An Evaluation of a Fluorescence Polarization Immunoassay of Thyroxin and Thyroxin-Uptake

R. G. Symons and R. F. Vining

We evaluated the fluorescence polarization immunoassay for total thyroxin (T4) and thyroxin-uptake (T-U) in the Abbott "TDx" Analyzer. Between-assay precision was good when we did once-fortnightly calibration and assayed samples in singleton. Measured T4 concentration was decreased in hemolyzed samples with obvious red coloration and undetectable in severely hemolyzed samples. The T-U assay was unaffected by hemolysis. Unlike the triiodothyronine-uptake methods, the T-U assay utilizes labeled T4 and measures a variable related to serum thyroxin-binding capacity rather than the concentration of unoccupied binding sites in serum. The T4 and T-U values of 422 samples correlated highly with a T4 (in-house) radioimmunoassay and a commercial assay for thyroxin-binding globulin, respectively. The free thyroxin ratio (ratio of T4 to T-U, FTI) correlated highly with free T4 concentration as measured by T4-analog-tracer radioimmunoassay (Tf4 analogous RIA). FTI and Tf4 values were discordant in late pregnancy (normal FTI and low Tf4) and euthyroid sick patients (above-normal or normal FTI and low Tf4), suggesting that the FTI gives fewer misleading results in these patients.

Additional Keyphrases: thyroid status · free thyroxin index variation, source of

Radioimmunoassay (RIA), currently the most popular technique for hormone analysis, is not well suited to automation. The need to use reagents at limiting dilutions to attain sufficient sensitivity, and the associated long incubation times; the technical problems of physically separating free and bound moieties; and the relatively cumbersome methods for quantification of radioactivity have all hindered development of a comprehensive automated radioimmunoassay system (1, 2). Homogeneous nonisotopic immunoassays overcome some of these difficulties (3–9). Because nonisotopic labels are used that emit a different signal when tracer is bound to antibody as compared with that emitted when tracer is free in solution, these assays bypass the associated technical problems of separation and thus remove a major source of immunoassay imprecision (10). Furthermore, nonisotopic immunoassays typically have response characteristics that can be measured optically (3–8) and have high signal-to-noise ratios (8, 9), making them more suited to automated hormone analysis than is RIA.

Fluorescence polarization immunoassay, a homogeneous immunoassay technique, has been successfully adapted to automated analyses for an extensive range of therapeutic drugs (11). This technique has recently been adapted to the measurement of serum thyroxin (T4) and thyroxin-uptake (T-U) by Abbott Laboratories.1 In both these assays, fluores-

cine-labeled T4 is used as tracer. The T4 assay is a conventional competitive-displacement assay; the T-U assay measures the degree of binding of tracer to the thyroid-binding proteins. The T-U assay differs from triiodothyronine (T3)-uptake techniques in that T4 is used as tracer rather than T3.

We have evaluated the claims of the manufacturer that the precision and stability of the instrument are such that the assays require calibration only once per fortnight, and that samples need not be assayed in duplicate. To determine the nature of the T-U assay, we studied the influence of variations in unoccupied binding-site capacity and total serum binding-site capacity on measured T-U values. We compared the serum T4, T-U, and calculated FTI values with data measured with an in-house T4 assay, with a thyroxin-binding globulin (Tbg) assay, and with an analog-T4-tracer RIA of free thyroxin (ft4 analog RIA), respectively.

Materials and Methods

Assays

Serum T4 by fluorescence polarization immunoassay: Serum T4 was so assayed with the Abbott "TDx" analyzer, (Abbott Laboratories, Irving, TX 75061) with fluorescence-labeled T4 as tracer. Thyroxin was released from its binding proteins in serum by pretreating the serum with a denaturing reagent (urea–sodium dodecyl sulfate–dithiothreitol) supplied by the manufacturer. Antibody-bound and unbound (free) fluorescein–T4 conjugate are differentiated by the difference in polarization of the emitted fluorescent light at 535 nm after exposure to exciting polarized light at 485 nm. The degree of polarization of fluorescent light is related to the degree of binding of tracer to the antibody (11).

Thyroxin-uptake assay by fluorescence polarization immunoassay. T-U was also assayed with the "TDx" Analyzer. This assay quantifies the binding of fluorescein-labeled T4 tracer to serum thyroid-hormone-binding proteins. The degree of polarization of fluorescent light is directly related to the proportion of tracer bound, or tracer uptake.

Free thyroxin index (FTI). FTI was calculated as the ratio of T4 to T-U, as recommended by the manufacturer.

Other thyroid-function tests. Thyroxin-analog-tracer radioimmunoassay of T4 (Amer sham International, Amer- sham, U.K.) was used to measure ft4 (analog RIA). Results correlate closely with T4 concentration as measured by equilibrium dialysis (12); the normal reference interval is 11 to 22 pmol/L. Tbg was measured by an immunoradiometric assay (Corning Medical, Medfield, MA 02052); the normal reference interval is 12 to 30 mg/L. T4, T3, and thyrotropin were assayed with radioimmunoassays established in the laboratory; the respective normal reference intervals are: 60 to 150 nmol/L, 1.2 to 2.8 nmol/L, and <6.0 milli-int. units/L.

Analytical Characteristics

Calibration curve precision profiles. The within-assay precision profiles for the T4 and T-U assays were calculated from duplicate data pooled from each of the five calibration

1 Nonstandard abbreviations: T3, 3,3',5-triiodothyronine; T4, thyroxin; ft4, free T4; Tf4 (analog RIA), free T4 measured by T4-analog-tracer radioimmunoassay; Tbg, thyroxin-binding globulin; FTI, free thyroxin index; T-U, thyroxin-uptake.

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curves we prepared during this study.

**Precision of singleton analysis.** In calibrating, we assayed standards in duplicate, but all other samples, including quality controls, were assayed singly. The quality controls supplied by the manufacturer (low, L; medium, M; high, H) were assayed singly in the first three (L1, M1, H1) and final three positions (L2, M2, H2) of the assay carousel. We assessed between-assay precision for samples assayed singly by treating the controls as six different quality controls (L1, L2, M1, M2, H1, H2). We compared this precision with the between-assay precision of duplicate analyses (calculated from the average values for each duplicate pair of quality controls, L1 & L2, M1 & M2, H1 & H2). We calculated within-assay precision by using Snedecor's equation (13).

Two pools of serum provided quality controls for the calculated PTI.

**Calibration curve stability.** The manufacturer recommends that assays be calibrated about once a fortnight. During this study we performed 43 T4 assays and 37 T-U assays. The instrument was calibrated five times for both the T4 and T-U assay at two-week intervals: the instrument automatically fits the standard curve (calibration curve) raw data and prints the parameters of the equation that is used to fit the standard curve; these parameters are then used in subsequent assays for the automatic calculation of T4 and T-U values of unknown samples from the raw-data values for these samples.

We assessed the T4 and T-U assay stability by manually recalculating the quality-control values from the raw data, using the parameters of the first calibration curve. This set of quality-control data, representing the quality-control values that would have been obtained during the 43 T4 or 37 T-U assays if the instrument had not been recalibrated during the entire study, were used as a measure of instrument drift or calibration curve stability. Similarly, we also manually recalculated the quality-control values, using the parameters of either the second, third, fourth, or fifth calibration curves.

**Effect of hemolysis on the T4 and T-U assays.** To investigate the effect of hemolysis, we prepared serum samples with various degrees of hemolysis by adding various volumes of erythrocyte lysate, prepared by mixing equal volumes of cells and distilled water, to a nonhemolyzed serum pool. After 5 min at room temperature, the samples were centrifuged at 1500 × g for 10 min to remove intact cells and debris. All hemolyzed serum samples were diluted to the same final volume with phosphate buffer, pH 7.8. Hemoglobin in the hemolyzed samples was determined spectrophotometrically from a standard curve of the difference in absorbance at 575 and 650 nm vs hemoglobin concentration of a series of standards.

**Effect of varying the concentrations of unoccupied thyroid hormone binding sites in serum.** To determine the nature of the T-U assay, we studied the influence of variations in unoccupied binding-site capacity. We prepared two series of serum samples with different degrees of occupancy of thyroid-hormone-binding sites by adding increments of T4. One series was prepared from a serum sample with an undetectable T4 concentration (<10 nmol/L), the other from a sample with a T4 concentration of 105 nmol/L. After we added T4, we incubated the samples for 3 h at room temperature, then assayed for both T3 uptake (MAA assay, Amersham International, Amersham, U.K.) and T-U.

**Patients**

Serum samples from 423 subjects were categorized into the following groups: 48 normal blood-bank volunteers, ages 18–55 years; 41 clinically euthyroid patients with normal serum T4, fT4 (analog RIA), T3, and thyrotropin concentrations; 19 clinically hypothyroid patients with subnormal fT4 (analog RIA) and T3, and above-normal thyrotropin; 27 clinically thyrotoxic patients with increased fT4 (analog RIA) and T3, and undetectable thyrotropin; 27 clinically euthyroid women in the third trimester of pregnancy; six clinically euthyroid subjects with thyroid-hormone-binding-protein abnormalities, including four with familial low TBG and two with familial dysalbuminemic hyperthyroxinemia; 35 cases of nonthyroidal illness with low T3 (euthyroid sick) and 22 with severe nonthyroidal illness (intensive care); and 35 patients who had had previous treatment with 131I-iodide or were currently receiving anti-thyroid medication for thyrotoxicosis, and were not currently receiving T4 replacement.

Clinically euthyroid subjects in the following groups were included: 79 patients on T4 replacement therapy; 39 patients with increased fT4 (analog RIA) as the only abnormal thyroid-function test result and not receiving T4 replacement (patients being treated for psychiatric illness, or being investigated for cardiac disease, hypertension, infertility, or diabetes); and 44 patients with compensated euthyroidism (low or normal fT4, normal T3, and above-normal thyrotropin).

**Results**

**Analytical Characteristics**

**Calibration curves and precision profiles.** The polarization decreased by only 30% over the range of T4 standards, but for the T-U test, increased by three- to fourfold over the range of T-U standards (Figure 1a).

Measurement of polarization was highly precise (Figure 1b), resulting in acceptable within-assay CVs of 1.8 to 7.8% for the T4 assay, and 1.0 to 4.4% for the T-U assay (Figure 1c).

**Precision of singleton analysis (Table 1).** Although duplicate analysis of T4 was no more precise than assaying single samples (CVs 3.5 to 5.9% vs 3.5 to 6.9%), duplicate measurements of T-U were marginally more precise than assaying samples singly (CVs 1.5 to 3.7% vs 1.5 to 5.8%).

**Calibration curve stability.** The T4 quality-control values recalculated by using the first calibration curve parameters drifted upwards during the two-month study (Figure 2a). The slope of the drift differed significantly from zero for each quality control (p <0.001; see legend to Figure 2). The drift was most pronounced at lower T4 values.

The between-assay standard deviations of the recalculated quality-control values were greater than those of the measured quality-control values obtained when the instrument was regularly recalibrated (Figure 2b). The between-assay standard deviation increased markedly at lower T4 values, consistent with the greater drift in the calculated values of the low-quality-control material.

Recalculated T-U values of the quality controls did not drift during the two-month study (Figure 3).

**Effect of hemolysis.** Measured T4 concentration decreased with increasing hemolysis (Table 2); T4 was less by 22% in a visibly red serum sample (1.2 g/L hemoglobin), and by 68% in a grossly hemolyzed sample (4.6 g/L hemoglobin).

The T-U, fT4 (analog RIA), and T4 (in-house) assays were unaffected by the highest degree of hemolysis studied.

**Reference Intervals**

We determined reference intervals for each assay by combining data from normal blood donors and clinically euthyroid patients with normal values for fT4 (analog RIA), T4, T3, and thyrotropin (Table 3). The following "normal"
reference intervals were adopted for routine diagnostic use: T4, 60 to 150 nmol/L; T-U, 0.60 to 1.25; FTI, 65 to 155.

Thyroxin-Uptake Assay

Thyroxin-uptake vs TBG. The T-U and TBG results correlated highly in the 422 subjects (T-U = 0.05 + 0.02 TBG, r = 0.88). Only 13% of the samples were differently classified by the two assays (discordant values). The discordant values were not associated with any one particular diagnostic category. Of the samples with discordant T-U and TBG values, 60% had slightly decreased TBG (9 to 11 mg/L) with normal T-U values (0.61 to 0.92).

Only four of the 38 samples with low TBG concentrations had very low concentrations (Table 4); T-U was commensurately decreased in only one of these samples. Two subjects with familial dysalbuminemic hyperthyroxinemia had normal T-U values.

Ratio of T4 to T-U vs ratio of T4 to TBG. We further investigated the relationship between T-U and TBG concentration by comparing the variation of the T4/T-U ratio (FTI) and T4/TBG ratio with increases in TBG concentration in all subjects without clinically obvious thyroid disease or nonthyroidal illness. The FTI decreased significantly with increasing TBG (r = -0.18, p < 0.05), but the T4/TBG ratio was even more affected (r = -0.55, p < 0.01) (Figure 4).

Effect on T-U of varying the concentration of unoccupied thyroxin-binding sites. T3 uptake rose by 50% to 55% above basal values when 500 nmol of T4 per liter was added to the samples (Figure 5). In contrast, the T-U values decreased when more binding sites were occupied by T4, but were much less sensitive to the addition of T4, changing by only 10%.

T4 Assay

The T4 (TDx) and T4 (in-house) values correlated highly (T4 TDx = 5.8 + 0.9 T4 in-house, r = 0.92), with only 9.2% of the samples being classified differently by the two assays.

Free Thyroxin Index

Although the FTI and fT4 (analog RIA) values correlated highly (FTI = 41 + 4.1 fT4, r = 0.86), 25% (n = 106) of the samples had discordant FTI and fT4 (analog RIA) values. Most (92/106) discordant values occurred for samples with low or high fT4 (analog RIA) values and normal FTI values (Table 5). The majority of these samples were from women in the third trimester of pregnancy, patients with severe nonthyroidal illness, patients on thyroxin replacement therapy, patients with an above-normal fT4 (analog RIA) as the only abnormal thyroid-function test result, or patients with compensated euthyroidism (Figure 6).

Discussion

The separation step of conventional RIA accounts for at least half of immunoassay imprecision (10). Automated homogeneous nonisotopic immunoassays eliminate the error associated with the separation step, and they further improve between-assay stability by automating the pipetting and the signal measurements, by providing precise timing of incubation, and by eliminating the error associated with measuring radioactivity. This greater stability should be reflected by a need for only infrequent calibration and analyses of single samples. Both the T4 and T-U assays we tested had good between-assay precision for singleton

<table>
<thead>
<tr>
<th>Table 1. Assay Precision</th>
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<td></td>
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<tr>
<td>Quality control</td>
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<tr>
<td>T4, nmol/L</td>
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<tr>
<td>L1</td>
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<tr>
<td>L2</td>
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<tr>
<td>M1</td>
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<td>M2</td>
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<tr>
<td>H1</td>
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<td>H2</td>
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<tr>
<td>1</td>
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<td>2</td>
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<td>T-U</td>
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<td>L1</td>
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<td>L2</td>
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<td>M1</td>
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<td>H2</td>
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<tr>
<td>2</td>
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<td>Free thyroxin index</td>
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<td>2</td>
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Precision was calculated from the values of controls included in 43 T4 assays and 37 T-U assays. Pools 1 and 2 (FTI controls) were assayed in 13 T4 and T-U assays. L, M, H: low-, medium-, and high-concentration samples.
Fig. 2. (a) Variation of the low (L), medium (M), and high (H) quality-control samples assayed in singleton in the 43 T4 assays and (b) between-assay precision of assay of the T4 quality controls
(a) The solid lines represent the values for the quality controls, which were manually calculated by using the first calibration curve parameters. The fine straight lines represent the mean and 2 SD range for the quality-control values calculated by the instrument (when used as recommended, i.e., with calibration at two-week intervals). Assays in which the instrument was re-calibrated are marked with an arrow-head. Analysis of drift: T4(L) = 55 + 0.3 (assay no), T4(M) = 100 + 0.3 (assay no), T4(H) = 198 + 0.5 (assay no). (b) Quality controls were assayed in singleton in the first three (L1, M1, H1) and last three (L2, M2, H2) locations of the carousel (x). ⊗, between-assay precision of assay of the same quality controls manually calculated by using the parameters of the first, second, third, fourth, or fifth calibration curves

analysis, comparable with that of duplicate analysis. Furthermore, the lack of drift in the T-U assay quality-control values suggested that the assay might need to be calibrated much less frequently than once each fortnight. However, once-fortnightly calibration was sufficient to correct for the drift in the T4 assay.

Of particular concern was the interference of hemoglobin in the T4 assay. Why there is interference in the T4 but not the T-U assay remains unknown.

Our data suggest that not only do the T-U and T3-U assays measure different aspects of thyroxin-binding protein, but also the T-U assay reflects the total binding capacity rather than the concentration of unoccupied binding sites. Although the TBG and T-U values correlated highly, the T-U values sometimes varied independently of changes in TBG concentration. Of the four subjects with familial reduced TBG, only one subject had low TBG and low T-U values; the remaining three had only moderately decreased T-U results, despite very low TBG concentrations.

Comparison of the ratio of T4 to T-U (FTI) and the ratio of T4 to TBG in clinically euthyroid subjects further suggests that T-U does not reflect TBG-binding capacity alone.

Table 2. Effect of Hemolysis on Results of T4 Assay
<table>
<thead>
<tr>
<th>Hemoglobin concn, g/L</th>
<th>Measured T4 concn, nmol/L</th>
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<tbody>
<tr>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td>0.03</td>
<td>90</td>
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<tr>
<td>0.40</td>
<td>86</td>
</tr>
<tr>
<td>0.63</td>
<td>81</td>
</tr>
<tr>
<td>1.20</td>
<td>68</td>
</tr>
<tr>
<td>2.25</td>
<td>56</td>
</tr>
<tr>
<td>4.60</td>
<td>32</td>
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Table 3. Reference Intervals (Mean ± SD)

<table>
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<tr>
<th>Normal persons (n = 48)</th>
<th>Patients with normal results for thyroid-function tests (n = 41)</th>
<th>Normal persons and patients with normal thyroid-function tests (n = 89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-U</td>
<td>0.89 ± 0.17</td>
<td>0.91 ± 0.17</td>
</tr>
<tr>
<td>T4, nmol/L</td>
<td>97 ± 21</td>
<td>102 ± 17</td>
</tr>
<tr>
<td>FTI</td>
<td>110 ± 13</td>
<td>111 ± 22</td>
</tr>
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Attwood and Atkin (14) have also reported a decrease in the T4/TBG ratio with increasing TBG concentrations, whereas the FTI decreased to a much smaller extent over the same range of TBG values. Others (12, 15–18) have also reported the lack of change in FTI (although the published FTI values were calculated from the T3 uptake values), and a decrease in the T4/TBG ratio during late pregnancy (12, 14–16, 19–21).
Table 4. Abnormalities in Thyroid Binding Protein

<table>
<thead>
<tr>
<th>Sample</th>
<th>TBG, mg/L</th>
<th>T4-U</th>
<th>TDx</th>
<th>In-house</th>
<th>FTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8</td>
<td>0.56</td>
<td>65</td>
<td>73</td>
<td>116</td>
</tr>
<tr>
<td>2</td>
<td>2.9</td>
<td>0.60</td>
<td>78</td>
<td>65</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>3.2</td>
<td>0.57</td>
<td>82</td>
<td>85</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>0.10</td>
<td>28</td>
<td>12</td>
<td>278</td>
</tr>
</tbody>
</table>

Thyroid-function tests in subjects with very low TBG concn

Familial dysalbuminemic hyperthyroxinemia

1 0.75 126 39 194
2 0.90 175 39 194

*Not assayed.

The T-U values in two subjects with familial dysalbuminemic hyperthyroxinemia demonstrated that the T-U is insensitive to changes in serum albumin concentration. Both samples had normal T-U values despite increased T4 binding capacity, as shown by increased T4 concentration.

We observed two diagnostic groups in which there were markedly different patterns of FTI and fT4 (analog RIA) values: late pregnancy and nonthyroidal illness. Although the increase in fT4 (analog RIA) in late pregnancy is well documented (12, 16, 17, 21), these changes have not been unequivocally demonstrated by others, who used the equilibrium dialysis FTI method (12, 18, 22, 23). The FTI values are consistent with the clinically euthyroid status of these subjects, but may not accurately reflect changes in fT4 concentration during pregnancy.

The fT4 values we observed in patients with nonthyroidal illness (normal or moderately increased FTI) are consistent with published data for both FTI (calculated from T3 uptake) and fT4 measured by equilibrium dialysis (12, 24, 25). The pattern of change in fT4 (analog RIA) in these patients was quite different, as is well documented in the literature (12, 26, 27). Whereas the interpretation of thyroid-function tests in patients with nonthyroidal illness remains controversial, the fT4 (analog RIA) values were less in keeping with their apparent clinically euthyroid status.

A comparison of the FTI and fT4 (analog RIA) values in the thyroxin-replacement, isolated increased fT4 (analog RIA), and compensated euthyroid groups revealed a high proportion of samples with discordant FTI and fT4 (analog RIA) values; these samples typically had moderately higher or lower fT4 (analog RIA) concentrations associated with normal FTI values. Again, the interpretation of abnormal fT4 (analog RIA) values in these groups is controversial, but the fT4 (analog RIA) assays may have greater diagnostic sensitivity than FTI in the patients receiving thyroxin-replacement. In a previous study (28) we observed a high incidence of above-normal fT4 (analog RIA) values in patients on thyroxin-replacement therapy who were clinically euthyroid and who had normal concentrations of serum T3.
We acknowledge the assistance of Abbott Laboratories, Diagnostic Division, Sydney, Australia, in providing kits at reduced cost and the instrumentation to carry out this study. We thank Mrs. Maria Gardiner for expert technical assistance in performing many of the radioimmunoassays reported in this study.

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These patients had a high incidence of suppressed thyrotropin responses to thyroliberin (thyrotropin-releasing hormone), which suggests that the increased FT4 (analog RIA) may indicate subclinical hyperthyroidism. Similarly, the association of moderately decreased FT4 (analog RIA), above-normal thyrotropin, and normal T3 concentrations in compensated euthyroid patients suggests the presence of subclinical hypothyroidism.

We conclude that the Abbott "TDx" analyzer provides fully automated, highly precise T4 and T-U immunoassays that are a practical and reliable alternative to RIA. Interference of hemolysis in the T4 assay indicates that hemolyzed samples cannot be reliably assayed with this instrument. The T-U most likely reflects the binding capacity of TBG and thyrotropin-binding prealbumin but not of albumin. The FT1 appeared to discriminate thyroid disease as effectively as FT4 (analog RIA), but was less sensitive than FT4 (analog RIA) to subclinical abnormalities in thyroid status. The FTI provided a better indication of euthyroid status in late pregnancy and nonthyroidal illness by giving fewer misleading results in these situations than did FT4 (analog RIA).