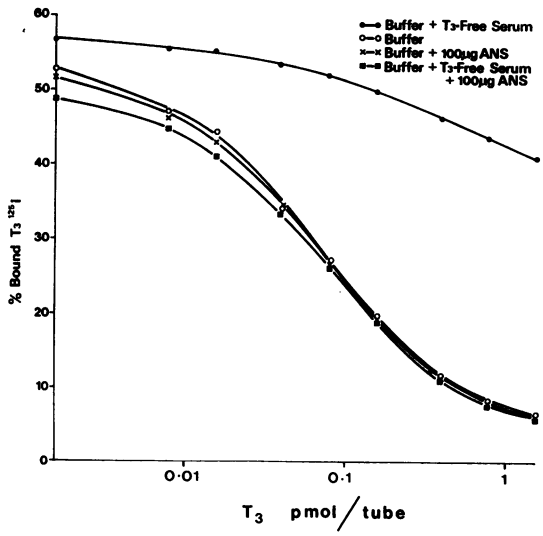

Fig 1 *Standard curve for T_3 using a large selection of antisera.*

intrinsic cross reaction of T_4 with many T_3 antisera. Tetrachlorthyronine (TCT) is a potent competitive inhibitor of TBG and has been employed successfully in the radioimmunoassay of T_3 (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 trichlorthyronine. Diphenylhydantoin will effectively inhibit T_3 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 merthiolate (Hesch, Hüfner, and Von Zu Mühlen Mühlen, 1972) have also been used; these compounds show minimal or no cross reaction with most T_3 antisera and have the added advantage of inhibiting T_3 binding to thyroxine-binding prealbumin (TBPA) (Larsen, 1971b). Of the compounds we have tested so far[1], 8-anilino-1-naphthalene sulphonic 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 T_3 and T_4 binding to TBG, showing no significant cross reaction with and causing no alteration in the equilibrium constants of a large selection of T_3 antisera[2] (figure 1).

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

Methods

ANTISERA

The T_3 antisera currently in use in our laboratory were raised in sheep against small doses of T_3 conjugate to bovine serum albumin distributed over multiple intracutaneous sites. Antisera harvested 10 weeks after primary immunization were usable in titres of 1/30000 to 1/150000. Cross reaction with highly purified T_4 was less than 0·1%.

DETAILS OF RADIOIMMUNOASSAY METHOD

The radioimmunoassay method is outlined in table II. It is essential to use serum free from T_3 and T_4 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 $^{125}I\,T_4$ 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 T_3 binding to TBG in most serum samples. All reagents are diluted in 0·05 M barbital buffer pH 8·6 to inhibit T_3 binding to TBPA. With most

1 Reagents
Barbitone buffer 0·05 M, pH 8·6 containing 0·05% bovine serum albumin
Serum treated with charcoal to remove T_3 and T_4
8-Anilino-1-naphthalene-sulphonic acid (ANS) 1 mg/ml
$^{125}I\,T_3$ (Amersham, 50-70 mCi/mg)
T_3 Standards—Serial dilutions in barbitone buffer, 0-1000 pg T_3/tube
T_3 Antiserum 1/100000 final dilution in barbitone buffer
2 Incubation Mixtures
0·1 ml antiserum 1/10 000
0·1 ml ANS (100 µg)
0·1 ml T_3 standards or buffer in standards and tests respectively
0·05 ml unknown serum or T_3-free serum in tests and standards respectively
0·1 ml $^{125}I\,T_3$ (30 pg)
Adjust final volume to 1·0 ml with barbitone buffer
For each tube set up a control without antiserum
3 Incubation
24 hours at 4°C
4 Separation
Charcoal
5 Count bound and free fractions

Table II *Protocol for T_3 radioimmunoassay*

[2]Malkus and Donabedian (*Clin. chim. Acta*, 1974, 51, 191) report interference by ANS with T_3-binding by two antisera. ED.

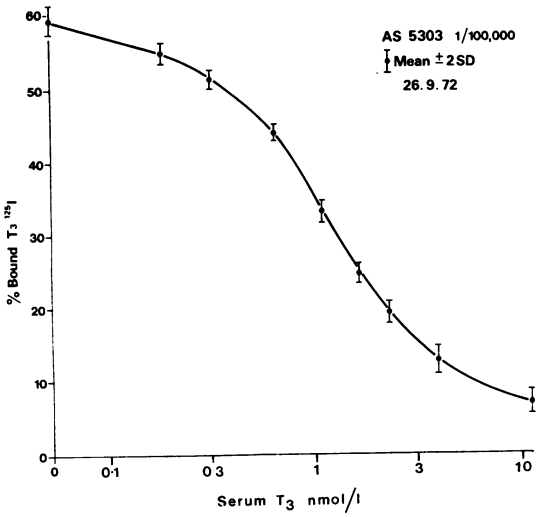

Fig 2 *T_3 standard curve.*

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 T_4 (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 T_3 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 T_3 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 ^{125}I T_3 in the absence of antiserum, is approximately 10% and does not vary with T_3 concentration. It is implied that ANS produces total inhibition of T_3 binding to endogenous TBG; theoretically this may not be true, but in practice the residual proportion of T_3 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 antiserum to act as its own control and thus exclude any error due to residual binding of T_3 to TBG. Within-batch precision calculated from replicates for a representative assay, expressed as the mean serum T_3 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 T_3 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

EUTHYROID SUBJECTS

Serum total T_3 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 signficant difference between males (mean serum T_3 1·83 ± 0·26 nmol/l) and females (mean serum T_3 1·82 ± 0·29 nmol/l). Females who were pregnant or taking oral contraceptives displayed higher serum T_3 levels which parallel the changes in serum T_4 in this group, presumably due to increased circulating TBG levels (figure 3). Twenty-two euthyroid inpatients with no evidence of thyroid disease exhibited serum T_3 levels within the normal range. The results of serum T_3 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 T_3 in whole serum. Subnormal T_3 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 T_3 levels found in cord blood, in the presence of normal serum T_4 levels, modestly elevated levels of thyrotrophin (TSH) and elevated TBG levels, should be similar to the serum T_3 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 T_3 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 T_3 concentration was 0·585 SD ± 0·38 nmol/l (38·1 ± 24·6 ng/100 ml). The serum T_3 level was below the lower limit of

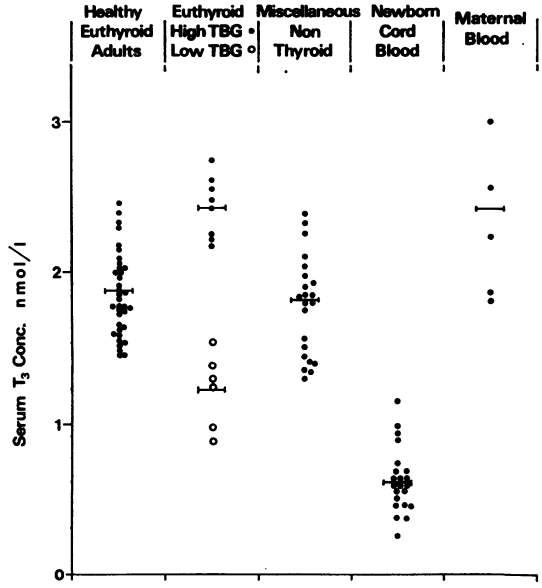

Fig 3 *Serum T_3 levels in healthy euthyroid adults*

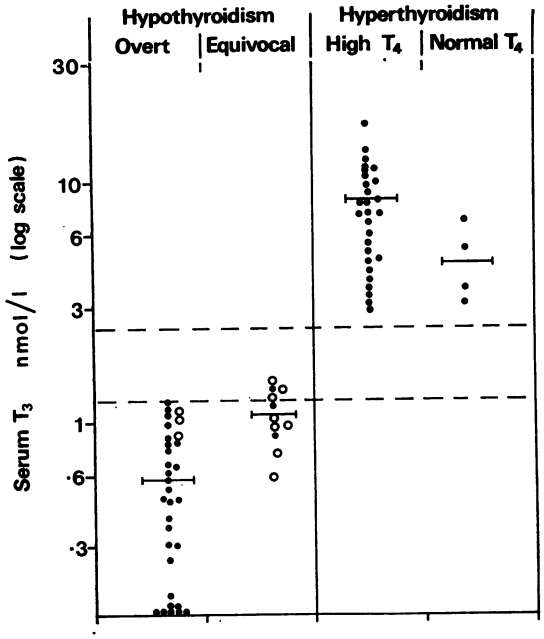

Fig 4 *Serum T_3 levels in cases of hypothyroidism and hyperthyroidism.*

the normal range in each patient (figure 4). In general there was a good correlation between the serum T_3 level and the severity of the hypothyroidism. Serum T_3 concentration paralleled serum T_4 concentration with the exception of three patients who exhibited subnormal T_3 levels but normal serum T_4 levels. Contrary to some other reports we have not encountered any patients with unequivocal clinical hypothyroidism in whom the serum T_3 level is within the normal range. Eleven patients were classified as equivocally hypothyroid on clinical grounds. Serum T_3 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 T_4 levels were within the normal range in eight of the 11 patients.

Acute falls in serum T_3 levels may occur in some patients in the absence of any clinical signs of hypothyroidism, eg, during the course of antithyroid drug therapy for thyrotoxicosis, or during the early postoperative period following pituitary or thyroid surgery. Subnormal T_3 values in these circumstances may represent transient changes in thyroid hormone output or may herald the onset of clinical hypothyroidism. Serial measurements of serum T_3 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 T_4 and a raised free thyroxine index the serum T_3 concentration ranged from 3·04 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 T_4 and free T_4 levels had serum T_3 levels ranging from 3·1 to 6·9 nmol/l (200 to 450 ng/100 ml) and a diagnosis of T_3-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 T_4 levels. This finding suggests that T_3-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 T_3-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 T_3 levels were within the normal range in a small series of clinically euthyroid patients with multinodular goitre, untreated endocrine exoph-

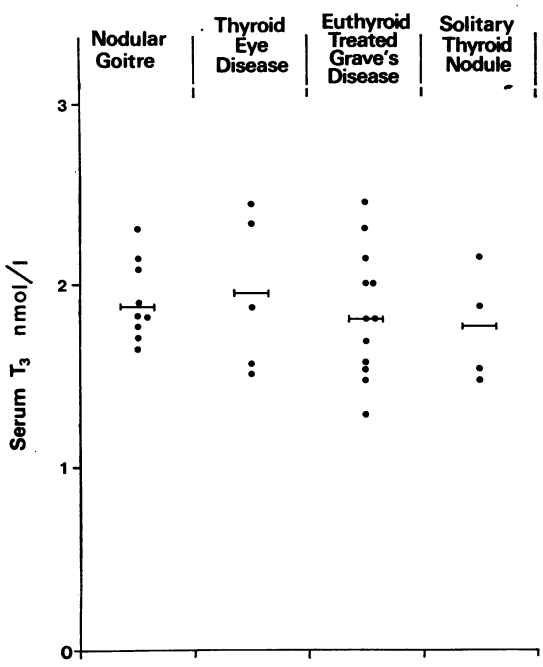

Fig 5 *Serum T_3 levels in a series of clinically euthyroid patients with various thyroid disorders.*

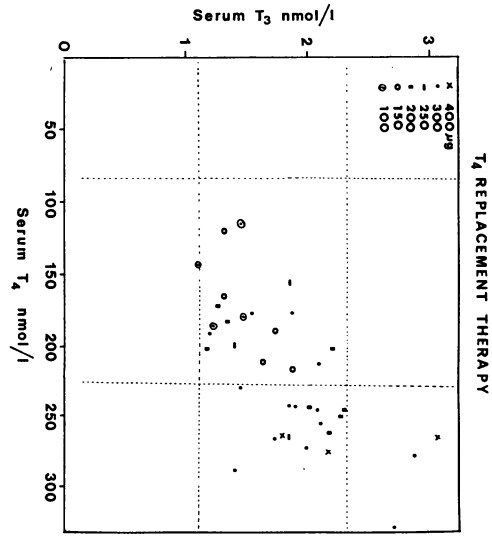

Fig 6 *Serum T_3 and T_4 levels in 37 patients receiving L-thryoxine replacement therapy.*

thalmos, treated Graves' disease, and solitary nodules proven by thyroid scintiscans (figure 5).

SERUM T_3 IN PATIENTS ON THYROXINE REPLACEMENT THERAPY

In patients on thyroxine replacement therapy serum T_4 estimations are not very helpful in assessing the optimal dose of T_4 for an individual patient. The daily production rate of T_4 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 T_4 in excess of this amount to maintain a state of euthyroidism. Serum T_4 levels in these patients are commonly within the upper part of the normal range or modestly elevated.

Serum T_3 and T_4 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 T_4 replacement dose varied from 100 μg to 400 μg per day. Serum T_3 and T_4 levels are shown in figure 6. Serum T_4 levels were raised in 18 out of 37 patients. The elevated T_4 levels were observed predominantly in patients taking 200 μg or more of thyroxine per day. By contrast, serum T_3 levels were elevated above the normal range in only three patients. Increases in serum T_3 were modest and less than the increases found in the patients with thyrotoxicosis. It is apparent that serum T_3, presumably derived from peripheral monodeiodination of T_4, more accurately reflects the metabolic status of the individual patient than does serum T_4. Although the factors responsible for this control system are poorly understood, the consistency of the T_4/T_3 ratios in the T_4-treated patients (mean ratio 83/1), at a higher level than those in the untreated euthyroid group (mean 70/1), suggests that conversion of T_4 to T_3 is dependent upon available T_4. Because the treated hypothyroid patients lack T_3 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 T_4 is required by these patients to maintain normal T_3 levels than is secreted by euthyroid subjects. This could explain the common finding of elevated serum T_4 levels[1] and higher T_4/T_3 ratios in the thyroxine-treated patients. The great variability in serum T_4 levels between patients taking the same dose of T_4 probably reflects individual variation in intestinal absorption

[1]Physicians who treat hypothyroidism with just enough thyroxine to suppress the raised TSH level have recently reported that this produces normal serum T_4 (and T_3) 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 T_4. Further studies are in progress to assess T_4 absorption and T_4 to T_3 conversion in treated hypothyroid patients.

Clinical Utility of Serum T_3 Determinations

At the present time the serum T_4 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 T_3 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 T_3 in serum, like that of T_4, 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 T_3 determinations is summarized in table III. Present evidence suggests

1 Diagnosis of thyrotoxicosis
2 ? Diagnosis of hypothyroidism
3 Assessment of acute changes in thyroid hormone secretion (a) during antithyroid drug therapy; (b) after thyroidectomy; (c) after hypophysectomy
4 Assessment of T_4 replacement therapy, especially in elderly patients with ischaemic heart disease and in young children
5 Assessment of thyroid gland autonomy eg, after TRH stimulation
6 Assessment of thyroid gland reserve, eg, after TSH stimulation

Table III *Clinical utility of serum T_3 determination*

that the measurement of serum T_3 is a valuable adjunct to the measurement of serum T_4 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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