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A New Method for Measuring the Free Thyroid Hormone in Human Serum and an Analysis of the Factors That Influence Its Concentration *

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There exists a great deal of evidence that it is the free or unbound portion of the circulating thyroid hormone that is accessible to the tissues and physiologically active, whereas the great majority of the hormone, which is protein bound, serves as a metabolically inert reservoir (1–4). If this is the case, then the concentration of free hormone in the plasma must be a major determinant of both the metabolic effectiveness and the rate of removal of the thyroid hormone in the blood.

Despite the probable importance of the free thyroid hormone, few reports have dealt with its measurement in the plasma. Certain methods for directly measuring the proportion of free thyroxine (T_4) in serum appear to have either technical or theoretical disadvantages (5, 6), although others have not been completely evaluated (7). On the other hand, there has been described a variety of methods, such as *in vitro* red blood cell or resin uptake techniques, which reflect the proportion of free thyroid hormone in the serum, but do not directly measure this function (8–11). Consequently, the absolute concentration of free hormone in serum cannot be calculated from such results, even though the total concentration of hormone in the serum is known.

The present report describes and evaluates a method for measuring the proportion of free thyroid hormone in serum that appears to be either simpler or more nearly indicative of the true value for this function than methods previously described. Both the proportion and absolute concentration of free T_4 in the serum of normal and abnormal patients have been determined and the causes of abnormal values in disease states evaluated. In addition, the proportion of free 3,5,3′-L-triiodothyronine (T_3) in normal serum has been measured. A preliminary description of the method and certain of the results obtained have appeared in abstract form (12).

Methods

Theoretical considerations. Theoretically, the per cent of free T_4[1] in the serum should be relatively simple to measure by equilibrium dialysis or ultrafiltration techniques. As has been recognized previously (5, 6), however, commercial preparations of I^{131}-labeled T_4 invariably are contaminated with sufficient radioactive iodide to preponderate over the free I^{131}-T_4 that passes the semipermeable membrane. Several methods have been described to meet or circumvent this problem (5, 6). As will be discussed below, these methods apparently have intrinsic difficulties. Therefore, a new method was sought for separating I^{131}-T_4 from I^{131}-iodide in equilibrium dialyzates of serum enriched with commercial labeled T_4. This method sought to take advantage of the very properties of I^{131}-T_4 and I^{131}-iodide that create analytic difficulties during the initial equilibrium dialysis. Thus, if the equilibrium dialyzate containing I^{131}-T_4 and I^{131}-iodide is

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[1] In the present report, for purposes of brevity, the proportion of free T_4 in serum, expressed as a per cent of the total, will be termed PFT_4; the absolute concentration of free T_4 will be termed AFT_4.

mixed with an equal volume of normal plasma and this mixture (equilibrium dialyzate-plasma, EDP) is itself subjected to dialysis, labeled I^- should be almost entirely free to diffuse into the dialyzate, whereas labeled T_4 should be almost entirely retained by the EDP within the dialysis sac. Simple dialysis would not remove I^{131}-iodide from the EDP completely, however, since labeled I^- is slightly bound by serum proteins (13). Complete removal of I^{131}-iodide should be achieved, however, by suspending an anion exchange resin in the buffer against which the EDP is dialyzed. Iodide dialyzed from the EDP would be absorbed to the resin and, in this way, a diffusion gradient for iodide would be maintained until all labeled I^- had been cleared from the EDP. Because of the intense association between T_4 and the proteins in the EDP, however, only negligible quantities of I^{131}-T_4 should become available to the resin during the time required to free the EDP of iodide.

Procedures. The method ultimately developed for measuring the PFT_4 in serum consists of two parts: *a*) a technique for obtaining an equilibrium dialyzate of the test serum enriched with I^{131}-labeled hormone, and *b*) a resin dialysis technique for freeing the equilibrium dialyzate of radioactive I^- so that its content of labeled hormone can be determined accurately. The first half of the method, equilibrium dialysis of serum, is a modification of that described by Sterling and Hegedus (5). The procedure employed was similar in most respects to that used by these workers, except that acid washing and soaking of dialysis tubing were not performed. Such treatment was found to lead not infrequently to small leaks in the tubing that produced spurious increases in measured values for the free T_4. Instead, dialyses were performed in tubing [2] that had been washed thoroughly in distilled water and soaked for 30 to 60 minutes in 0.07 M sodium phosphate buffer, pH 7.4 (standard phosphate buffer) before use. The method employed is as follows:

1) When labeled T_4 arrives from a commercial source,[3] it is immediately made up in 5% human serum albumin (HSA) to a concentration of approximately 160 μc per ml. This solution can usually be stored in the refrigerator or freezer for 1 week without excessive deterioration.

2) To 6.0 ml of the serum to be tested is added 25 μl of the labeled T_4 in HSA. This is allowed to stand for a short period. At the usual specific activity of the commercial I^{131}-T_4, this increases the T_4 concentration of the sample by approximately 2 μg per 100 ml.

3) Five ml of the test serum containing labeled T_4 is pipetted into a dialysis sac. This is then sealed, care being taken to avoid contaminating the outside of the sac.

4) The dialysis sac is rinsed briefly under running tap water and is placed inside a 40- or 50-ml cellulose nitrate centrifuge tube containing 5.0 ml of standard phosphate buffer. The tube is tightly sealed, and its lower portion is inserted into a shaking water bath at 37° C for 20 hours.

[2] Visking casing, 23 mm in diameter, Union Carbide Corp., Chicago, Ill.

[3] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

5) After dialysis is complete, 3.0 ml of the dialyzate is pipetted into a test tube, and 3.0 ml of any serum or plasma is added (indifferent plasma; outdated blood bank plasma may be used). Two mg of KI is added, and the contents of the tube are mixed thoroughly. This constitutes the equilibrium dialyzate-plasma mixture (EDP).

6) Five ml of the EDP is pipetted into a dialysis sac, which is then sealed.

7) The sac containing the EDP is placed into a cellulose nitrate centrifuge tube containing 17 ml of standard phosphate buffer and 17 ml of Amberlite IRA-400 anion exchange resin previously equilibrated with the same buffer.[4]

8) The tube is tightly sealed and is placed on a rotating mixer.

9) After 2 hours, the tube is removed from the mixer, the sac removed and opened, and 3.0 ml of its contents pipetted into a counting tube (counting sample A).

10) From the original test serum, 100 μl is pipetted into a counting tube, and the volume is brought to 3.0 ml (counting sample B).

With each group of determinations a sample from a large pool of normal serum is analyzed. Additional specimens ("correction standards") are run in order to correct both for I^{131}-iodide added to the test serum as a contaminant and for osmotic dilution of the EDP during resin dialysis. These are prepared as follows:

1) To 6.0 ml of a 1:1 mixture of standard phosphate buffer and indifferent serum or plasma are added 25 μl of I^{131}-T_4 in HSA and 2.0 mg of KI.

2) One hundred μl is pipetted into a counting tube, and the volume is brought to 3.0 ml (counting sample C).

3) Five ml is pipetted into a dialysis sac, which is then sealed.

4) The sac is placed inside a cellulose nitrate centrifuge tube containing 17 ml of standard phosphate buffer and 17 ml of IRA-400 resin. The tube is sealed and placed in a rotating mixer for 2 hours.

5) After this period, the sac is removed from the tube and opened. One hundred μl of its contents is pipetted into a counting tube, and the volume is made to 3.0 ml (counting sample D).

Correction factors. To obtain maximal accuracy, it is necessary to correct for several sources of error. First, since the commercial preparation of I^{131}-T_4 is invariably contaminated with I^{131}-iodide, which is later removed from the equilibrium dialyzate, it is necessary that the counting rate of the standard used in calculating the final PFT_4 not include counts added to the test serum as iodide. Secondly, osmotic dilution of the EDP occurs during the resin dialysis procedure. This decreases the concentration of I^{131}-T_4 remaining in the EDP and lowers calcu-

[4] By means of a pipette from which the tip has been removed, a suspension of resin in buffer is transferred into a 50-ml graduated centrifuge tube. This process is continued until the volume of the sedimented resin is 17 ml. The volume of the supernatant buffer is then adjusted to an additional 17 ml, and the entire contents are then transferred to the cellulose nitrate centrifuge tube.

lated values for PFT_4. Both of these difficulties are overcome, however, by utilizing a correction standard. A sample of plasma is diluted with buffer to simulate an EDP, labeled T_4 is added, and the mixture is subjected to resin dialysis. Since both osmotic dilution and complete removal of iodide occur, the counting ratio of pre- to postdialysis specimens always exceeds unity and constitutes a factor that corrects for both osmotic dilution and contamination of the original I^{131}-T_4 with I^{131}-iodide.[5]

Let cpm_A, cpm_B, cpm_C, and cpm_D represent the radioactivity in counting samples A, B, C, and D, respectively. $Cpm_A \times 5/1.5 =$ total free I^{131}-T_4 in equilibrium with test serum. $Cpm_B \times 50 =$ total I^{131} added to test serum. $100 \times (cpm_A \times 5/1.5) / (cpm_B \times 50) = PFT_4$ (uncorrected). $Cpm_C/cpm_D =$ correction factor for osmotic dilution and I^{131}-iodide contamination. PFT_4 (uncorrected) $\times cpm_C/cpm_D = PFT_4$.[6] Absolute concentration of free $T_4 =$ protein-bound iodine (PBI) $\times PFT_4/0.65$ (millimicrograms T_4 per 100 milliliters).

Electrophoretic methods. In many cases, a sample of the serum containing the labeled T_4 that was used for measurement of the free T_4 was subjected to filter paper electrophoresis in Tris-maleate buffer, pH 8.6, by methods previously described (4). The distribution of such small quantities of I^{131}-T_4 is believed to reflect the approximate apportionment of endogenous T_4-binding proteins in serum.

Miscellaneous methods. Values for serum PBI were performed by a modification of the method of Zak (14). Statistical analyses were performed according to methods described by Snedecor (15).

Patient material. Sera in the normal group were obtained from donors whose ages ranged between 2 and 84 years. Sera from patients in various abnormal categories were obtained from patients in the wards or outpatient clinics of the Boston City Hospital or St. Elizabeth's Hospital. Sera were quickly separated from clotted blood and were kept frozen until used.

In the patients with hyper- or hypothyroidism, diagnoses were based upon clinical examination and upon values for serum PBI and thyroid radioactive iodine uptake and, occasionally, of basal metabolic rate. Patients designated as having nonthyroid illness ("sick patients") comprised a group with malignancy, myocardial or cerebral infarction, severe infection, and other severe acute or chronic systemic disorders.

Results

Evaluation of the resin dialysis procedure. Initial experiments were performed to determine the efficacy of the resin dialysis procedure in eliminating I^{131}-iodide from the EDP. In these experiments, 3.0 ml of standard phosphate buffer was mixed with an equal volume of human plasma to prepare a simulated EDP. This mixture was enriched with 20 μc of inorganic I^{131} and 2.0 mg of KI and subjected to the resin dialysis treatment. In 18 experiments, an average of 99.85% of the added I^{131}-iodide was removed from the EDP (range, 99.30 to 99.96%). Prolongation of the resin dialysis treatment or transfer of the sac containing the EDP to another tube for a second resin dialysis treatment did not appreciably improve the removal of I^{131}-iodide from the EDP. Removal of labeled I^- appeared to decrease slightly (never to less than 99.30%) as the solution of I^{131}-iodide employed aged progressively after arrival from a commercial source. In these instances, prior addition of thiouracil, cysteine, or ascorbic acid reduced the quantity of I^{131}-iodide retained in the EDP. In two experiments in which large quantities of I^{131}-iodide (100 μc) were employed, chromatography of the EDP after resin dialysis treatment revealed a small radioactive peak at the origin and no radioiodine in the iodide zone, suggesting that I^{131} that had not been removed from the EDP was firmly bound to the plasma proteins. Very likely this represented an actual iodination of the proteins by oxidized products in the I^{131}-iodide employed.

Additional experiments were performed to determine whether significant losses of labeled T_4 from the EDP would occur during the resin dialysis procedure. To accomplish this, it was necessary either to eliminate completely or to ascertain correctly the proportion of the I^{131}-iodide with which the preparation of I^{131}-T_4 was contaminated. Three methods were used. First, filter paper chro-

[5] The extent of dilution of the EDP during resin dialysis treatment was directly assessed by measuring the OD_{280} of the EDP before and after treatment. In 115 pairs of samples, optical density ratios averaged 1.095 ± 0.040 (mean $\pm$ SD). The validity of utilizing such ratios was verified in other experiments in which I^{131}-labeled HSA, previously freed of I^{131}-iodide, was added to several EDP. Ratios of OD_{280} and of I^{131} in pre- and postresin dialysis specimens agreed closely.

[6] Values obtained with this calculation and presented in the present report are only half those described in Reference 12, since values therein were based on the total free T_4 in the entire system, rather than the total free T_4 in the dialysis sac. As pointed out to the authors by Dr. George C. Schussler, the concentration of free T_4 at equilibrium will be a function only of the binding interaction within the sac and, within broad limits, will be independent of the volume outside the sac. Therefore, the pertinent quantity of the free T_4 is not that within the entire system, but only that within the sac where the interaction of free with protein-bound T_4 occurs.

matography was employed to determine the proportion of I^{131}-iodide contaminating the I^{131}-T_4 added to the simulated EDP, and calculated values for recovered radioactive T_4 were corrected for this contamination. In other experiments, preparative paper chromatography of the commercial I^{131}-T_4 was performed. With the aid of radioautographs, the I^{131}-T_4 zone was excised, and small segments thereof were eluted directly into the simulated EDP. This was then subjected to the resin dialysis treatment. In the third series of experiments, serum was obtained from three patients given an intravenous injection of commercial I^{131}-T_4 several days earlier. Since previous studies have shown that I^{131}-iodide and other labeled products of deterioration of I^{131}-T_4 are removed from the blood more quickly than I^{131}-T_4 itself, the radioactive T_4 given to the patients would have been purified biologically (16). In these experiments, 3.0 ml of the patients' sera was mixed with 3.0 ml of phosphate buffer to simulate the EDP, and this mixture was then subjected to the resin dialysis treatment. In all three types of experiments, totaling 14 in number, an average of 99.9% (range, 99.4 to 104.0%) of I^{131}-T_4 was recovered in the EDP after the resin dialysis procedure.

Experiments were also performed to determine the extent to which T_3 would be lost from the EDP during the resin dialysis treatment. In four experiments, recovery of chromatographically purified T_3 from a simulated EDP after resin dialysis averaged 97.2% (range, 95.7 to 98.8%).

Evaluation of the equilibrium dialysis method. A variety of experiments was performed to evaluate the characteristics of the system for equilibrium dialysis of the test serum. In the system described by Sterling and Hegedus (5), to which the present system closely adhered, distribution equilibrium for free T_4 was achieved in 16 hours. In the present studies, successive values of PFT_4 obtained at 16, 20, and 24 hours in equilibrium dialyzates of the same serum were virtually identical, indicating that equilibrium had been achieved.

Sterling and Hegedus also reported that negligible quantities of I^{131}-T_4 were lost by adsorption to either the dialysis sac or to the wall of the cellulose nitrate tube in which equilibrium dialysis had been performed (5). This conclusion was based on calculation of the total recovery of I^{131} and necessitated estimation of the volume of fluid both within and outside the dialysis sac at the completion of dialysis, a procedure in which accuracy of measurement must necessarily be limited. In the present studies, an alternative method was employed. To enhance the accuracy of counting, serum was enriched with several times the usual concentration of I^{131}-T_4. At the end of 16, 20, and 24 hours of dialysis, the entire tube and its contents were counted by a clinical scintillation probe. The contents of the tube were then removed, the tube was rinsed three times quickly, but thoroughly, with distilled water, and the empty tube was counted. Thereafter, a solution of 1% HSA was introduced into the tube and allowed to stand for 15 minutes. The tube was then emptied, rinsed thoroughly, and counted again. In three experiments, between 15 and 20% of the initial total radioactivity was found affixed to the tube after the distilled water wash. No difference between the quantity of I^{131} adsorbed to the tube at 16, 20, and 24 hours could be detected. This radioactivity was largely removed by 1% HSA, indicating that it had been reversibly adsorbed to the surface of the tube. Thus, it appears that although significant quantities of T_4 within the dialysis system became adsorbed to the tube in which equilibrium dialysis is performed, the entire contents of the dialysis system came into equilibrium within 16 hours. In other experiments, it was found that adsorption of I^{131}-T_4 from the dialyzate was not decreased by treating the cellulose nitrate tube with silicone, or by using a glass tube, whether or not it had been siliconized.

A third series of experiments was performed to ascertain the effects of dilution of the test serum upon the measured values of PFT_4. A pool of fresh normal serum was enriched with I^{131}-T_4 and serial dilutions with standard phosphate buffer, ranging between 1:5 and 1:300, were prepared. Samples of whole and dilute sera were then analyzed for PFT_4 by the method described above. Values for the actual PFT_4 in each specimen were divided by the dilution factor. This calculation is equivalent to that employed by Oppenheimer, Squef, Surks, and Hauer to obtain an estimate of the PFT_4 in undiluted specimens from analyses of dilute specimens (6). As this calculation has been thought to correct for the effects of dilution, the resulting values will be termed "corrected" values for PFT_4.

In four experiments, corrected values for PFT_4 calculated in this manner were consistently influenced by dilution. Dilutions of 1:5 resulted in corrected values for PFT_4 that were approximately 55% of those obtained in undiluted sera. With further dilution, however, corrected values for PFT_4 did not decrease further (Figure 1).

Reproducibility of the method. In 11 consecutive analyses of a pool of normal serum, values for PFT_4 averaged 0.049 ± 0.005 (mean $\pm$ SD). In another pool of serum, 12 consecutive analyses yielded values for PFT_4 that averaged 0.051 ± 0.003. In a single experiment, concomitant analysis for PFT_4 in quintuplicate samples yielded values within the narrow range of 0.049 to 0.051.

Values in various clinical states (Table I). In 105 normal patients ranging in age between 2 and 84 years, values for PFT_4 averaged 0.050 ± 0.009 (mean $\pm$ SD). In 21 patients with myxedema, values were significantly decreased, averaging 0.037 ± 0.010. Here, however, considerable variability and overlap with the normal range was evident. Values for PFT_4 in 7 of the 21 patients with myxedema were within 1 SD of the normal mean. A more consistent decrease in PFT_4 (0.026 ± 0.006) was observed in 14 patients with normal pregnancy. As anticipated, the mean value for PFT_4 (0.111 ± 0.072) was significantly increased in 44 patients with thyrotoxicosis. Although great variability was evident in the extent of increase, values within 1 SD of the normal mean were found in only seven. A significant increase in PFT_4 was also evident in patients with severe nonthyroidal illness in whom values averaged 0.078 ± 0.033. Overlap with values in both the normal and thyrotoxic range was seen in this group.

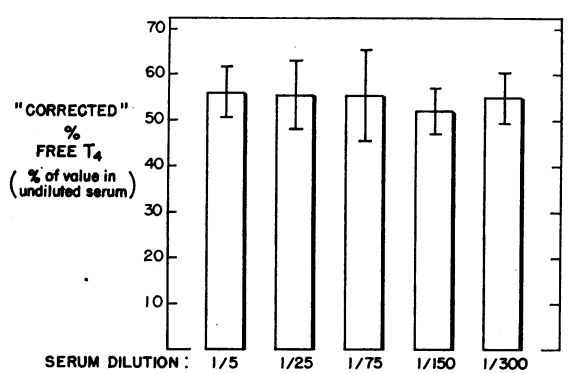

FIG. 1. EFFECT OF DILUTING SERUM ON CALCULATED ("CORRECTED") VALUES FOR THE PROPORTION OF FREE THYROXINE (PFT_4). In diluted serum, corrected per cent of free T_4 was obtained by dividing the measured value for PFT_4 by the dilution factor, a calculation analogous to that employed by Oppenheimer and colleagues (6). Values shown represent mean $\pm$ standard deviation of results in four separate experiments.

TABLE I

*The per cent (PFT_4) and absolute concentration (AFT_4) of free thyroxine, and the protein-bound iodine (PBI) in the serum of normal patients and of patients with various abnormal states**

Diagnosis	PFT_4	PBI	AFT_4
	% total	*μg/100 ml*	*mμg/100 ml*
Normal	0.050±0.009 (105)	5.4±0.8 (87)	4.03± 1.08 (87)
Myxedema	0.037±0.010† (21)	1.6±0.5† (21)	0.88± 0.52† (21)
Pregnancy	0.026±0.006† (14)	8.0±0.9† (12)	3.21± 0.56† (12)
Thyrotoxicosis	0.11±0.072† (44)	12.9±2.9† (43)	20.56±13.07† (43)
Sick	0.078±0.033† (27)	4.6±1.4‡ (22)	5.12± 2.99§ (22)

* Values presented represent mean ± standard deviation. Numbers in parentheses indicate number of observations in different patients.
† Significance of difference from normal value, $p < 0.001$.
‡ Significance of difference from normal value, $p < 0.025$.
§ Significance of difference from normal value, $p < 0.050$.

Values for AFT_4 in normal subjects averaged 4.03 ± 1.08 mμg per 100 ml. A significant decrease was evident in patients with myxedema, largely as a result of the low concentration of hormone in the serum, although low values for PFT_4 were also contributory. In accord with the findings of Sterling and Hegedus (5), but contrary to the findings of Oppenheimer and colleagues (6), mean values for AFT_4 in the sera of pregnant women (3.21 ± 0.56) were significantly less than normal, although individual values in the two groups overlapped considerably. In keeping with the findings of other workers (5–7), values for AFT_4 were consistently increased in patients with thyrotoxicosis, often strikingly so. In patients with nonthyroidal illness, a slight, but significant, increase in the mean AFT_4 (5.12 ± 2.29) was also seen; however, this increase was not so great as the increase in PFT_4, since values of the PBI in this group were significantly subnormal. This increase in AFT_4 in sick patients is in accord with the findings of Oppenheimer and colleagues (6), but does not agree with the findings of Sterling and Hegedus (5), whose patients may have been less seriously ill. In the present studies, the difference between AFT_4 in normal and sick patients was small and would not have achieved statistical

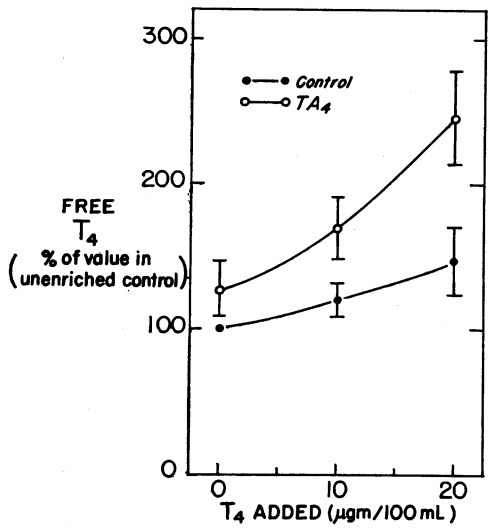

FIG. 2. EFFECT OF ENRICHING NORMAL SERUM WITH THYROXINE (T_4) AND 3,5,3',5'-TETRAIODOTHYROACETIC ACID (TA_4) ON THE PROPORTION OF FREE T_4. Open circles indicate sera enriched with 466 μg per 100 ml of TA_4, a concentration shown to inhibit completely the binding of T_4 by thyroxine-binding prealbumin (18). Values shown represent mean ± standard deviation of results in four separate experiments.

significance but for the large group of normal patients studied.

In a single patient with an idiopathic decrease in thyroxine-binding globulin (TBG), reported elsewhere in detail (4), PFT_4 was increased to 0.120, but because the PBI was low (2.0 μg per 100 ml), AFT_4 was within the normal range (3.67 mμg per 100 ml). In a single patient with an idiopathic increase in TBG (17), PFT_4 (0.025) was decreased into the range of normal pregnancy, and PBI was elevated, with the result that AFT_4 (3.67 mμg per 100 ml) was normal. In four male patients treated with diethylstilbestrol, 40 mg daily for 3 weeks, values for PFT_4 averaged 0.022, but PBI was elevated, and the mean AFT_4 (3.72 mμg per 100 ml) was within the normal range.

Effect of enriching normal and abnormal sera with stable T_4. Experiments were performed to determine the effect on PFT_4 of enriching normal sera with concentrations of T_4 ranging between 5 and 20 μg per 100 ml. In seven experiments, a distinct but gradual increase in the PFT_4 occurred over this range of enrichment, and values of PFT_4 in the most highly enriched samples averaged 153 ± 35% of those obtained in unenriched specimens. Nevertheless, despite the fact that PBI values in enriched specimens were at least as high as those found in patients with thyrotoxicosis, values for PFT_4 in T_4-enriched normal sera were well below those usually found in the sera of thyrotoxic patients.

To investigate this apparent discrepancy, two additional groups of experiments were performed. In the first, samples of normal sera were enriched with 3,5,3',5'-tetraiodothyroacetic acid (TA_4) to a concentration of 466 μg per 100 ml, a concentration shown earlier to prevent completely binding of T_4 by thyroxine-binding prealbumin (TBPA) (18). After addition of I^{131}-T_4, values for PFT_4 were determined. In 20 such experiments, PFT_4 in TA_4-enriched sera averaged 147 ± 41% of values in unenriched controls. Experiments were then performed to compare the effect of progressive loading with stable T_4 (0, 10, and 20 μg per 100 ml) on the PFT_4 of sera with and without added TA_4 (466 μg per 100 ml). Values for PFT_4 were determined in duplicate, and a portion of each sample was also subjected to filter paper electrophoretic analysis. In sera containing TA_4, electrophoretic analysis revealed no binding of T_4 by TBPA, regardless of whether stable T_4 had also been added. Here, the proportion of I^{131}-T_4 bound by both TBG and albumin was greater than in sera containing no TA_4. In sera containing TA_4, progressive enrichment with T_4 produced displacement of I^{131}-T_4 from TBG to albumin. Far less displacement of I^{131}-T_4 to albumin was produced by T_4 in sera containing no TA_4; in these specimens, I^{131}-T_4 displaced from TBG became associated largely with TBPA. In four experiments shown in Figure 2, enrichment of control sera with T_4 produced a gradual increase in PFT_4. In the absence of added T_4, sera containing TA_4 displayed higher values for PFT_4 than did controls, and values for PFT_4 increased more sharply in the former when T_4 was added.

In the second group of experiments, the effect of enrichment with stable T_4 on the PFT_4 in the serum of normal and sick patients was compared. In the latter samples, in which binding of T_4 by TBPA was greatly reduced, enrichment with stable T_4 to a maximal concentration of 20 μg per 100 ml increased PFT_4 far more than in comparably enriched normal sera. In two experiments performed in duplicate, values for PFT_4 in

normal sera were increased 24% by enrichment with stable T_4. In sera of sick patients, comparable enrichment increased PFT_4 by 102%. Thus, values for PFT_4 in T_4-enriched sera of sick patients averaged 2.7 times those found in comparably enriched normal controls.

The per cent of free T_3 in normal serum (PFT_3). In 12 sera from normal subjects, PFT_4 and PFT_3 were concomitantly assessed. In all instances, values for PFT_3 were much larger than for PFT_4, the ratio of the two averaging 9.6 ± 2.4.

Discussion

The present report describes a simple, reproducible method for measuring the proportion of free T_4 and other labeled thyroid hormones in human serum. This method appears to have major advantages over those that have previously been fully described (5, 6). The first phase of the present method, equilibrium dialysis of the test serum, is essentially the same as that described by Sterling and Hegedus and would be expected, therefore, to share any shortcomings with the method described by those workers (5). In the present studies, a substantial proportion of the I^{131}-T_4 was found to be adsorbed to the tube in which equilibrium dialysis was carried out. Failure of Sterling and Hegedus to find such adsorption can probably be ascribed to the indirect method they employed to study this question. Adsorption is not progressive, however, since the studies indicate that an equilibrium is established between the test serum, the dialyzate, and the wall of the cellulose nitrate tube. Nevertheless, such adsorption, in effect, lowers the total concentration of T_4 in the system and would be expected to decrease values for PFT_4 somewhat. From the rather low rate of increase in PFT_4 caused by addition of T_4 (Figure 2), however, it may be presumed that this effect would be small.

Values for the PFT_4 in normal serum obtained by the present method are substantially lower than those reported in the original method of Sterling and Hegedus, in which resin columns were employed to separate I^{131}-iodide from I^{131}-T_4 in the equilibrium dialyzate (5). The higher values those workers obtained can probably be ascribed to incomplete separation of I^{131}-T_4 from the preponderating I^{131}-iodide in dialyzates, especially as our values are very similar to those obtained with the magnesium precipitation method, which Sterling and Brenner have recently described in abstract form (19).

Another difference between the present results and those reported by Sterling and Hegedus is that these workers found little or no increase in PFT_4 when normal serum was enriched with stable T_4 in concentrations as high as 20 μg per 100 ml. In the present studies, such enrichment of normal serum led to clearly increased values for PFT_4. The magnitude of the increase was quite variable, however, especially at the highest concentration of T_4 employed. At this concentration, the total thyroxine in the system would be approximately 28 μg per 100 ml, a value at the upper limit or in excess of the normal range for the T_4-binding capacity of TBG (1). Thus, the sera in which PFT_4 increased most markedly at this level of enrichment may have been those in which the binding capacity of TBG had been exceeded. Walfish, Britton, Volpé, and Ezrin placed a similar interpretation upon the rapid increase in the *in vitro* red cell uptake of labeled T_3 that occurs as serum is enriched with T_4 in concentrations similar to those herein employed (20).

The second previous method for directly estimating the PFT_4 in serum is that described by Oppenheimer and colleagues (6). This method involves the equilibrium dialysis of extensively diluted samples of serum. Such dilution increases the proportion of unbound T_4 in the system and thus makes possible adequate separation of I^{131}-T_4 from I^{131}-iodide by trichloroacetic acid precipitation of the dialyzate after addition of protein. In their original report, Oppenheimer and colleagues made the assumption that absolute values for PFT_4 in dilute sera would be increased above those in undiluted sera by a factor equal to the extent of dilution (6),[7] and in a later communication an extensive mathematical analysis was presented to support this assumption (21). Thus, the corrected values for the PFT_4 of undiluted serum

[7] Oppenheimer and colleagues did not actually present values for PFT_4. Rather, they calculated a "dialyzable fraction (DF)," representing the fraction of total T_4 in their entire system that was dialyzable. From the published conditions, however, values for corrected PFT_4 analogous to those herein described can readily be calculated.

could be calculated as the quotient of the PFT_4 in diluted serum divided by the dilution factor. In these studies, no difference was found between corrected values for PFT_4 in samples diluted 1:8, 1:30, and 1:150. Although values for PFT_4 in undiluted sera, assayed by ultrafiltration, were consistently higher than corrected values in dilute sera, this was ascribed to inaccuracies of the ultrafiltration technique (21). The present findings suggest an alternative explanation, that use of dilute sera yields corrected values for the PFT_4 of undiluted sera, which are spuriously low. Thus, concomitant analyses of undiluted and variously diluted sera by the present technique revealed that dilution of serum by as little as 1:5 decreased values for corrected PFT_4 by approximately 50%, although no further effect was evident at higher dilutions. It is probably significant, furthermore, that the average value for AFT_4 in normal sera reported by Oppenheimer and colleagues is approximately 50% lower than that obtained by the present technique. In view of both the apparent effects of diluting serum and the ease and reliability with which the resin dialysis technique separates I^{131}-iodide and I^{131}-T_4, analysis of diluted serum seems less desirable than use of undiluted specimens.

The concept that the interaction between T_4 and the thyroxine-binding proteins of serum is a reversible binding equilibrium would dictate that increases in the absolute concentration of T_4, such as are produced by *in vitro* enrichment, should increase the PFT_4 (1). The failure of other workers to note appreciable increases over a physiologic range of T_4 concentrations was puzzling in this light (5), but the present data, which do demonstrate such increases in PFT_4, tend to support traditional concepts. Nevertheless, values for PFT_4 in normal sera in which the T_4 concentrations were increased to levels characteristic of thyrotoxicosis were lower than values for PFT_4 usually seen in sera from patients with this disease. This suggests that an increase in hormonal concentration cannot alone account for the high values for PFT_4 that patients with thyrotoxicosis often demonstrate. Conceivably, a decrease in the activity of the thyroxine-binding proteins could accentuate the increase in PFT_4 produced by an increased T_4 concentration. The T_4-binding capacity of TBG in thyrotoxicosis is most often found to be normal or slightly increased (1, 6, 20). It seemed reasonable, therefore, to suspect that the other major T_4-binding protein, TBPA, might be implicated. A decreased binding capacity of TBPA in many patients with thyrotoxicosis was first noted by Richards, Dowling, and Ingbar (22) and has recently been confirmed by Oppenheimer and his colleagues (6). The former workers, employing red cell uptakes of I^{131}-T_4 and I^{131}-T_3, found that T_4-enrichment of sera from sick patients with low T_4-binding capacities of TBPA produced uptake values within the thyrotoxic range, but did not do so in sera with normal TBPA. In the present studies, this relationship was directly tested and verified in two ways. First, enrichment of sick sera with low T_4-binding capacities of TBPA produced significantly greater increases in PFT_4 than in normal sera, and values in the thyrotoxic range were obtained. Second, when T_4-binding by TBPA was obliterated by the addition of TA_4, PFT_4 in unenriched sera increased to values similar to those seen in sick patients whose binding of T_4 by TBPA was comparably low. When sera containing TA_4 were enriched with T_4 to concentrations similar to those found in thyrotoxicosis, a sharper rise in PFT_4 than in normal sera was noted, and values for PFT_4 in the thyrotoxic range were found. These findings suggest that the pronounced increase in PFT_4 noted in the serum of some patients with thyrotoxicosis may be due to both an increase in the concentration of the ligand (i.e., T_4) and a decrease in available binding sites through a reduction in the T_4-binding capacity of TBPA. The possibility that other, as yet undiscovered, factors may be operative cannot be excluded, however.

By the same token, decreased T_4 binding by TBPA would appear to contribute at least in part to the increased values for PFT_4 noted in sera from patients with a variety of severe illnesses. Such changes, first evident in the red blood cell uptake studies of Richards and co-workers (22), have recently been confirmed by dialysis of dilute serum by Oppenheimer and co-workers (6). Similar changes in TBPA have been noted in patients severely ill with myxedema, and this factor may account for the present observations that values for PFT_4 in patients with myxedema are not infrequently in the normal range. These direct measurements are consistent with the several re-

ports that indirect indexes of PFT_4, such as resin and blood cell uptakes, are often unexpectedly high in patients with myxedema (23–29).

In the present studies, despite considerable variability, values for AFT_4 in patients with nonspecific illness were significantly increased as a result of increases in PFT_4. This change in AFT_4 contrasts strikingly with the normal values for AFT_4 in those disorders in which PFT_4 is altered as a result of a primary, uncomplicated change in T_4 binding. Examples of the latter are patients with idiopathic increase or decrease in TBG and patients given estrogen. In such patients, total hormonal concentration varies directly with the change in T_4 binding so that the alteration in PFT_4 is overcome and AFT_4 is normal. In the patients with nonspecific illness, in contrast, values for PBI were somewhat lower than normal, but this decrease was insufficient to prevent a significant increase in AFT_4 for the group as a whole. One may wonder, therefore, whether in such patients homeostasis is driven to provide increased quantities of physiologically active hormone to the peripheral tissues. Such a hypothesis is consistent with observations that demonstrate an increased rate of T_4 degradation in both febrile and afebrile patients with a variety of illnesses (30, 31).

It is generally considered that the T_4 in the plasma turns over at a relatively slow rate, and this is true when the entire circulating hormone is considered. T_4 clearance rates from the plasma in normal adults average approximately 1 L per day (32). If it is true, however, that only the free T_4 is available to the cells, then the value for PFT_4 would indicate that the clearance of free T_4 normally averages approximately 2,000 L per day or more than 80 L per hour. Turnover of free T_4 is thus seen to be exceedingly rapid, and the profound effect of hormonal binding on over-all kinetics of hormonal metabolism is evident.

Finally the present studies have provided the first data concerning the relationship between PFT_4 and PFT_3, the latter being nine to ten times the former. The majority of data in the literature indicates that I^{131}-T_3, *in vivo,* turns over at the rate of approximately 55% per day (33–37), although one report indicates that the rate of turnover may be somewhat slower (38). Although few, if any, data are available concerning the volume of distribution of T_3, inspection of published observations indicates that the distribution space may be approximately 12 L (37, 38). This value is only slightly greater than that for T_4 (32), a conclusion consonant with the suggestion that the per cent of free hormone is a major determinant of the intracellular component of its distribution space (2). Thus, although PFT_3 is approximately ten times PFT_4, approximately 99.95% and 99.50% of T_4 and T_3 in the blood are protein bound, respectively.

From the foregoing figures it may be estimated that the over-all clearance of T_3 in adults is approximately 7 L daily. Thus the clearance of free T_3, which comprises approximately 0.50% of the total T_3, would be approximately 1,400 L per day, a value less than the calculated rate of clearance of free T_4. This finding may be related to a lesser ability of cellular mechanisms to degrade T_3, since most *in vitro* deiodinating systems for thyroid hormones degrade T_3 less rapidly than T_4 (39–41).

Summary

A simple, reproducible method has been developed and evaluated for measuring the per cent of free thyroid hormone in human serum (PFT_4). In normal patients, the free thyroxine (T_4) in serum averaged 0.050% of the total, whereas the proportion of free triiodothyronine was nine to ten times as large. Significant decreases in PFT_4 were found in the serum of patients with myxedema or pregnancy and significant increases in patients with thyrotoxicosis or a variety of severe nonthyroidal illnesses (sick patients). In the several diagnostic categories, however, considerable overlap of individual values with the normal range was observed.

Values for the absolute concentration of free thyroxine in serum were decreased in patients with myxedema and increased in patients with thyrotoxicosis. Small but significant decreases and increases, respectively, were found in absolute concentrations of free T_4 in the sera of pregnant and sick patients.

Use of dilute, rather than whole, serum was found to yield spuriously low values for the per cent of free T_4. Enrichment of normal sera with sufficient thyroxine to bring protein-bound iodine into the thyrotoxic range increased PFT_4. Nev-

ertheless, values for the per cent of free T_4 in enriched specimens of normal serum were less than those usually seen in patients with thyrotoxicosis. Enrichment with thyroxine of sera in which thyroxine binding by prealbumin (TBPA) was decreased, either as a result of systemic illness or of the addition of tetraiodothyroacetic acid, produced far greater increases in the per cent of free T_4 than it did in normal serum, and values in the thyrotoxic range resulted.

The data provide further evidence that TBPA contributes significantly to thyroxine binding in normal serum and suggest that the increased values for the per cent of free T_4 found in serum from thyrotoxic patients are due, at least in part, both to an increase in thyroxine concentration and a decrease in TBPA.

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