

Measurement of the Plasma Free Thyroxine Level as a Test of Thyroid Function

MAURICE WELLBY,* M.D., M.Sc., M.C.P.A.; M. W. O'HALLORAN,† M.Sc.

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The concept that the small amount of unbound or free thyroxine (T_4) is the factor in determining the true thyroid status of the individual (Robbins and Rall, 1957) has been supported by evidence in recent studies (Christensen, 1960; Sterling and Hegedus, 1962; Lee *et al.*, 1964; Ingbar *et al.*, 1965). Nevertheless, the measurement of either the plasma protein-bound iodine (P.B.I.) or the resin uptake of labelled triiodothyronine (T_3) should suffice to confirm a diagnosis in most cases of primary myxoedema and primary thyrotoxicosis (Wellby and O'Halloran, 1966).

However, in many conditions in which there is an abnormality in T_4 binding by serum proteins there are often deviations from the normal in both P.B.I. and resin uptake of T_3 without associated changes in the thyroid status (Robbins and Rall, 1960). The serum level of free T_4 should be of value in diagnosing the thyroid status of patients with such conditions.

The procedures for the estimation of free T_4 utilize either dialysis (Sterling and Hegedus, 1962; Ingbar *et al.*, 1965) or Sephadex filtration (Lee *et al.*, 1964) to separate the free from the protein-bound T_4 .

Clark and Horn (1965) have used the mathematical product of P.B.I. and resin uptake of T_3 as a "free T_4 index," and justify this approach on the mathematical relationship that exists between free T_4 , P.B.I., and resin uptake when certain assumptions are fulfilled.

The purpose of this paper is to investigate the comparative value of two methods of assessing the free T_4 level of serum—namely, the Sephadex-filtration technique of Lee *et al.* (1964) and the "free T_4 index" of Clark and Horn (1965).

Methods

The normal subjects were volunteers or outpatients without evidence of serious disease. The patients with thyroid disorders were restricted to those with untreated primary thyrotoxicosis or myxoedema. The thyroid status was assessed on the usual clinical criteria, and invariably independently of the results of the P.B.I. or resin uptake of T_3 . Studies were made also on patients with abnormal thyroid-binding proteins in the serum. Patients with a clinical diagnosis of either nephrosis or macroglobulinaemia, or who were currently being treated with androgen or high doses of corticosteroids, or who had serum albumin levels of less than 3.6 g./100 ml., were classified as having decreased thyroid-binding. Subjects undergoing normal pregnancy or who were being treated with oestrogen-containing drugs were classified as having increased thyroid-binding. The uptake of ^{131}I by the thyroid gland and the response to therapy were used where applicable. Blood was collected and heparinized and the plasma separated under iodine-free conditions. The plasma was stored frozen until the day of assay.

The P.B.I. was determined by the alkaline incineration procedure of Acland (1957). Assays were performed in dupli-

cate and the results discarded if the differences exceeded 0.5 $\mu\text{g.}/100$ ml. The resin uptake of T_3 was performed in duplicate according to modifications (Taylor *et al.*, 1964) of the method of Woldring *et al.* (1961). The results, expressed as a percentage of a pool of normal plasma, were discarded if the differences between duplicates exceeded 10% of their mean value. The "free thyroxine index" of Clark and Horn (1965) was calculated from the mathematical product of P.B.I. and resin-uptake percentage divided by 100.

The Sephadex filtration method of estimating free T_4 was performed by modifications of the method of Lee *et al.* (1964). The ^{131}I - T_4 of specific activity of 25 to 40 $\mu\text{Ci}/\mu\text{g.}$ was sent by air freight from Abbott Laboratories, and on receipt stored at 4° C. and used for no more than 10 days after receipt. Approximately 0.5 μCi of ^{131}I - T_4 is added to duplicate 1-ml. aliquots of plasma. The enriched plasma is placed on a small column of Sephadex G-25 (coarse bead type), and the protein-bound T_4 is washed through with 0.5 M saline and collected in a 100-ml. volumetric flask. The volume is adjusted to 100 ml., and 3-ml. aliquots are counted in a scintillation detector fitted with a well-type crystal. The free T_4 remains on the Sephadex, and the intact column is wrapped in parafilm, placed in a counting-tube, and counted. Sufficient counting-time is allowed to collect 5,000 to 10,000 counts above background.

The free T_4 value as $\text{m}\mu\text{g.}$ of $T_4/100$ ml. of plasma is calculated from:

$$\text{Free } T_4 = \frac{F \times \text{P.B.I.}}{0.654 \times B}$$

where F = net count rate of Sephadex-bound ^{131}I

B = net count rate of protein-bound ^{131}I

P.B.I. is expressed in $\text{m}\mu\text{g.}/100$ ml. plasma.

The percentage of total T_4 present in the free state is calculated from $F \div B \times 100$, assuming the protein-bound T_4 to approximate the total T_4 .

Results

Euthyroid Subjects and Patients with Primary Thyroid Diseases (Table I).—There was no overlap between the three groups in the P.B.I. values but there was the expected overlap in resin-uptake values, particularly between the hypothyroid and the euthyroid groups. There was a similar overlap in the free T_4 percentages: for the hypothyroid group values ranged from 0.025 to 0.078, for the euthyroid group from 0.047 to 0.085, and for the hyperthyroid group from 0.081 to 0.144. There was no overlap in the free T_4 index or in the values for Sephadex-free T_4 concentration (Table I). There was a highly significant ($P < 0.001$) correlation between the results obtained with the "free T_4 index" and the Sephadex-bound T_4 in the three groups of subjects without binding abnormalities (Fig. 1).

Subjects with Binding Abnormalities.—The results (except for the hyperthyroid subjects in pregnancy or on oestrogen therapy) are shown in Table II and Fig. 2. The normal ranges for T_3 , "free T_4 index," and Sephadex-free T_4 concentration

* Clinical Biochemist, Queen Elizabeth Hospital, Woodville, South Australia.

† Biochemist, Queen Elizabeth Hospital, Woodville, South Australia.

are derived from the mean ± 2 standard deviations of a normal population of 150 subjects, including the 23 normal subjects of this investigation. Fig. 2 shows at a glance that neither the P.B.I. nor the resin uptake of T_4 is accurate in diagnosing the euthyroid status of subjects with binding abnormalities. The resin-uptake value was outside the normal range in 57% of the subjects with decreased binding and in 63% with increased binding. The P.B.I. was incorrect in 71% of subjects with decreased and in all of the subjects with increased binding. By contrast, both the "free T_4 index" and the free T_4 concentration values were within the normal range in all subjects with decreased binding. In the subjects with increased binding the "free T_4 index" and the free T_4 concentration were correct on 75 and 87.5% of occasions respectively.

There were eight subjects who were clinically thyrotoxic and who, in addition, had increased plasma protein T_4 binding due either to pregnancy or to oestrogen therapy. The values found were as follows: resin uptake, a range of 76.0 to 105.8% of the normal pool (normal range, 76 to 117); P.B.I. range from 10.3 to 18.0 $\mu\text{g./100 ml.}$ (normal 3.8 to 7.0); "free T_4 index," range from 9.2 to 17.9 (normal 3.4 to 6.8); and for free T_4 concentration, range from 9.3 to 18.6 $\mu\text{g./100 ml.}$ (normal 3.4 to 7.3).

Discussion

The mean free T_4 concentration in normal plasma estimated with Sephadex was found in the present study to be 5.4 $\mu\text{g.}$

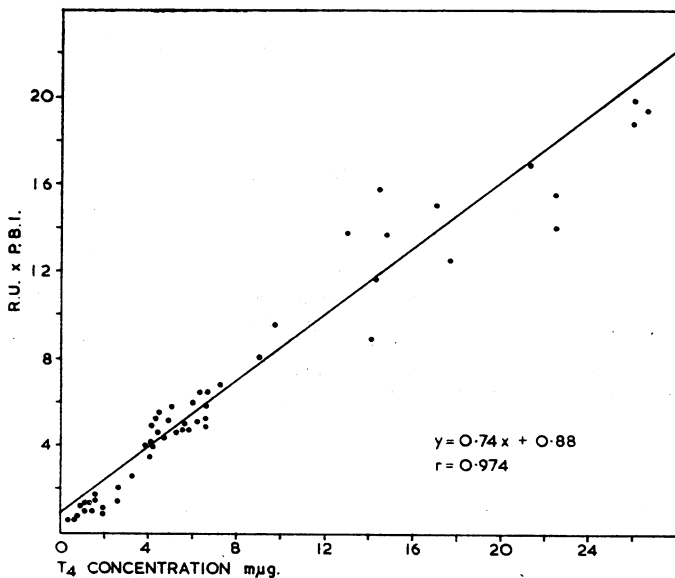


FIG. 1.—Correlation of results for "free thyroxine index" and for free thyroxine concentration ($\mu\text{g./100 ml.}$) in hyperthyroid, euthyroid, and hyperthyroid subjects.

of T_4 per 100 ml. of plasma, which is between the value of 3.1 $\mu\text{g./100 ml.}$ found with Sephadex by Lee *et al.* (1964) and the value of 9.9 $\mu\text{g./100 ml.}$ of Sterling and Hegedus (1962) and 9.1 $\mu\text{g./100 ml.}$ of Liewendahl and Lamberg (1965) obtained by dialysis through cellophane. Ingbar (1958) reported a figure of 4.0 $\mu\text{g./100 ml.}$ in a series of 87 normal patients, using the technique of dialysis followed by a second dialysis combined with ion exchange to purify the free T_4 from contaminating inorganic iodine. As Lee *et al.* (1964) suggest, one possible reason for differing results is a dissociation of a small amount of T_4 from the T.B.P. T_4 complex, either before or during the assay process. Lee *et al.* assume that, as the values with Sephadex are less than those obtained with a dialysis procedure, then the Sephadex does not cause any dissociation of T_4 . However, this assumption is valid only if the recoveries of free T_4 in the Sephadex method are near 100%, and if the values obtained in the dialysis procedure are not artifactually elevated by contaminating radioactive inorganic iodide. When special precautions are taken to eliminate this contamination the values obtained are considerably reduced (Ingbar *et al.*, 1965).

The present investigation confirms others (Sterling and Hegedus, 1962; Liewendahl and Lamberg, 1965; Ingbar *et al.*, 1965) in demonstrating clear-cut differences between the total free T_4 values obtained in euthyroid, hyperthyroid, and hypothyroid subjects. This observation supports the theory that it is the unbound form of T_4 which crosses the cell membrane and determines the physiological thyroid status of the individual. However, there seems little point in performing a free T_4 esti-

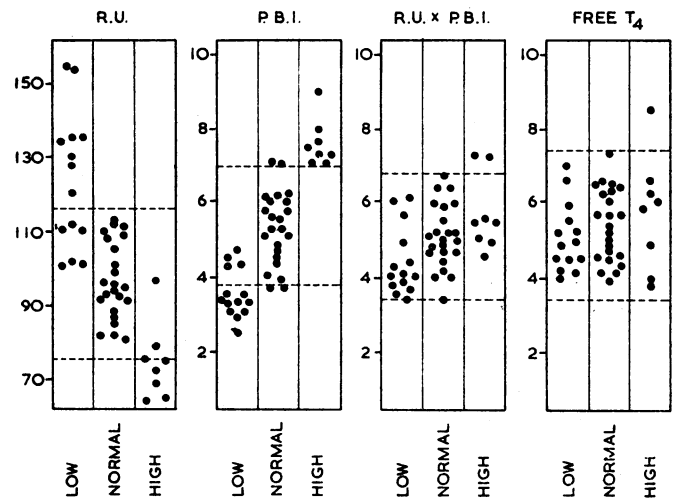


FIG. 2.—Results for resin uptake (R.U.) (percentage of normal), protein-bound iodine (P.B.I., $\mu\text{g./100 ml.}$), "free thyroxine index" (R.U. \times P.B.I.), and free thyroxine concentration ($\mu\text{g./100 ml.}$) in normal subjects and in patients with decreased (low) and increased (high) plasma thyroid-binding proteins. The dotted lines indicate the limits of the normal ranges as explained in the text.

TABLE I.—Values (Mean and Standard Deviations) for Thyroid Function Tests in Normal Subjects and in Patients with Untreated Primary Thyroid Disorders

Group	P.B.I. ($\mu\text{g./100 ml.}$)	Resin Uptake (Percentage of Normal)	Free T_4 Index ($(T_4/100) \times \text{P.B.I.}$)	Free T_4 Concentration ($\mu\text{g./100 ml.}$)	Free T_4 Percentage
Hypothyroid (15)	1.89 \pm 0.69	72.5 \pm 12.1	1.34 \pm 0.51	1.71 \pm 0.73	0.05 \pm 0.01
Euthyroid (23)	5.33 \pm 0.98	96.5 \pm 10.2	5.09 \pm 0.84	5.43 \pm 0.99	0.07 \pm 0.01
Hyperthyroid (15)	11.06 \pm 2.40	131.4 \pm 16.8	15.37 \pm 3.90	19.01 \pm 6.25	0.11 \pm 0.02

Figures in parentheses indicate number of subjects in group.

TABLE II.—Values (Mean and Standard Deviations) for Thyroid Function Tests in Normal Subjects and in Patients with Abnormalities in Thyroxine Binding by Plasma Protein

Group	P.B.I. ($\mu\text{g./100 ml.}$)	Resin Uptake (Percentage of Normal)	Free T_4 Index ($(T_4/100) \times \text{P.B.I.}$)	Free T_4 Concentration ($\mu\text{g./100 ml.}$)	Free T_4 Percentage
Decreased binding (14)	3.58 \pm 0.67	123.7 \pm 18.2	4.41 \pm 0.91	5.06 \pm 0.90	0.095 \pm 0.02
Normal (23)	5.33 \pm 0.98	96.6 \pm 10.2	5.09 \pm 0.84	5.43 \pm 0.99	0.07 \pm 0.01
Increased binding (8)	7.57 \pm 0.64	75.1 \pm 10.6	5.71 \pm 0.97	5.86 \pm 1.60	0.05 \pm 0.01

Figures in parentheses indicate number of subjects in group.

mation in order to confirm a diagnosis of uncomplicated, untreated thyrotoxicosis or myxoedema when this could be accomplished by performing P.B.I. and resin uptake of T_3 estimations (Wellby and O'Halloran, 1966).

In the presence of iodine contamination, the free T_4 concentration is invalidated, as a P.B.I. is needed for its calculation, whether this be by a direct Sephadex method or the arbitrary "free T_4 index." Possibly the percentage of free T_4 which is derived entirely from measurements of radioactivity could be of value in this situation. It would be necessary first to determine whether the property of the Sephadex in partitioning the free from the protein-bound T_4 is interfered with by excess inorganic or organic iodine. According to the results of this investigation, the value of estimating the percentage of free T_4 is minimized by the large overlap between the myxoedema and the normal group of subjects, which reflects the overlap in resin uptake of T_3 between myxoedematous patients and normal subjects. There is no significant overlap, however, between the normal and the thyrotoxic group. In view of the good correlation observed between the "free thyroxine index" (resin uptake \times P.B.I.) and Sephadex free T_4 concentration (percentage free $T_4 \times$ P.B.I.) the correlation between resin uptake and percentage free T_4 is not unexpected.

The free T_4 assay has an even greater value in diagnosing the thyroid status of patients with disturbances in the binding of T_4 by plasma proteins. The inaccuracies of the P.B.I. and the resin uptake in such subjects are now well documented. There are few data in the literature as yet on the values for free T_4 concentration in these conditions. Lee *et al.* (1964) found normal concentrations in three euthyroid pregnant subjects. A small though significant diminution in the free T_4 concentration in pregnancy has been found with dialysis procedures (Sterling and Hegedus, 1962; Ingbar *et al.*, 1965). It is apparent from the present study that either the "free T_4 index" or the free T_4 concentration in euthyroid patients with binding abnormalities produces values within the range found for euthyroid subjects without binding abnormalities. This confirms and extends the findings of Clark and Horn (1965), who found the "free T_4 index" to be in the normal range in 23 euthyroid pregnant subjects and in four euthyroid patients with nephrosis. The value of free T_4 estimation is further demonstrated by the elevated values found in each of the six pregnant thyrotoxic subjects. Other studies from this laboratory have shown that the "free T_4 index" of Clark and Horn (1965) is of value in assessing the thyroid status of patients who have had thyrotoxicosis treated with ^{131}I (Wellby and O'Halloran, 1966).

The usefulness of the free T_4 assay in investigating the thyroid status of patients with binding abnormalities having been established, the question of which is the more useful technique to perform remains to be answered. One advantage of the Sephadex method is that it provides data on the percentage of T_4 in the free form as well as the free T_4 concentration, and this could be useful in studying the changes occurring in thyroid hormone kinetics in the circulation of patients undergoing treatment for thyrotoxicosis, in the study of patients with familial disturbances in the thyroid-binding proteins, and in other situations.

Although the "free T_4 index" is only an arbitrary index of free T_4 it has the distinct advantage that it utilizes techniques

that should exist in the average diagnostic laboratory. Furthermore, this investigation demonstrates that there is very good correlation between the "free T_4 index" and a Sephadex estimation of free T_4 . It seems justifiable to conclude that the "free T_4 index" is a useful addition to the established methods in the diagnosis of thyroid disorders.

Summary

Comparative studies on two methods of estimating the free thyroxine (T_4) level in plasma are described, one being the Sephadex-gel filtration technique and the other the mathematical product of protein-bound iodine (P.B.I.) and the resin uptake of triiodothyronine (T_3)—that is, the "free T_4 index" of Clark and Horn.

The results of free T_4 assays on plasma from normal subjects are: Sephadex method, range 3.6–7.4 $m\mu\text{g.}$; "free T_4 index," 3.4–6.8 units. The values obtained in hypothyroid and in thyrotoxic patients are sharply differentiated from those in euthyroid subjects.

Studies were made also on eight euthyroid subjects with increased plasma protein binding of T_4 due to pregnancy or oestrogen therapy and in 14 euthyroid subjects with decreased binding secondary to hypoalbuminaemia or nephrosis. Whereas a high percentage of the P.B.I. and the resin-uptake values were outside the normal ranges, both methods of estimating the free T_4 levels produced normal results with only one exception. Elevated levels were found in the six thyrotoxic subjects with binding abnormalities.

It is concluded that free T_4 assays are useful in assessing thyroid function, particularly in patients with T_4 binding abnormalities in the plasma, where the interpretation of P.B.I. and resin uptake of T_3 can be difficult.

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