Direct and Indirect Techniques for Free Thyroxin Compared in Patients with Nonthyroidal Illness I. Effect of Free Fatty Acids

Gyorgy Csako, Mark H. Zweig, Janice Glickman, Jane Kestner, and Mark Ruddel

We examined the effect of endogenous free fatty acids (FFA) on the measurement of free thyroxin (FT4) by five different methodologies represented in 16 different assays in a large number of patients with nonthyroidal illness (NTI). Some, but not all, one-step (analog) FT4 RIAs negatively correlated with FFA concentration. All two-step FT4 RIAs, equilibrium dialysis FT4, and the dialyzable (free) fraction of T4 positively correlated. In contrast, a binding-rate-based FT4 RIA, FT4 indices based on T3 macroaggregated albumin uptake, and T4/TBG ratios did not correlate. We also analyzed the FT4–FFA relationship with a second, more sensitive approach by correlating test results with FFA/albumin molar ratio as an estimate of the "excess" (nonalbumin bound) FFA. We found that all FT4 RIAs, equilibrium dialysis FT4, FT4 indices based on T3 uptake, the dialyzable fraction of labeled T4 in equilibrium dialysis, the fraction of labeled T4 bound to solid phase antibody in the binding-rate-based RIA, and T3 uptake correlated with the FFA/albumin molar ratio. This FFA dependency was comparable among all the various techniques and was relatively small. Thus, increases or decreases in FT4 results due to varying FFA (and albumin) concentrations are highly likely with most currently available methods (only the T4/TBG ratio did not reveal FFA-dependency), but the magnitude of changes varies with the "excess" FFA.

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

The direct measurement or indirect estimation of free (nonprotein bound) thyroxin (FT4) in serum is considered to be a reliable indicator of thyroid status.1 Several new commercial methods have become available for determining FT4 in the last few years. There are, however, reports that various serum components may interfere with the assay of FT4 (1–15). Free (non-glycerin-bound) fatty acids (FFA), also referred to as nonesterified fatty acids (NEFA), are now thought to represent a major factor responsible for altered FT4 results (6–15). Depending on the type of method, both abnormally high and low FT4 results have been described in the presence of increased concentrations of FFA (7–15). Recently, some manufacturers modified their FT4 kits to eliminate the interference arising both from varying concentrations of endogenous T4 binding proteins in serum and from FFA. However, the validity of serum FT4 determinations with various methods remains controversial in different clinical situations. In this work we studied the performance of 16 FT4 methods with respect to a possible interference by endogenous FFA. We examined these methods in patients whose results generate the most controversy in thyroid testing, those with nonthyroidal illness (NTI).

Materials and Methods

Patients

In all, 115 serum specimens were collected from 104 patients with NTI in the Warren G. Magnuson Clinical Center, NIH, Bethesda, MD, and stored at −20 °C until use (within six months). Most patients (n = 95) were seriously or critically ill and were treated in medical (n = 23) and surgical (mainly for cancer) (n = 41) intensive-care units or in a cardiac-surgery recovery room (n = 31) at the time of blood drawing. After completion of thyroid-function tests on the sera, we concluded that two patients represented cases of primary hypothyroidism (above-normal FT4 by two different high-resolution methods, and subnormal FT4 by all techniques) and that a third patient had primary hyperthyroidism (subnormal thyrotropin by two different high-resolution methods, and above-normal FT4 by all methods). We therefore excluded from further analysis three serum samples from these three patients.

Determination of FT4, T3 Uptake, Total T4 and Thyroxin-Binding Globulin (TBG)

We tested the sera with all FT4 assays commercially available in the U.S.A. in 1987. Principles, procedures, and reference intervals of the various direct FT4 kits are summarized in Tables 1 and 2. In the case of the Magic 1-step FT4 assay, we tested both the commercially marketed version (hereafter referred to as "A") and an investigational version (hereafter referred to as "B") of the kit, the latter incorporating undisclosed change(s) in the formulation of reagent(s). The SimulTRAC kit simultaneously measures TSH (125I-labeled) and FT4 (60Co-labeled) in serum. All the other direct assays for FT4 use 125I for labeling T4 or T3 analogs. In addition, we also tested the performance of indirect FT4 methods in patients with NTI. We calculated FT4 indices by multiplying the T3 uptake fraction (determined with macroaggregated albumin; Amersham Corp., Arlington Heights, IL 60005) with the total T4 concentration obtained by the GammaCoat (Clinical Assays, Dade, Baxter Travenol Diagnostics, Inc., Cambridge, MA 02139) method (hereafter referred to as "A") and the Immophase (Ciba Corning Diagnostics Corp., Medfield, MA 02052) method (hereafter referred to as "B"). We calculated ratios of T3 to TBG by using the total T4 concentration obtained with method "A" and method "B", respectively, and TBG concentrations obtained by an immunoradiometric method (Immophase, Ciba Corning Diagnostics). The reference interval for MAA T3 uptake fraction is 0.255–0.344, for total T4 is 65–155 mmol/L (A) or 61–159 mmol/L (B), and for TBG it is 12–26 mg/L. From the T3 uptake and total T4 intervals, the reference interval for FT4 index is estimated as 16–53 (A) or 16–55 (B), and for T4/TBG ratio is estimated as 2.3–12.9 (A) or 2.2–13.2 (B).
Other Assays

We used commercial RIA kits for measuring total T₃ (Quanticoat T₃; Kallestad Diagnostics, South Austin, TX 78746), and reverse T₃ (DAB/PEG kit; Serono Diagnostics Inc., Norwell, MA 02061). We determined thyrotropin by two different immunoradiometric techniques. Besides the SimulTRAC method ("A"), we also used the TSH₃ MAIA Clone kit method ("B") (Serono Diagnostics Inc., Norwell, MA 02061). We measured albumin by the bromocresol purple dye-binding method with an acet III (DuPont Co., Wilmington, DE 19888) and prealbumin (transthyretin) by rate nephelometry with the Array Protein System (Beckman Instruments, Inc., Brea, CA 92621). FPA were determined by a colorimetric method (18) at MetPath Laboratories, Kensington, MD 20895.

Table 1. Comparison of One-Step RIAs with Labeled T₄ Analogs ("Tracer") for Estimating FT₄

<table>
<thead>
<tr>
<th>Separation system</th>
<th>Procedure*g</th>
<th>Magnetic sepn, h</th>
<th>Magnetic particle</th>
<th>Solid Phase Component System*</th>
<th>SimulTRAC FT₄/TSH₄*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amerlex-M*</td>
<td>Sample vol, μL 100</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Tracer vol, μL 500</td>
<td>1000</td>
<td>1000</td>
<td>200</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Antibody vol, μL 500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Incubation at 37 °C, h 1</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Other</td>
<td>Magnetic sepn, 15 min</td>
<td>9.4–25.2</td>
<td>10.3–25.8</td>
<td>11.6–32.3</td>
<td>9.0–21.9</td>
</tr>
<tr>
<td>Reference range, pmoL*h</td>
<td>(n = 876)</td>
<td>(n = 251)</td>
<td>(n = 121)</td>
<td>(n = 550)</td>
<td>(n = 135)</td>
</tr>
<tr>
<td></td>
<td>(n = 9.4–25.2)</td>
<td>(n = 9.0–21.9)</td>
<td>(n = 10.7–21.9)</td>
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<td></td>
</tr>
</tbody>
</table>


Table 2. Comparison of Two-Step RIAs, Equilibrium Dialysis, and Binding-Rate-Based RIA, with Labeled T₄ ("Tracer") for Estimating FT₄

<table>
<thead>
<tr>
<th>Assay system</th>
<th>GammaCost 2-Step RIA*</th>
<th>Phase II RIA*</th>
<th>Spiro RIA*</th>
<th>Equilibrium dialysis*</th>
<th>Immophase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sequential RIA</td>
<td>Sequential RIA automated for KinetiCount 48</td>
<td>Sequential RIA</td>
<td>Dialysis cell</td>
<td>Binding rate-based RIA</td>
</tr>
<tr>
<td>Separation system</td>
<td>Coated tube</td>
<td>Solid phase receptacle</td>
<td>Macrobead</td>
<td>Membrane/MgCl₂</td>
<td>Porous glass particles</td>
</tr>
<tr>
<td>Procedure*h</td>
<td>Sample size</td>
<td>50 μL sample 1 mL buffer</td>
<td>100 μL sample 500 μL buffer</td>
<td>100 μL sample 1 mL antibody</td>
<td>200 μL sample 6 mL buffer</td>
</tr>
<tr>
<td></td>
<td>First incubation Decant 1 mL buffer</td>
<td>15 min 37 °C</td>
<td>45 min RT with rotation</td>
<td>4 mL buffer</td>
<td>5 min labeled sample dialyzed against 5 mL buffer</td>
</tr>
<tr>
<td></td>
<td>Second incubation Decant 1 h RT</td>
<td>15 min 37 °C</td>
<td>45 min RT with rotation</td>
<td>4 mL buffer</td>
<td>18 h 37 °C</td>
</tr>
<tr>
<td></td>
<td>Final step Decant, count Rinse, count Aspirate, count Decant, count</td>
<td>Decant, count</td>
<td>11.6–23.0</td>
<td>14.2–29.7</td>
<td>12.9–29.7</td>
</tr>
<tr>
<td>Reference range, pmoL</td>
<td>7.7–20.6</td>
<td>(n = 222)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
</tr>
</tbody>
</table>

Statistical Analysis

We analyzed the data by simple linear regression. We eliminated a maximum of two outliers from the regression analyses by omitting data points that generated residuals greater than four times the standard error of the estimate (4 $S_{y|x}$) (19).

Results

Concentration of FFA in Serum

Ninety-five percent (107/112) of the results for FFA concentration in patients with NTI were below the upper limit, and two-thirds of them (74/112) were below the lower limit of the reference interval (0.45–0.90 mmol/L). The results were skewed towards the low concentration, with a mean of 0.38 mmol/L (Figure 1).

Correlation of $FT_4$ with FAA

In the regression analysis, the binding-rate-based $FT_4$ RIA, all one-step $FT_4$ RIAs, and the $FT_4$ indices produced negative slopes with FFA concentration (Figure 2, rows 1, 2, 4). But of the seven one-step $FT_4$ RIAs only four (Amerlex-M, GAMMacoat 1-step, Magic 1-step [A], and Solid Phase Component System) had slopes significantly different from zero ($r = -0.25$ to $-0.42, P < 0.001$). On the other hand, the three two-step $FT_4$ RIAs ($r = 0.28–0.46$) and equilibrium dialysis ($r = 0.25$) all exhibited highly significant ($P < 0.01$) positive correlations with FFA concentration (Figure 2, row 3). Like $FT_4$ indices, the $T_4$/TBG ratios did not significantly correlate with FFA concentration (Figure 2, row 4). Based on the slopes, changes in the concentration of FFA were, in general, associated with a greater change in $FT_4$ results obtained by the two-step RIAs and equilibrium dialysis (8.6–12.8 pmol of $FT_4$ per liter per 1 mmol of FFA per liter) than in those obtained by the one-step methods (from −3.4 pmol of $FT_4$ per liter with the Solid Phase Component System to −10.7 pmol of $FT_4$ per liter with the Magic 1-step [A] method per 1 mmol of FFA per liter) (Figure 2).

The dialyzable (free) fraction (expressed as %) of total $T_4$ in equilibrium dialysis showed a significant positive correlation with FAA ($y = 0.026x + 0.029, S_{xy} = 0.0215, P < 0.0099, r = 0.24, n = 110$). In contrast, the fraction of labeled $T_4$ bound to a solid phase antibody (A or red tube) during the 30-min incubation ("rate of binding") in the Immophase (binding-rate-based $FT_4$ assay) did not reveal a statistically significant association with FAA.

Correlation of $FT_4$ with the FFA/Albumin Molar Ratio

Because recent reports have suggested a stronger correlation between $PT_4$ concentration and the molar ratio of FFA to albumin than between $FT_4$ concentration and FFA concentration ($I3, I4$), we also examined the former relationship. The FFA/albumin molar ratios ranged from 0.02 to 3.45 (mean = 0.90, n = 112) in our patients with NTI (Figure 1). Least-squares regression analysis of the $FT_4$ results and FFA/albumin molar ratios disclosed negative slopes for the binding rate-based method and all one-step $FT_4$ RIAs (Figure 3, rows 1, 2). Even $FT_4$ methods that did not correlate significantly with FFA concentration (Immophase, Coat-A-Count, the new version of Magic 1-step [B], and SimulTRAC) (Figure 2) showed statistically significant ($P < 0.05$) associations with the FFA/albumin molar ratio (Figure 3). The one-step (analogue) $FT_4$ methods all highly significantly ($P < 0.007$) correlated with the FFA/albumin molar ratio, whereas the binding rate-based method correlated less ($P < 0.02$). As above with FFA concentration, the two-step $FT_4$ RIAs and equilibrium dialysis $FT_4$ positively correlated with the FFA/albumin molar ratio (Figure 3, row 3). Interestingly, the correlation for $FT_4$ measured by equilibrium dialysis changed from highly significant ($P < 0.007$) with FFA concentration to weakly significant ($P < 0.02$) with FFA/albumin molar ratio. Unlike FFA concentration, changes in the FFA/albumin molar ratio had comparable magnitude (but opposite direction) of effects on the binding-rate-based method (Immophase) (−2.3 pmol of $FT_4$ per liter per 1 unit ratio), one-step RIAs (−1.8 to −5.1 pmol of $FT_4$ per liter per 1 unit ratio), two-step RIAs (3.1 to 4.1 pmol of $FT_4$ per liter per 1 unit ratio), and equilibrium dialysis (2.7 pmol of $FT_4$ per liter per 1 unit ratio). The $FT_4$ indices showed statistically significant ($P < 0.05$) negative correlations, whereas the $T_4$/TBG ratios remained unassociated with the FFA/albumin molar ratio (Figure 3, row 4).

The dialyzable (free) fraction (expressed as %) of total $T_4$ equilibrium dialysis and the fraction of labeled $T_4$ bound to a solid phase antibody (A or red tube) during the 30-min incubation ("rate of binding") in the Immophase (binding-rate-based $FT_4$ assay) were both positively correlated with the FFA/albumin molar ratio ($y = 0.0126x + 0.0264, S_{xy} = 0.0230, P < 0.0001, r = 0.37, n = 111$; and $y = 0.0045x + 0.0248, S_{xy} = 0.0118, P < 0.0024, n = 109$, respectively).

Relationship of FFA Concentration or FFA/Albumin Molar Ratio to Other Thyroid Function Tests and $T_4$ (or $T_4$ Analog) Binding Proteins

We found no statistically significant associations between FFA concentration and $T_3$ uptake (Figure 4), total $T_4$ measured by two different methods (A and B), total $T_3$, reverse $T_3$, TSH measured by two different "ultrasensitive" methods (A and B), TBG, albumin, or prealbumin. The FFA/albumin molar ratio also failed to correlate with reverse $T_3$, TSH (A and B), and prealbumin. In contrast, we found statistically significant positive correlation between $T_4$ uptake and the FFA/albumin molar ratio (Figure 4), and negative correlations between total $T_4$ (nmol/L) ([method A] ($y = -11.63x + 86.17, S_{xy} = 38.65, P < 0.0218, r = -0.22, n = 112$) or (method B) ($y = -12.75x + 79.64, S_{xy} = 38.51, P < 0.0133, r = -0.24, n = 109$), total $T_3$ (pmol/L) ($y = -0.19x + 0.84, S_{xy} = 0.52, P < 0.0059, r = -0.26, n = 112$), albumin (g/L) ($y = -4.78x + 36.43, S_{xy} = 8.54, P < 0.0001, r = -0.38, n = 112$), or TBG (mg/L) ($y = -2.25x + 18.17, S_{xy} = 5.27, P < 0.0013, r = -0.30, n = 112$) and the FFA/albumin molar ratio ("x").

Discussion

In 1967, Hollander et al. (20) proposed that nonesterified FFA might influence the blood concentration of $FT_4$ in vivo.
Indeed, subsequent studies confirmed that, by competing for the same or closely-located protein binding sites, FFA may act as T₄ (and likely T₃ analog) binding inhibitors (6, 21–26). This mechanism is intriguing in patients with NTI in whom both low and high FT₄ results have been described (1–3, 13, 14, 27–30). Nevertheless, the roles of FFA in regulating FT₄ concentration in vivo and in affecting the measured FT₄ in vitro remain controversial (7–15, 20–24, 31, 32). Without distinguishing between in vitro and in vivo mechanisms, our present work in patients with NTI indicates that FFA alone or with co-inhibitors of T₄ (or T₃ analog) binding to proteins (such as drugs (33)) affect the measured FT₄ concentration by most, but not all, methods.

In accord with most previous observations (13, 14, 26), we found normal or subnormal FFA concentrations in the serum of the overwhelming majority (95%) of patients with NTI who were, in general, seriously or critically ill at the time of testing. Unusually high FFA concentrations appear to be rare in patients with NTI and occur only in extreme conditions [e.g., an FFA concentration of 8 mmol/L was reported in a child with glycogen storage disease (9)]. Even in 10 patients with severe, uncontrolled diabetes, the concentration of FFA in serum was only 1.11 (SD 0.80) mmol/L as compared with 0.33 (SD 0.12) mmol/L in 12 healthy euthyroid controls (6). The finding (24) that FFA concentrations often exceed 3 mmol/L in patients with NTI has been

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Fig. 2. Effect of FFA concentration on the apparent FT₄ in patients with NTI

*P < 0.01, **P < 0.001, and ***P < 0.0001
attributed by others (13) to in vivo (heparin administration (6–11)) and in vitro (increase in FFA during blood collection and during storage (6, 34, 35)) artifacts. Most recently, however, Liesenfeld et al. (32) cautioned against overinterpretation of the in vitro increase in the concentration of FFA. These authors found no difference in serum FFA 1 and 3 h after collection of blood samples (storage temperature not stated). Likewise, in their hands, storage of serum samples frozen (at an unspecified temperature) for as long as three months did not cause a significant increase in the concentration of FFA (or in FT₄ concentration by equilibrium dialysis). Furthermore, comparing the serum FFA concentrations before and after overnight equilibrium dialysis at 37 °C, they found relatively small increments, ranging, on average of groups, from 0.19 to 0.23 mmol/L in the sera of NTI patients (n = 30), pregnant women in late pregnancy (n = 14), and healthy individuals (n = 23) (32). Regarding in vivo causes of increased FFA concentration in serum in patients with NTI, Davies and Turney (36) recently showed that even after high intravenous doses of heparin the peak mean FFA concentration reaches only about 1.5 mmol/L (baseline: ~0.2 mmol/L) in uremic patients undergoing hemodialysis (n = 6). In euthyroid healthy volunteers, only extreme manipulations such as a fat meal followed by heparin injection produced a rapid and transient rise from a baseline of 0.33 (SD 0.12) mmol/L to ~2 mmol/L serum FFA concentration (6, 11). Our findings of generally low or normal serum FFA concentrations in NTI patients, most of whom were being cared for in various intensive-care units and probably exposed to some heparin at the time of testing,

Fig. 3. Effect of FFA/albumin molar ratio on the apparent FT₄ in patients with NTI

*P <0.01, **P <0.001, and ***P <0.0001
are consistent with these reports.

Although most of the FFA concentrations were below the upper limit of the reference interval in our patients with NTI, there was a more than 10-fold difference between the lowest and highest results (Figure 1). The relatively wide scattering of FFA results allowed us to study correlations between FFA and the apparent FT₄. We found disparate effects of FFA on FT₄ as measured by various techniques in the sera of NTI patients. We noted negative correlations of FFA concentrations with some of the one-step (analogue) FT₄ RIAs, positive correlations of FFA concentrations with two-step RIAs and equilibrium dialysis, whereas we saw no significant correlations of FFA concentrations with a binding-rate-based FT₄ RIA and indirect FT₄ methods such as FT₄ indices and T₃/TBG ratios. These observations are at variance with previous reports (7, 10, 12-14). For correct interpretations of the observed correlations we should make several additional comments:

(a) **FFA/albumin molar ratio as an estimate of “excess” (non-albumin bound) FFA.** Based on studies in vitro, Chopra et al. (12) proposed that the concentrations of albumin and other T₄ (and likely T₃) analog binding proteins in serum modulate the interference by FFA in the measurement of FT₄. Two other groups of investigators confirmed this by showing that the effect of either exogenous FFA added in vitro (13) or endogenous FFA in patients with NTI (14) is modified by the concentration of albumin. These findings are not surprising, because almost all the circulating FFA are bound to albumin (22, 37). Mendel et al. (13) concluded that FFA will interact with other serum proteins and thereby modulate the measured FT₄ concentration only after the high-affinity FFA binding sites on albumin become saturated at an FFA/albumin molar ratio of ~5. In another work (14), FT₄ measured by equilibrium dialysis correlated with the FFA/albumin molar ratio better than with FFA concentration in patients with NTI. Here we found greater and more correlations between FT₄ measured by a variety of methods and the FFA/albumin molar ratio than between FT₄ and FFA concentration. All these observations are consistent with the proposed modulating role of serum albumin concentration (12-14). We also agree with previous studies (13, 31), which found that a high (>5) FFA/albumin molar ratio rarely occurs in patients with NTI. Of 112 specimens we found none with a ratio >3.5. Mendel et al. (13) reported two out of 11 NTI patients with a ratio >2.5 and none with >4, but the mean FFA/albumin molar ratio was higher (1.53 ± 0.41) in their patients (n = 11) than in ours (0.90, n = 112). Liewendahl et al. (14) documented only two of 61 patients with NTI who had an FFA/albumin molar ratio >4.

(b) "Improved" one-step (analogue) FT₄ RIAs. We found that not all one-step (analogue) FT₄ RIAs exhibit a statistically significant negative correlation with FFA. At least two manufacturers of one-step FT₄ RIAs have claimed that their currently available kits (after undisclosed modifications) are not interfered with by varying endogenous concentrations of FFA. With linear regression analysis against FFA concentration we could confirm the validity of this claim in patients with NTI for the Coat-A-Count kit but not for the Amerlex-M FT₄ assay. By adding various fatty acids to normal human serum, Tikanoja and Liewendahl (15) could, however, affect results for FT₄ obtained with the Coat-A-Count kit. Furthermore, we should emphasize that we detected FFA-dependency of all one-step FT₄ RIAs by regression analysis against the FFA/albumin molar ratio as an estimate of "excess" (nonalbumin bound) FFA.

(c) **The mechanism of interference.** The finding of correlations between FFA and measured FT₄ would not imply necessarily a cause-and-effect relationship. Nevertheless, as discussed above, current evidence indicates that FFA probably interfere with FT₄ measurements by competing with T₄ (and likely T₃ analogues) for identical or closely located binding sites on proteins (21-26). We found negative correlations between FFA/albumin molar ratio and total T₄ measured by two different methods or total T₃ concentrations. In contrast, we observed positive correlations between the dialyzable (free) fraction of labeled T₄ in equilibrium dialysis and FFA or FFA/albumin molar ratio. All these findings are consistent with a competitive inhibition by FFA in patients with NTI. But because of a concomitant decrease of major thyroxin-binding proteins such as albumin and TBG, only the increase in the dialyzable (free) fraction of labeled T₄ in response to an increase in FFA may be taken as evidence for the presence of T₄ binding inhibitors in these patients. The possibility of a conformational change of binding proteins due to association with FFA also has been suggested (25). Whatever the mechanism, of importance is that only unsaturated FFA (binding potency increasing with the number of double bonds) are active for inhibition (6, 25, 26). Because ~60% of total FFA (26) are unsaturated, correlations of total FFA or FFA/albumin molar ratio with FT₄ may approximate the correlation between unsaturated FFA or unsaturated FFA/albumin molar ratio and FT₄. "Perfect" correlations based on "true" binding inhibitory potencies would, however, require determination of individual unsaturated FFA species, especially oleic acid (24-26).

(d) Effect of total T₄. Several FT₄ methods require the simultaneous determination of total T₄. (i) The FT₄ concentration and the dialyzable fraction of labeled T₄ measured by equilibrium dialysis was positively correlated, whereas total T₄ was negatively correlated with the FFA/albumin molar ratio. It follows then that the "excess" (nonalbumin bound) FFA exerted a relatively greater effect on the dialyzable fraction of labeled T₄ than on total T₄ (FT₄ = dialyzable fraction × total T₄). (ii) We found a negative correlation of FT₄ indices with the FFA/albumin molar ratio. This occurs because of a comparatively lesser effect of "excess" FFA on T₃ macroaggregated albumin uptake (positive correlation) than on total T₄ (negative correlation) (FT₄ index = T₃ uptake × total T₄). (iii) We found the same pattern (negative correlation) between the binding-rate-based FT₄ RIA (Imnomphase) and the FFA/albumin molar ratio. But the fraction of labeled T₄ bound to the solid phase antibody during the 30-min incubation positively, whereas total T₄ negatively correlated with the same ratio. These observations imply a comparatively greater effect of "excess"
FPA on total T₄ than on the bound fraction ("rate of binding") of labeled T₄ (FT₄ = bound fraction × total T₄). Our observation that the binding-rate-based FT₄ RIA (Imnophasé) and the FT₄ indices based on T₃ macroaggregated albumin uptake are affected similarly to FPA is not surprising, because the former method is essentially identical to the FT₄ index methods, differing primarily in that T₄ antibody is used instead of macroaggregated albumin, resin, or other materials to measure labeled T₄ uptake, and in being calibrated with a set of standards of known FT₄ concentration. (ii) We detected no correlation between T₄/TBG ratios and FPA/albumin molar ratio at the time when both total T₄ and TBG were negatively correlated with the FPA/albumin molar ratio. This indicates that total T₄ and TBG changed in the same proportion with changing FPA/albumin molar ratio in our patients with NTI.

(e) Pre-analytical and intra-assay increase in serum FPA concentration. One of the aspects of the interference of FPA with FT₄ measurements is related to possible in vitro artifacts. We already have discussed the controversy regarding possible increases of FPA in serum or plasma during blood clotting, processing, storage, and testing (6, 13, 31, 32, 34, 35). Here, we want to further comment on the testing phase only. Tables 1 and 2 show the technical steps involved in direct FT₄ assays. Despite identical incubation requirements of three one-step (analog) FT₄ RIAs (Table 1), results by Amerlex-M and the "old" version (A) of Magic 1-step were strongly correlated, whereas those by Cost-A-Count and the "new" version (B) of Magic 1-step did not exhibit a statistically significant association with FPA (Figure 2). Conversely, different incubation conditions (Table 2) comparably influenced the FT₄ results with various two-step RIAs (Figure 2). Although these observations do not rule out possible intramethod increases of FPA, they suggest that for expression of FPA-dependence the formulation of kits may be more important than the duration or temperature of incubation. We should recall, however, that the use in the regression analysis of FPA/albumin molar ratio instead of FPA concentration virtually eliminated the interkit differences within a given type of methodology for FT₄ (one- or two-step RIA).

In the final analysis, the most important question is the clinical relevance of the observed phenomena. To answer this question, several findings need to be reiterated. First, the relative concentrations of FPA and albumin rather than the concentration of FPA alone determine the extent of FPA effect on measured FT₄. Second, of five different FT₄ methodologies tested, only the T₄/TBG ratio is devoid of FPA-dependency in patients with NTI. One-step RIAs, binding-rate-based RIA (which really is based on T₄ uptake by T₄ antibody), and FT₄ indices based on T₃ macroaggregated albumin uptake correlated negatively, whereas two-step RIAs and equilibrium dialysis were positively correlated with the FPA/albumin molar ratio. Third, according to the slopes (FPA/albumin molar ratio vs FT₄), various RIA methods and equilibrium dialysis change approximately one-fifth (range: 1/3 to 1/8), whereas the FT₄ indices change approximately one-tenth of the reference interval for each unit change in FPA/albumin molar ratio. Fourth, FPA/albumin molar ratios >3 appear rarely in patients with NTI. Thus, though in these patients positive or negative effects (depending on the methodology) on FT₄ results are common, the magnitude of effect is usually small, varying with the "excess" FPA. Based on the finding of a slight but significant positive correlation between "excess" FPA and FT₄, measured by techniques that have the firmest physico-chemical basis for the belief that they genuinely measure FT₄ (i.e., two-step methods and equilibrium dialysis), it is intriguing to suggest that this correlation may reflect the true in vivo status of the patients and is not the result of methodological artifact. However, a definite answer requires additional studies.

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References