Simultaneous Measurement of Percentage Free Thyroxine and Triiodothyronine: Comparison of Equilibrium Dialysis and Sephadex Chromatography

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An equilibrium dialysis technique was used to measure simultaneously the proportion of free thyroxine (%FT_4) and free 3,5,3′-triiodothyronine (%FT_3) in sera from patients with diverse states of thyroid function and abnormal levels of plasma T_4-binding proteins. In general, the correlation between %FT_4 and %FT_3 values was excellent in the entire group of patients studied. Studies were also conducted to ascertain whether Sephadex columns could be employed to obtain simultaneous measures of plasma binding of T_4 and T_3. Mixtures of diluted serum and ^{125}I-T_4 and ^{131}I-T_3 were applied to columns of Sephadex in order to separate "bound" and "free" fractions. The values for percent free T_4 and T_3 yielded by the Sephadex process (%FT_4S and %FT_3S), although far greater numerically, correlated closely with %FT_4 and %FT_3 measured directly by equilibrium dialysis. When %FT_4 and %FT_3 were multiplied by their respective serum concentrations, the resulting free T_4 and free T_3 indices provided good separation of hyperthyroid and hypothyroid groups from the euthyroid group. As in the dialysis method, %FT_4S closely correlated with %FT_3S.

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3,5,3′-Triiodothyronine (T_3) has been recognized as a major determinant of thyroid hormone action within the organism. From estimates of relative biologic potencies and production rates, it seems clear that the metabolic contribution of T_3 is at least as great as that of thyroxine (T_4), and perhaps much greater. As with T_4, the free fraction of T_3 in plasma is probably the metabolically active moiety. Despite the great attention paid to measuring the proportion and absolute concentration of free T_4, similar measurement of free T_3 in abnormal states has been relatively neglected.

The present study compares the values of percent free T_4 and T_3 determined directly by equilibrium dialysis (%FT_4 and %FT_3) in sera from patients with diverse states of thyroid function or with abnormalities of T_4-binding proteins. In addition, since gel filtration is a far simpler technique than equilibrium dialysis for separating free from bound thyroid hormones, we assessed the correlation between %FT_4 and %FT_3 values obtained by dialysis and those yielded by gel filtration on Sephadex (%FT_4S and %FT_3S).

MATERIALS AND METHODS

Sera obtained from 54 individuals were studied. Nine subjects were euthyroid and clinically well. Four other euthyroid subjects had increased serum thyroxine-binding globulin (TBG) concentrations secondary to administered estrogens. The study group also included 10 euthyroid patients with nonthyroid illness, 12 patients with hypothyroidism, and 19 with hyperthyroidism. Three of the hyperthyroid

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patients were receiving estrogenic medications and had increased serum TBG concentrations.

Dialysis method. Measurements of $\%FT_4$ and $\%FT_3$ in serum were made by a modification of the equilibrium dialysis technique of Sterling and Brenner (*1*), using ^{125}I-labeled T_4 and ^{131}I-labeled T_3 from a commercial source. Just before use, the labeled hormones were diluted to the desired concentration in a solution of 0.125 gm of human serum albumin in 100 ml of standard phosphate buffer (potassium phosphate buffer, pH 7.4, r/2 = 0.15, containing 0.001 *M* sodium azide). Aliquots of the solution containing both labeled T_4 and T_3 were then mixed briefly with Iobeads (0.1 gm/ml; Technicon Co., Ardsley, N.Y.) to remove most (above 95%) of the contaminating ^{125}I and ^{131}I ions. Specimens of serum were diluted with two parts of standard phosphate buffer and were then enriched with 50 ml of the stock solution of labeled hormones. This resulted in ultimate enrichment of endogenous hormones with approximately 0.62 μCi/ml and 10 ng/ml of labeled T_4 and 0.90 μCi/ml and 10 ng/ml of labeled T_3. Aliquots (3.0 ml) of diluted serum specimens (1 ml of serum, 2 ml of standard phosphate buffer) were then pipetted into a multichambered Plexiglas dialysis apparatus and dialyzed against 5 ml of the phosphate buffer, using cellulose dialysis membrane that had been soaked in a buffer but not acid-washed. Each sample was analyzed in duplicate. Dialyses were allowed to proceed for 17 hr at 37°C. At that time, 2.0 ml of carrier T_4 solution (1.0 mg/ml) was added to 3.0 ml of dialysate, and precipitation of labeled T_4 and T_3 was carried out using $MgCl_2$ (*1*). In recovery experiments, more than 91% of ^{131}I-T_3 and 95% of ^{125}I-T_4 were recovered in the precipitate; adding carrier T_3 did not improve the recovery of labeled T_3.

The content of ^{131}I and ^{125}I was measured both in magnesium precipitates and in the original dialysands, corrections being made for crossover of counts from ^{131}I into the ^{125}I range. The $\%FT_4$ and $\%FT_3$ were calculated as previously described (*1*). Observed values were "corrected" for the threefold dilution of serum by dividing them by 3. With each batch of analyses performed, specimens of a normal serum pool were assayed and corrected values of $\%FT_4$ and $\%FT_3$ in the pool were calculated as described above. Values in test sera were expressed as a fraction of those found in the normal serum pool ("normalized" $\%FT_4$ and $\%FT_3$).

Sephadex method. The method of preparing dilute serum containing both labeled T_4 and T_3 was identical to that described to prepare specimens for dialysis. A Tetralute column (Ames Co., Elkhart, Ind.) was brought to room temperature, the overlying alkaline fluid was decanted, and the column was washed twice with 4.0 ml of standard phosphate buffer. The final pH of the second eluate was 7.4. One-half milliliter of labeled diluted serum was added to the column and allowed to enter the column bed. The column was washed with 4.0 ml of phosphate buffer and the resulting eluate was collected as the "bound" fraction (B). A new collecting tube was placed under the column; 1.0 ml of pooled normal plasma was added and allowed to enter the column bed. The column was again washed with 4.0 ml of buffer, and the eluate was collected as the "free" fraction (F). These "bound" and "free" fractions (B and F) were counted in a two-channel gamma scintillation counter, with corrections made for crossover counts from ^{131}I into the ^{125}I range, and expressed in counts per minute. Values for $\%FT_4S$ and $\%FT_3S$ were calculated according to the following formulae:

$$\%FT_4S = \frac{F\ (cpm)\ ^{125}I\text{-}T_4 \times 100}{F\ (cpm)\ ^{125}I\text{-}T_4 + B\ (cpm)\ ^{125}I\text{-}T_4},$$

$$\%FT_3S = \frac{F\ (cpm)\ ^{131}I\text{-}T_3 \times 100}{F\ (cpm)\ ^{131}I\text{-}T_3 + B\ (cpm)\ ^{131}I\text{-}T_3}.$$

Results were normalized by dividing by the corresponding values obtained in the specimen of pooled serum. Each sample of serum was analyzed in duplicate.

The total serum T_4 concentration was determined by a competitive protein-binding method described previously (*2*). The total serum T_3 concentration was measured by radioimmunoassay in 36 patients (lack of serum prevented its measurement in some cases). For each hormone, a free hormone index was calculated by multiplying serum total hormone concentration by the normalized value of the corresponding free fraction, as measured by dialysis or Sephadex.

RESULTS

Standardization of methods. Preliminary experiments were conducted to determine properties of the Tetralute columns with respect to adsorption and elution of the radioiodine-labeled hormones and their contaminants. The bed volume of the column was approximately 1.3 ml. Specimens of dilute serum (1:3) enriched with radioiodine-labeled T_4 and T_3 were prepared in the manner described above, except that the contaminating iodides were not removed by Iobeads. One-half milliliter of the mixture was allowed to enter the column, with the initial eluate being discarded. The column was washed with standard phosphate buffer, successive fractions of the eluate (three drops/fraction) being collected and

their radioactivity measured. Two peaks of radioactivity containing both ^{131}I and ^{125}I were observed. The initial peak appeared in Fractions 10–12 at the void volume of the column, and the smaller second peak appeared between Fractions 14 and 21. Figure 1 is a representative elution pattern in which only data for ^{125}I-T_4 are shown; the concordant peaks for ^{131}I-T_3 are omitted. The first peak was identified as protein-bound hormone (in a separate experiment radioiodinated serum albumin appeared at the same position). The second peak was identified as inorganic radioiodide contaminating the original labeled hormone, since radioactivity in these fractions was not protein-precipitable and since prior treatment of the labeled hormone mixture with Iobeads reduced the size of the second peak by at least 80%. After adding 1.0 ml of normal serum to the column, a third peak appeared, representing "free" hormone previously adsorbed to Sephadex and now eluted by proteins of the added undiluted serum.

Seven separate experiments were run. The Sephadex method displayed less variability than did the dialysis technique. Interassay coefficients of variation were 29.3% for %FT_4 as compared to 14.3% for %FT_4S, and 19.0% for %FT_3 as compared to 10.2% for %FT_3S. Intra-assay variability was substantially smaller and was similar for these two methods (%FT_4, 0.81%; %FT_4S, 2.0%; %FT_3, 0.13%; %FT_3S, 0.2%).

Relation between free T_4 and free T_3. Over the range of patients studied, normalized values for %FT_4 and %FT_3 (dialysis method) were closely correlated ($r = +0.95$). The line of regression calculated by the method of least squares closely approximated the line of identity (Fig. 2). A close linear relationship (Fig. 3) was also evident when %FT_4S and %FT_3S (Sephadex method) were compared ($r = +0.94$). In neither regression was the slope significantly different from 1.0.

Comparison of dialysis and Sephadex methods. The values for %FT_4 and %FT_4S were closely correlated (Fig. 4), as were values for %FT_3 and %FT_3S (Fig. 5). Correlation coefficients were +0.84 for T_4 and +0.87 for T_3.

Table 1 presents values for free T_4 index and free T_3 index in subjects on whom sufficient serum was available for measurement of total hormone concentration as well as free hormone percentage by both the dialysis and Sephadex methods. Both hormone indices by either technique showed little or no overlap among the normal, hypothyroid, and hyperthyroid groups. One normal subject had the lowest free T_4 index and the highest free T_3 index in that group, resulting in some overlap in the free T_3 index ranges

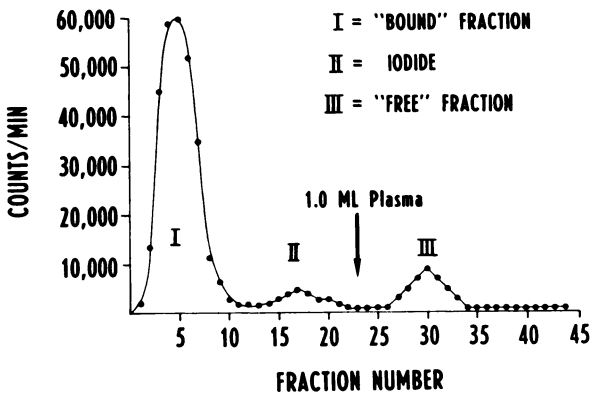

FIG. 1. Elution of radioactivity from Sephadex column after application of ^{125}I-T_4 in normal serum. Each fraction contained three drops of eluate. Peak I corresponds to protein-bound ^{125}I-T_4; Peak II, to inorganic ^{125}I; Peak III, to "free" ^{125}I-T_4 (eluted by adding 1.0 ml of unlabeled plasma).

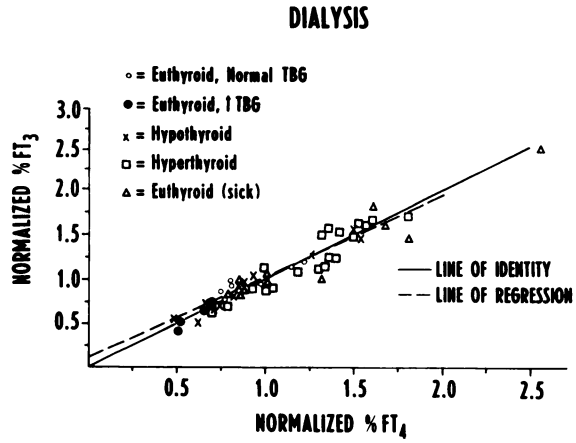

FIG. 2. Comparison of proportions of free T_3 and free T_4, each expressed in terms of normal serum value, by equilibrium dialysis. Correlation coefficient $r = +0.95$.

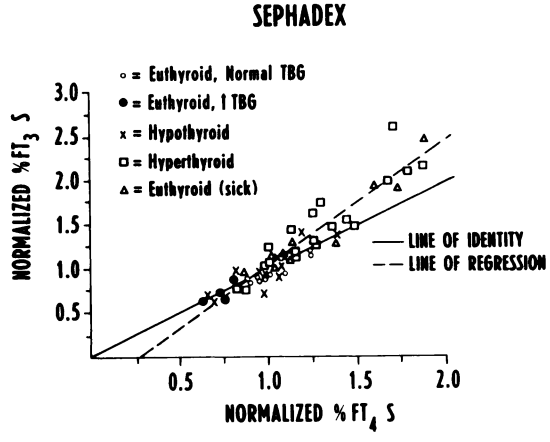

FIG. 3. Comparison of proportions of free T_3 and free T_4 (normalized) by Sephadex column method ($r = +0.94$).

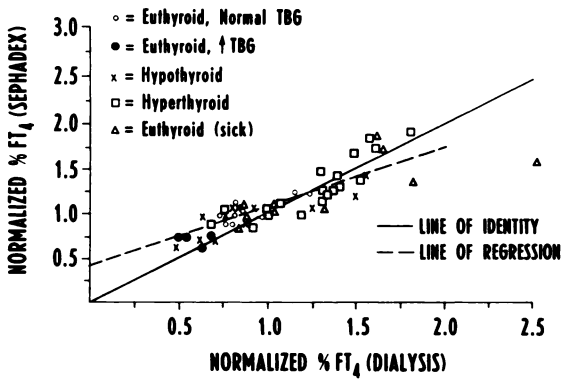

FIG. 4. Comparison of Sephadex and dialysis methods for proportion of free T_4 in various sera ($r = +0.84$).

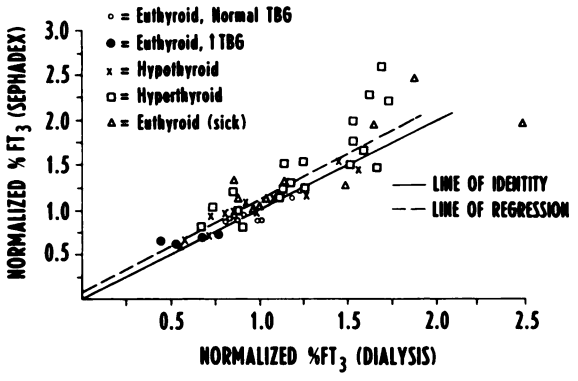

FIG. 5. Comparison of Sephadex and dialysis methods for proportion of free T_3 ($r = +0.87$).

between normal and hyperthyroid groups. The mean free T_3 index in the hyperthyroids, however, was nearly five times the normal mean. As expected, the results in the high-TBG euthyroid subjects were close to normal. In patients with nonthyroid illness, mean values were not significantly different from normal, but the range overlapped with that of the hypothyroid and hyperthyroid groups.

DISCUSSION

The present results show an excellent correlation between $\%FT_4$ and $\%FT_3$ measured by equilibrium dialysis, over a wide range of plasma binding activities. This remarkably close agreement is consistent with the widely held view that TBG is the predominant plasma binding protein for both hormones. Izumi also found good correlation between $\%FT_4$ and $\%FT_3$ using a dialysis method in a series of patients over a wide range of plasma hormone-binding activities (*3*).

The Sephadex column method yields reasonably good relative estimates of $\%FT_4$ and $\%FT_3$. Others have employed Sephadex for the rapid separation of "free" from protein-bound forms of thyroid hormones (*4–12*). An interaction between aromatic compounds and dextran gel (*13–15*) is the most likely explanation for the adsorption of iodothyronines to Sephadex. Thus, there is competition for thyroid hormones between low-affinity high-capacity Sephadex and higher-affinity binding proteins of serum, particularly TBG. Adsorption of hormones by Sephadex accounts for the fact that the percent Sephadex uptake is severalfold higher than the percent free hormone by dialysis. The normalized Sephadex values, based on results in a normal serum pool as a reference standard, allow use of the Sephadex method for determination of relative changes in $\%FT_4$ and $\%FT_3$. This more convenient procedure yields results that correlate closely with those obtained by the laborious dialysis method. For routine diagnostic use, either the $\%FT_4$ or the $\%FT_3$ can be used to calculate *both* a free T_4 index and a free

TABLE 1. FREE T_4 INDEX AND FREE T_3 INDEX BY TWO METHODS*

Condition	Free T_4 index		Free T_3 index	
	Sephadex method	Dialysis method	Sephadex method	Dialysis method
Normal	5.64	4.63	108	109
(n = 9)	(4.90–6.47)	(3.73–6.49)	(89–153)	(90–151)
Hypothyroid	1.29	1.16	49	45
(n = 5)	(0.24–1.47)	(0.28–2.23)	(8–90)	(9–84)
Hyperthyroid	11.37	13.44	586	471
(n = 11)	(8.35–35.7)	(8.50–33.6)	(188–1020)	(130–1145)
Nonthyroid illness (Eu)	3.84	4.12	98	87
(n = 7)	(1.35–9.83)	(1.29–9.98)	(20–180)	(25–154)
High TBG (Eu)	5.24	4.35	101	94
(n = 4)	(4.77–5.80)	(3.35–5.40)	(94–110)	(65–116)

* Free hormone index was computed by multiplying the total hormone concentration (in micrograms of T_4 per 100 ml or nanograms of T_3 per 100 ml) by the proportion of "free" hormone, normalized to the value obtained in the normal serum pool. For each group, the mean and range of values are shown.

T_3 index from respective values for total T_4 and total T_3 concentration in serum. The method also permits simultaneous determination of the proportion of "free" T_3 for the purposes of clinical investigation.

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