We studied the correlation of thyroxin (T₄) binding proteins with the apparent free T₄ (FT₄) in 101 patients with nonthyroidal illness (NTI). Most patients (95%) were seriously ill at the time of blood collection. Concentrations of T₄-binding prealbumin (transthyretin), albumin, and T₄-binding globulin (TBG) often were low in the sera of these patients. Albumin was the most frequently subnormal. FT₄ in serum was determined by five methods represented in 16 different assays. With few exceptions, analog (one-step) FT₄ RIAs—both the binding-rate-based RIA and the related FT₄ indices (calculated from triiodothyronine-macroaggregated albumin uptake and total T₄)—and T₄/TBG ratios correlated positively and usually highly significantly (P < 0.01) with concentrations of prealbumin, albumin, and TBG. Equilibrium dialysis values for FT₄ did not correlate with prealbumin concentrations but showed a weakly (P < 0.03) positive correlation with albumin and a highly significant (P < 0.002) positive correlation with TBG. Of the three two-step FT₄ RIAs tested, the only statistically significant but weakly (P < 0.02) positive correlation with T₄-binding proteins was between Spiria FT₄ and TBG. Thus, in these NTI patients, FT₄ estimates vary with methodology and, to a lesser extent, with the particular assay used. The results from two-step FT₄ RIAs are least associated with binding protein concentrations.

Additional Keyphrases: radioimmunoassay · thyroxin analog · equilibrium dialysis · free thyroxin index · thyroxin/thyroxin-binding globulin ratio

Almost all the thyroid hormones in blood are bound to transport proteins such as thyroxin-binding globulin (TBG) (~80% of thyroxin (T₄) and ~90% of triiodothyronine (T₃)), albumin (~5% of both T₄ and T₃), and (thyroxin-binding) prealbumin (transthyretin) (~15% of T₄ and ~5% of T₃) (1). Hence, changes in the concentration of thyroid-hormone-binding proteins profoundly affect the total hormone concentrations in serum (1, 2). There is a dynamic equilibrium between the large protein-bound fractions (~99.98% of T₄ and ~99.7% of T₃) and the minute free fractions (~0.02% of T₄ and ~0.3% of T₃) of thyroid hormones (2). Although researchers have proposed that albumin- and TBG-bound thyroid hormones are directly available to some tissues (3), it is believed that free thyroid hormone is the fraction that enters the cells and exerts the principal metabolic effect. Indeed, the protein-free, dialyzable fraction of these hormones has been shown to bear a closer relationship to a subject’s metabolic status than does the total thyroid hormone concentration (1, 2). Also, because of the variable concentrations of the transport proteins, the total concentrations of thyroid hormones in serum can frequently change in the absence of thyroid disease (1, 2).

Along with thyrotropin (thyroid-stimulating hormone, TSH), measurement of free thyroxin (FT₄) in serum is increasingly used for screening and diagnosis of thyroid abnormalities (2, 4). Several reports indicate, however, that assays for FT₄ might be affected by changes in the concentrations of thyroid-hormone-binding proteins (5–19). Because patients with nonthyroidal illness (NTI) often exhibit changes in the concentrations of these proteins (8–14, 20, 21), we analyzed how these alterations correlate with FT₄ as estimated in patients with NTI by 16 different assays, based on a variety of methodologies.

Materials and Methods

Subjects

We studied 112 serum samples from 101 patients. All patients had NTI, and most of them (95%) were seriously or critically ill, in intensive-care units, at the time of blood sampling. We reported further details previously (22).

Thyroid-Function Tests

We tested the performance of 16 FT₄ methods, including:

(a) a binding-rate-based RIA (Immophase; Ciba Corning Diagnostics Corp., Medfield, MA 02052);

(b) seven one-step (analog) RIAs: Amerlex-M (Amer sham Corp., Arlington Heights, IL 60005), Coat-A-Count (Diagnostic Products Corp., Los Angeles, CA 90045), GammaCoat one-step (Clinical Assays, Dade, Baxter Trav enol Diagnostics, Inc., Cambridge, MA 02139), a commercially marketed (method A) and an investigational (method B) version of Magic one-step (Ciba Corning Diagnostics), Solid-Phase Component System (Becton-Dickinson Immunodiagnostics, Orangeburg, NY 10962), and SimulTRAC (Becton-Dickinson Immunodiagnostics);

(c) three two-step RIAs: GammaCoat 2-Step ( Clinical Assays), Phase II with KinetiCount 48 (Medical and Scientific Designs, Inc., Rockland, MA 02370), and Spiria (International Immunoassay Labs, Inc., Santa Clara, CA 95054);

(d) equilibrium dialysis (SmithKline Bio-Science Labs, Inc., Van Nuys, CA 91405);

(e) two FT₄ indices based on T₃ macroaggregated albumin uptake and total T₄; and

(f) two T₄/TBG ratios for the estimation of FT₄.

Details of the FT₄ assays are described elsewhere (22). We measured T₃ uptake by macroaggregated albumin (MAA; Amer sham Corp., Arlington Heights, IL 60005); total T₄ by two different RIAs [method A: GammaCoat (Clinical Assays), method B: Immophase (Ciba Corning Diagnostics)]; total T₃ by RIA (Quanticoat; Kallestad Diagnostics, South Austin, TX 78746); reverse T₃ by DAB/PEG kit (Serino Diagnostics Inc., Norwell, MA 02061); and
TSH by two different immunoradiometric assays (IRMA) (method A: SimulTRAC (Becton-Dickinson Immunodiagnostics), and method B: TSH MAIA Clone kit (Serono Diagnostics)).

We also determined the concentration of the major thyroid-hormone-binding proteins: prealbumin, by rate nephelometry in an Array Protein System (Beckman Instruments, Inc., Brea, CA 92621); albumin, by brom cresol purple dye binding in an aca (DuPont Co., Wilmington, DE 19898); and TBG, by IRMA with Immaphase (Ciba Corning Diagnostics).

Statistical Analysis

We used simple linear regression for analysis of relationships between various serum components. We excluded up to seven outliers from the analyses by omitting data points that generated residuals greater than 4 S_{xy} (23).

Results

Measurement and correlation of concentrations of prealbumin, albumin, and TBG in serum. The concentrations of prealbumin and albumin in serum were low in 44% and 70% of patients, respectively. The overall mean concentrations were subnormal: 159 mg/L (reference interval: 170–420) and 32 g/L (reference interval: 38–49) for prealbumin and albumin, respectively (Figure 1). In fact, some serum prealbumin concentrations were below the lower limit of linearity (70 mg/L) of the method used (normal "C" dilution). Arbitrarily, we set these prealbumin results to 70 mg/L in plotting the frequency distribution, in calculating the mean (which is thus somewhat overestimated) (Figure 1), and in analyzing the relationship of prealbumin to thyroid-function tests (see below). The concentration of TBG in serum was normal in 84% of the cases, the overall mean being 16 mg/L (reference interval: 12–28) (Figure 1).

Correlations between prealbumin (mg/L) and albumin (g/L) were statistically highly significant (P <0.0001) and positive: y_{PreAlb} = 3.5327x_{Alb} + 47, S_{xy} = 57, r = 0.49, n = 88; and between albumin (g/L) and TBG (mg/L): y_{Alb} = 0.8901x_{TBG} + 18, S_{xy} = 8, r = 0.53, n = 112. Prealbumin was not correlated with TBG.

Correlation of FT₄ with prealbumin. The binding-rate-based RIA (Immaphase) (r = 0.36), six of seven one-step (analog) RIAs (r = 0.25–0.40), and all indirect methods (FT₄ indices (r = 0.31–0.37) and T₄/TBG ratios (r = 0.31–0.41)) positively correlated (probability of slope = 0 from <0.0250 to <0.0001) with the prealbumin concentration in serum (Figure 2, rows 1, 2, and 4). In contrast, the SimulTRAC one-step FT₄ RIA, the three two-step FT₄ RIAs, and equilibrium dialysis FT₄ showed no statistically significant association with prealbumin (Figure 2, rows 2 and 3). According to the slopes for these regressions, the binding-rate-based RIA and one-step RIAs that correlated with prealbumin changed by 2–3 pmol of FT₄ per liter (~1/6 to 1/8 of the reference interval) for a 100 mg/L change in prealbumin. The corresponding changes in FT₄ indices were by 5–6 units (~1/6 to 1/7 of the reference interval) and in T₄/TBG ratios were by 0.7–1.0 unit (~1/11 to 1/15 of the reference interval).

Correlation of FT₄ with albumin. The binding-rate-based FT₄ RIA (r = 0.61), all seven one-step FT₄ RIAs (r = 0.57–0.78), both FT₄ indices (r = 0.62), and both T₄/TBG ratios (r = 0.34–0.46) showed statistically highly significant (P <0.0003) positive correlation with the serum albumin concentration (Figure 3, rows 1, 2, and 4). The three two-step FT₄ RIAs were not associated, whereas equilibrium dialysis FT₄ weakly (r = 0.21, P <0.03) correlated with the serum albumin concentration (Figure 3, row 3). The regression slopes indicate that the binding-rate-based and one-step RIAs were comparably affected by changing serum albumin concentrations by 3–5 pmol of FT₄ per liter (~1/3 to 1/4 of the reference interval) for a 10 g/L change in albumin, whereas equilibrium dialysis was less affected, i.e., by 1.8 pmol of FT₄ per liter (~1/9 of the reference interval) for that albumin change. The rate of change for the FT₄ indices was approximately 8 units (~1/4 to 1/5 of the reference interval) and for the T₄/TBG ratios was approximately 0.7 unit (~1/13 to 1/17 of the reference interval) per 10 g/L change in albumin.

Correlation of FT₄ with TBG. The binding-rate-based FT₄ RIA (r = 0.65), all seven one-step FT₄ RIAs (r = 0.41–0.65), equilibrium dialysis FT₄ (r = 0.37), both FT₄ indices (r = 0.70–0.74), and one of the T₄/TBG ratios (B) (r = 0.30) showed highly significant (P <0.002) positive correlation, whereas one of the three two-step FT₄ RIAs (Spiria) showed weakly significant (P <0.02) positive correlation (r = 0.22) with the serum TBG concentration (Figure 4). Of 16 methods tested, only the GammaCoat and Phase II two-step FT₄ RIAs and a T₄/TBG ratio (A) based on GammaCoat total T₄ did not correlate with the TBG concentration (Figure 4, rows 3, 4). From the slopes of these regressions, we conclude that those RIAs that significantly correlated with TBG and the equilibrium dialysis FT₄ method changed by 3.6 to 8.2 pmol of FT₄ per liter (~1/2 to 1/6 of the reference intervals) for every 10 mg/L change in

![Fig. 1. Frequency distribution of the serum concentration of major thyroid-hormone-binding proteins in patients with NTI](image-url)
TBG, the FT₄ indices changed by about 17 units (~1/2 to 1/3 of the reference intervals), and the T₄/TBG ratio (B) changed by 0.9 unit (~1/12 of the reference intervals) for the same change in TBG.

Relationship of the free fraction of total T₄ and other thyroid-function test results to thyroid-hormone-binding proteins.

The dialyzable fraction of total T₄ in equilibrium dialysis and the fraction of total T₄ bound to solid-phase-bound antibody (A or red tube) during the 30-min incubation "rate of binding" in the Immophase FT₄ RIA were not associated with the prealbumin concentration (Table 1). However, both fractions showed highly significant (P < 0.0001) negative correlations with albumin and TBG (Table 1).

Similarly, the T₃-macroaggregated albumin uptake fraction did not correlate with prealbumin but exhibited highly significant (P < 0.0001) negative correlations with albumin and TBG (Figure 5).

The concentrations of total T₃ (measured by either method) and total T₃ all highly significantly (P < 0.01) and positively correlated with all three major thyroid-hormone-binding proteins (Table 2). On the other hand, the concentration of reverse T₃ correlated highly significantly (P < 0.0005) and positively with prealbumin but did not correlate with albumin and TBG (Table 1).

Interestingly, results by the two "ultrasensitive" TSH IRMA we tested showed highly significant (P < 0.0001) positive correlations with serum albumin and, to a lesser extent (P < 0.01), with TBG concentration (Table 1). They did not correlate, however, with the prealbumin concentration.
Discussion

Using sera from a large number of seriously or critically ill patients with NTI, we demonstrated that results by most methods for measurement of FT₄ correlate with the concentration of the major thyroid-hormone-binding proteins: prealbumin, albumin, and TBG. This may be ascribed to at least two factors. First, because the non-protein-bound fraction of T₄ is only about 0.02% (2), small changes in the sequestration of labeled T₄ or T₃ analog can result in comparatively large changes in the free fraction. As indicated in previous reports (8-14, 20, 21) and our current findings, changes (mostly decreases) in the concentration of thyroid-hormone-binding proteins in serum are common in patients with NTI. We found the frequency of abnormally low concentrations to be in the order albumin > prealbumin > TBG. Second, except for the two-step FT₄ RIA's, all other FT₄ methodologies we tested involve an incubation step in which labeled T₄, T₃ analog, or T₃ is mixed with the serum to be analyzed (see Tables 1 and 2 in 22). The concomitant presence of thyroid-hormone-binding proteins along with binding inhibitors of T₄ (and likely of T₃ analog), such as drugs (24), free fatty acids (22, 25-27), and other endogenous substances (28), can thus affect the sequestration of labeled T₄, T₃ analog, or T₃. A concentration-related increase of the FT₄ fraction in equilibrium dialysis has been well documented for certain drugs (24). Although initially thought to bind to proteins (particularly TBG) negligibly, T₃ analogs, like their parent compound T₃ (1, 2), are now known to bind with different affinities to thyroid-binding proteins (29). Consequently, in the absence of...
antibody, 2.2% of (e.g.) the Amerlex T₄ analog is bound to prealbumin, 71.0% to albumin, and 25.1% to TBG (29).

Our results for 16 FT₄ assays, representing five different methodologies (the binding-rate-based RIA is conceptually related to the FT₄ index, based on T₃ uptake and total T₄), demonstrate that the dependence of FT₄ measurements on the concentration of thyroid-hormone-carrier proteins is linked to the methodology involved rather than to the formulation of a particular assay (kit). The one-step (analog) FT₄ RIAs (except for the SimulTRAC assay with respect to prealbumin), the binding-rate-based RIA, the FT₄ indices, and one of the two T₄/TBG ratios positively correlated with all three thyroid-hormone-binding proteins in our patients with NTI. Equilibrium dialysis results positively correlated with albumin and TBG. Consistent with a setup that prevents contact between labeled T₄ and the patient’s serum, only the three two-step FT₄ RIAs had no highly significant associations with the concentration of thyroid-hormone-binding proteins. In fact, only one of the three two-step assays, the Spiria FT₄ RIA, showed any statistically significant correlation with thyroid-hormone-binding proteins: a weakly (P < 0.03) positive correlation with TBG.

These results are at variance with our previous findings (7) and other reports regarding the dependence of FT₄ measurements on thyroid-hormone-binding proteins (5–19). Positive, negative, and no correlation have all been reported between thyroid-hormone-binding proteins and FT₄ as measured by various techniques (5–19). We explain the sometimes-discordant observations, in part, by differences in the patient populations studied. For instance, in contrast to the present work, where 96% of the patients

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**Fig. 4.** Correlation of endogenous TBG concentration with the apparent FT₄ values, as obtained by various methods in patients with NTI.
Table 1. Linear-Regression Analysis of Major Thyroid-Hormone-Binding Proteins with the Dialyzable and Antibody-Bound Fractions of $T_4$, Total $T_4$, Total and Reverse $T_3$, and TSH in Patients with NTI

<table>
<thead>
<tr>
<th>$y$</th>
<th>Slope</th>
<th>Intercept</th>
<th>$S_{y.x}$</th>
<th>$P &lt;$</th>
<th>$r$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x = \text{prealbumin, mg/L}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzable fraction of $T_4$</td>
<td>$-0.00005$</td>
<td>$0.05$</td>
<td>$0.02$</td>
<td>$0.1251$</td>
<td>$0.17$</td>
<td>$87$</td>
</tr>
<tr>
<td>Antibody-bound fraction of $T_4$</td>
<td>$-0.00003$</td>
<td>$0.03$</td>
<td>$0.01$</td>
<td>$0.1180$</td>
<td>$0.17$</td>
<td>$86$</td>
</tr>
<tr>
<td>Total $T_4$, nmol/L</td>
<td>$0.1858$</td>
<td>$47$</td>
<td>$35$</td>
<td>$0.0021$</td>
<td>$0.32$</td>
<td>$89$</td>
</tr>
<tr>
<td>Total $T_4$ (B), nmol/L</td>
<td>$0.2013$</td>
<td>$34$</td>
<td>$36$</td>
<td>$0.0004$</td>
<td>$0.37$</td>
<td>$87$</td>
</tr>
<tr>
<td>Total $T_3$, pmol/L</td>
<td>$0.0033$</td>
<td>$0.10$</td>
<td>$0.47$</td>
<td>$0.0001$</td>
<td>$0.45$</td>
<td>$89$</td>
</tr>
<tr>
<td>Reverse $T_3$, pmol/L</td>
<td>$-0.0023$</td>
<td>$1.08$</td>
<td>$0.41$</td>
<td>$0.0005$</td>
<td>$0.38$</td>
<td>$83$</td>
</tr>
<tr>
<td>TSH (A), milli-int. units/L</td>
<td>$0.0029$</td>
<td>$1.7$</td>
<td>$1.5$</td>
<td>$0.2038$</td>
<td>$0.14$</td>
<td>$89$</td>
</tr>
<tr>
<td>TSH (B), milli-int. units/L</td>
<td>$0.0022$</td>
<td>$1.1$</td>
<td>$1.2$</td>
<td>$0.2451$</td>
<td>$0.13$</td>
<td>$82$</td>
</tr>
<tr>
<td>$x = \text{albumin, g/L}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzable fraction of $T_4$</td>
<td>$-0.00014$</td>
<td>$0.08$</td>
<td>$0.02$</td>
<td>$0.0001$</td>
<td>$0.55$</td>
<td>$110$</td>
</tr>
<tr>
<td>Antibody-bound fraction of $T_4$</td>
<td>$-0.0007$</td>
<td>$0.05$</td>
<td>$0.01$</td>
<td>$0.0001$</td>
<td>$0.51$</td>
<td>$109$</td>
</tr>
<tr>
<td>Total $T_4$, nmol/L</td>
<td>$2.7329$</td>
<td>$-12$</td>
<td>$30$</td>
<td>$0.0001$</td>
<td>$0.64$</td>
<td>$112$</td>
</tr>
<tr>
<td>Total $T_4$ (B), nmol/L</td>
<td>$2.8813$</td>
<td>$-23$</td>
<td>$33$</td>
<td>$0.0001$</td>
<td>$0.63$</td>
<td>$110$</td>
</tr>
<tr>
<td>Total $T_3$, pmol/L</td>
<td>$0.0429$</td>
<td>$-0.71$</td>
<td>$0.36$</td>
<td>$0.0001$</td>
<td>$0.74$</td>
<td>$112$</td>
</tr>
<tr>
<td>Reverse $T_3$, pmol/L</td>
<td>$-0.0029$</td>
<td>$0.82$</td>
<td>$0.45$</td>
<td>$0.5634$</td>
<td>$0.06$</td>
<td>$103$</td>
</tr>
<tr>
<td>TSH (A), milli-int. units/L</td>
<td>$0.0832$</td>
<td>$-0.4$</td>
<td>$1.6$</td>
<td>$0.0001$</td>
<td>$0.43$</td>
<td>$112$</td>
</tr>
<tr>
<td>TSH (B), milli-int. units/L</td>
<td>$0.0594$</td>
<td>$-0.3$</td>
<td>$1.3$</td>
<td>$0.0001$</td>
<td>$0.42$</td>
<td>$100$</td>
</tr>
</tbody>
</table>

* By equilibrium dialysis.
** Fraction of labeled $T_4$ bound to solid-phase-bound antibody (A or red tube) during the 30-min incubation ("rate of binding") in the Immophase FT4 RIA.

were seriously or critically ill, fewer than half our previous study (15) population belonged to this category, the others being healthy volunteers, known or suspected thyroid patients, and ambulatory patients with NTI. Abnormalities of $T_3$-binding capacities or of binding affinities have been demonstrated in patients with NTI (30).

Particularly noteworthy is that in the present study with NTI patients even those analog methods (e.g., Amerlex-M, Coat-A-Count) that are modified to avoid interference from variations in concentrations of endogenous thyroid-hormone-binding proteins exhibited statistically significant positive correlations with prealbumin, albumin, and TBG. Similar results have been reported by some investigators (15-19) but denied by others (31-33).

We found positive correlations between FT4 as estimated by the binding-rate-based RIA ($T_4$ fraction bound × total $T_4$) or by the closely related FT4 indices ($T_4$ uptake fraction × total $T_4$) or equilibrium dialysis (dialyzable $T_4$ fraction × total $T_4$) and thyroid-hormone-binding proteins. Because there were statistically significant negative correlations between $T_4$ or $T_3$ fractions and thyroid-hormone-binding proteins, we must assume that the positive correlations between total $T_4$ or $T_3$ and thyroid-hormone-binding proteins (see below) were comparatively stronger.

Highly statistically significant ($P < 0.01$) correlations between total $T_4$ or $T_3$ and TBG are not surprising, because

![Fig. 5. Correlation of endogenous thyroid-binding protein concentrations with $T_3$ macroaggregated albumin uptake fraction in patients with NTI](image-url)
TBG usually carries more than 80% of the total thyroid hormones (T). These findings accord with our previous observations on NTI patients (12), where we also confirmed a positive correlation between total T₄ or T₃ and albumin and demonstrated that both total T₄ and T₃ are positively correlated with the concentration of prealbumin. Thus, the often-low concentrations of total T₄ and T₃ in our patients are at least partly attributable to the concomitantly low concentrations of the respective binding proteins. Additional mechanisms may involve accumulation of T₄⁻ and T₃⁻-binding inhibitors such as drugs and endogenous metabolic products, e.g., free fatty acids (22, 24–28). The statistically highly significant negative correlation between reverse T₃ and prealbumin (but not albumin or TBG) in our patients suggests that a worsening nutritional status of a patient, as evidenced by a progressive decrease in the concentration of prealbumin (34), is associated with decreasing de-iodination of reverse T₃, as evidenced by its increasing serum concentration (1, 2). Our finding of more often decreased albumin than prealbumin concentration and of a positive correlation between these proteins is in variance with previous observations in patients with NTI (34)—perhaps owing to differences in patient population and in turnover rates of prealbumin and albumin (e.g., early or advanced stage of malnutrition).

It is intriguing that both albumin and TBG (of which the former in particular was often in decreased concentration in serum) positively correlated with TSH, measured by either of two different "ultrasensitive" methods in seriously or critically ill patients with NTI. This phenomenon may be related to decreased secretion of TSH by NTI patients (35).

In summary, we found that, except for the two-step methods, results by most (if not all) currently used commercial FT₄ techniques are highly significantly correlated with concentrations of one or more of the three major thyroid-hormone-binding proteins in NTI patients. Second, the relative impact of the changing thyroid-hormone-binding protein concentrations on FT₄ measurements could be further assessed by expressing the change in FT₄ in terms of a fraction of the respective reference interval. For all three major binding proteins, the impact was the greatest on the one-step (analog) FT₄, RIAs, the binding-rate-based RIA, and the related FT₄ indices (T₄ uptake fraction × total T₄). In comparison, the impact on T₄/T₃ ratios was two to threefold less and, though statistically highly significant, was negligible in magnitude. Third, because subnormal TBG concentrations appear less often than subnormal albumin and prealbumin concentrations in patients with NTI, changes in the concentration of the latter two binding proteins are more likely to be associated with changes in measured FT₄.

The manufacturers of the FT₄ assay kits examined here generously provided us with materials, information, and advice, for which we are most grateful. We thank Mrs. Ruth Chealer for technical assistance and Mrs. Joyce Lee for secretarial help.

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