Differentiating Various Abnormalities of Thyroxin Binding to Serum Proteins by Radioelectrophoresis of Thyroxin and Immunoassay of Binding Proteins

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Using the simple method of protein analysis described here, we could identify thyroxin (T₄)-binding-protein abnormalities in euthyroid patients with hyperthyroxinemia or hypothyroxinemia. Serum incubated with [¹²⁵]I-thyroxin was analyzed by agarose gel electrophoresis, with bromphenol blue staining of protein. The relative distribution of radioactive T₄ was determined for each binding protein—thyroxin-binding globulin (TBG), transthyretin, albumin, and T₄-binding immunoglobulin (when present)—and the mass of T₄ bound to each was determined. We also used sensitive immunoassays to quantify TBG, transthyretin, and albumin concentrations, then calculated the mass of T₄ (as determined by electrophoresis) bound per unit mass of the respective binding protein. When the concentration of binding proteins was altered (e.g., TBG excess or TBG deficiency), the T₄ binding/mass ratio for each protein remained within the expected range; but when the functional affinity of a binding protein was altered—as in dysalbuminemic hyperthyroxinemia and in low-T₄ euthyroid illness—this ratio was abnormal. This procedure can be used to help identify TBG excess, TBG deficiency, dysalbuminemic hyperthyroxinemia, prealbumin-associated hyperthyroxinemia, variant TBG with reduced affinity for T₄, euthyroid sickness, and T₄-binding autoantibodies.

Additional Keyphrases: electrophoresis, agarose gel • thyroid function • autoantibodies • reference interval

Laboratory differentiation of patients with euthyroid hyperthyroxinemia from those with hyperthyroidism is sometimes difficult, especially if the hyperthyroidism is pituitary in origin. Euthyroid hyperthyroxinemia may result from an increase in the concentration of thyroxin-binding globulin (TBG) (1, 2), increased concentrations of transthyretin (prealbumin) (3), or the presence of molecular variants of transthyretin (4) or of albumin with increased affinity for thyroxin (T₄) (5, 6). The last-mentioned disorder is referred to as "familial dysalbuminemic hyperthyroxinemia" (FDH). Distinguishing euthyroid hyperthyroxinemia from hyperthyroidism is also difficult, particularly for the central forms of hyperthyroidism, secondary and tertiary hyperthyroidism. Euthyroid hyperthyroxinemia can result from a decreased TBG concentration (7), the presence of molecular variants of TBG with reduced T₄-binding affinity (8), or the euthyroid hyperthyroxinemia of illness (the low-T₄ state of nonthyroidal illness). For making those differential diagnoses, it would be useful if one could determine the distribution of T₄ among the binding proteins and the concentration of protein-bound T₄ per unit mass of the respective protein.

In individuals with abnormal T₄ concentrations whose results for free T₄, thyrotropin, and thyrotrphin stimulation tests are within normal limits, the assessment of T₄ per unit mass of the binding protein will help in confirming protein-binding abnormality and in distinguishing molecular variants of binding protein from abnormal concentrations of binding protein.

The presence of circulating autoantibodies to thyroid hormones (T₃ or triiodothyronine) in serum also can complicate the interpretation of thyroid-function tests (9). Depending on the RIA procedures used, such antibodies can produce increases or decreases in the apparent concentration of thyroid hormones.

Methods for assessing protein-binding abnormalities have been described for patients with euthyroid hyperthyroxinemia and euthyroid hypothyroxinemia (10, 11), and there are methods for detecting the presence of thyroid-hormone-binding antibodies (12). Most methods for assessing protein binding are limited by the inability to differentiate molecular variants of the binding proteins. Hence, we describe here a simple radioelectrophoretic method to determine the percentage of T₄ bound to each of the normal thyroid-hormone-binding proteins and to abnormal thyroid hormone-binding immunoglobulins, when present.

We calculated the mass of T₄ bound to each protein and determined the mass of each protein to differentiate altered binding affinity from altered protein concentration. We also described how to accurately quantify the T₄ concentration in T₄-autoantibody-containing samples.

Materials and Methods

Materials

Highly purified TBG was obtained from American Biosystems, Inc., Stillwater, MN; transthyretin from Calbiochem, La Jolla, CA; human serum albumin, bovine serum albumin, and agarose were from Sigma Chemical Co., St. Louis, MO; and high-specific-activity radiolabeled thyroxin (¹²³I-T₄, 4400 kCi/mol) was from DuPont, Wilmington, DE. We prepared radiiodinated TBG, transthyretin, and albumin, using Chloramine T as originally described by Hunter and Greenwood (13).

Procedures

Incubation of serum with ¹²³I-T₄: The serum or plasma samples were incubated with radioactive T₄ before electrophoresis. For a typical assay, 100 µL of sample was incubated with 10 µL of high-specific-activity ¹²³I-T₄ (3.3 µCi) and 1 µL of bromphenol blue solution (10 g/L as marker for electrophoresis) at 37 °C for 30 min; we then stopped the interaction by chilling to 1 °C. We electrophoresed 1 µL of all the samples immediately, but in some experiments we

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removed unbound radioactive T₄ by charcoal separation before electrophoresis. However, this separation provided no advantage in the electrophoresis, so we did not use charcoal separation routinely.

**Agarose gel electrophoresis:** For slab gel electrophoresis we used a 12 g/L agarose gel at pH 8.7, with 40 mmol/L sodium borate buffer containing 1 mmol of calcium lactate per liter. We applied to the loading gel 1 μL of the sample (with the bromphenol blue) and electrophoresed the sample in a vertical electrophoresis unit with a water cooling system. To each plate we applied 20 mA for 2.5 h. At the end of the electrophoresis, the gels were carefully removed and dried under a 500-W heat lamp for 1 h. The dried gels were sliced into 3-mm segments and the radioactivity of each piece was counted in a gamma counter. Figure 1 shows a plot of radioactivity vs gel slice number.

The radioactivity in the electrophoretogram was also scanned in an automated system (Auto Microbiology System, Inc., San Diego, CA). In some experiments, the gels were also autoradiographed and the peaks were measured with a densitometer. The radioactive peaks coincided in all the three methods. We identified the specific protein peaks by using pure proteins and staining with Amido Black or by using radiiodinated proteins: TBG, albumin, or transthyretin. To localize the various binding proteins, we routinely ran on each plate nonradioiodinated human serum albumin and serum and calculated the R₅ values for each binding protein.

This electrophoresis system reliably separated the various binding proteins clearly, with R₅ values of 0.66, 0.47, 0.34, and 0.1 for transthyretin, albumin, TBG, and anti-T₄ IgG, respectively. The average analytical recovery of radioactivity, determined routinely in each run, was 75%.

**Quantification of T₄-binding proteins:** Albumin and transthyretin were determined with an automated protein analyzer ("Array"; Beckman Instruments, Brea, CA). This is a rate nephelometric assay in which specific antibodies are used for the binding proteins (14). The intra-assay coefficients of variation (CV) for albumin and transthyretin were 1% and 1.2%; the interassay CVs were 2.0% and 2.6%, respectively. We estimated TBG content by direct radioimmunoassay with use of 125I-labeled TBG and antibodies to TBG (15). The assay is highly specific; there is <0.01% cross-reactivity with albumin and transthyretin. The intra- and interassay CVs were 5% and 8%, respectively.

**Quantification of T₄:** To measure total T₄ in the serum samples, we used a radioimmunoassay procedure (16) involving 125I-T₄ and anti-T₄ antibodies in the presence of a T₄-binding inhibitor (anilinonaphthalenesulfonic acid). For the bound/free separation, we used a 250 g/L solution of polyethylene glycol (125 g/L final concentration). The assay is sensitive and specific for T₄ (16). Intra- and interassay CVs were 4% and 7%, respectively. However, this assay could not be used for samples containing anti-T₄. Use of either a second-antibody separation step or a high concentration of anilinonaphthalenesulfonic acid allowed accurate determination of total T₄ in the presence of T₄-binding autoantibodies. Thus, T₄ bound to anti-T₄ was released by using HCl (0.1 mol/L), and the T₄ antibody was precipitated with polyethylene glycol, as previously described for the assay of insulin in sera containing insulin antibodies (17). The mixture was centrifuged, and the supernate was neutralized to pH 7.0 with 0.1 mol/L sodium hydroxide and assayed by RIA as above. Complete release of T₄ from T₄ antibody was checked by use of 125I-T₄, and the recovery was 85% as assessed with use of either radioactive or nonradioactive T₄.

**Distribution of T₄ in various binding proteins:** Using the total T₄ concentration and the percentage of 125I-T₄ radioactivity in each protein peak, we calculated the mass of T₄ bound to each binding protein. For example, albumin-bound T₄ = (% of 125I-T₄ bound to albumin × T₄ concentration in the sample)/100, and albumin-bound T₄/mg of albumin = T₄ bound to albumin/mg of albumin in the sample.

**Clinical samples:** Serum or plasma samples were obtained from normal volunteers at Nichols Institute. Hypothyroid, hyperthyroid, TBG-deficient, TBG-excess, and T₄-antibody-containing samples were taken from specimens sent to the Reference Laboratory.

Euthyroid hypothyroxinemia of illness (low-T₄ state), FDH, and transthyretin-deficient samples were obtained from White Memorial Medical Center, Los Angeles, CA. All patients with the euthyroid hypothyroxinemia of illness had normal thyrotropin concentrations as determined by sensitive thyrotropin assay (18) and normal free T₄ concentrations as determined by direct equilibrium dialysis of undiluted serum (19).

**Results**

Radioactive thyroxin was observed to bind maximally to the binding proteins at 37 °C within 30 min. We could reliably separate the T₄-binding proteins by agarose gel electrophoresis (Figure 1). All three T₄-binding proteins—TBG, transthyretin, and albumin—could be demonstrated in normal human serum samples (Figure 1A). All three peaks were resolved with no overlap. The radioactive peak

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**Figure 1. Distribution of radioactive 125I-T₄ in normal human serum (A) or serum containing antibodies to T₄ (B) after electrophoresis.**

The arrows indicate the mobility of various purified protein markers, as determined by electrophoresis of radiiodinated binding proteins. The CPM/mg of slice is shown as a function of the agarose gel slice number.
corresponded to the standard protein peaks as run in the same gel and stained with Amido Black. An additional radioactive peak could be demonstrated in the gamma globulin region in samples containing antibodies to Tg (Figure 1B).

Total T4 was quantified by direct radioimmunoassay in all samples except those with autoantibody to Tg. In the latter, the samples were treated with HCl to release T4, and antibody to Tg was removed by precipitation with polyethylene glycol. After antibody removal the T4 concentration was determined by radioimmunoassay. This procedure resulted in accurate assessment of T4 concentration; values for T4 in the treated samples, when diluted, paralleled those on standard curve for T4.

Table 1 shows the range of values found for 28 normal adult controls for the percentage distribution of radioactive T4 among the various binding proteins as well as the mass of T4 bound to each protein. The clinical disorders we studied included hypothyroidism, hyperthyroidism, Tg excess, dysalbuminemic hyperthyroxinemia, the euthyroid hyperthyroxinemia of illness, patients with T4 autoantibodies, a transthyretin variant, and a variant Tg. Figure 2 shows the Tg-bound T4, transthyretin-bound T4, and albumin-bound T4 in these various clinical conditions. The concentration of Tg-bound T4 was high in the serum of patients with hyperthyroidism or Tg excess. Low values for Tg-bound T4 were observed with Tg deficiency, variant Tg, hyperthyroidism, and the euthyroid hyperthyroxinemia of illness. A high concentration of transthyretin-bound T4 was observed in one patient with a transthyretin variant.

Similarly, sera from patients with FDH showed high concentrations of albumin-bound T4. Low concentrations of all three forms of protein-bound T4 were seen in samples from hypothyroid patients, in samples containing T4 autoantibody, and in samples from the euthyroid hyperthyroxinemia of illness.

Table 1 lists the ranges found for Tg, transthyretin, and albumin in the controls. Figure 3 shows the concentrations in the various clinical disorders we studied. Tg concentrations were abnormal only in samples from patients with Tg deficiency or excess conditions. Albumin and transthyretin were low in some patients with the euthyroid hyperthyroxinemia of nonthyroidal illness. It has been shown that FDH and Tg variant proteins have an altered affinity for T4 (6, 20). To determine whether the present methods can distinguish abnormalities of protein

Table 1. Analysis of T4-Binding Proteins in 28 Normal Individuals

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Range (mean ± 2 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-T4 bound to Tg, %</td>
<td>67-87</td>
</tr>
<tr>
<td>125I-T4 bound to transthyretin,%</td>
<td>5-27</td>
</tr>
<tr>
<td>125I-T4 bound to albumin, %</td>
<td>3-10</td>
</tr>
<tr>
<td>Tg-bound T4, µg/L</td>
<td>46-91</td>
</tr>
<tr>
<td>Transthyretin-bound T4, µg/L</td>
<td>5-24</td>
</tr>
<tr>
<td>Albumin-bound T4, µg/L</td>
<td>3-9</td>
</tr>
<tr>
<td>Tg-bound T4/albumin, mg/g</td>
<td>2.5-4.7</td>
</tr>
<tr>
<td>Transthyretin-bound T4/transthyretin, µg/g</td>
<td>20-90</td>
</tr>
<tr>
<td>Albumin-bound T4/albumin, µg/g</td>
<td>0.07-0.22</td>
</tr>
<tr>
<td>Tg concn, mg/L</td>
<td>12-30</td>
</tr>
<tr>
<td>Transthyretin concn, mg/L</td>
<td>140-420</td>
</tr>
<tr>
<td>Albumin concn, g/L</td>
<td>32-55</td>
</tr>
</tbody>
</table>

Fig. 2. Distribution of T4 in serum samples from normal subjects and from patients with various clinical conditions

The amount of T4 bound to each binding protein was calculated by multiplying the percentage of radioactivity in each binding protein fraction by the total T4 concentration. Shaded area: the normal reference interval

concentration from abnormalities of affinity in protein variants, T4 concentration in each protein fraction was expressed per mass of the respective binding protein. Table 1 shows the normal ranges for Tg-bound T4 per gram of Tg, transthyretin-bound T4 per gram of transthyretin, and albumin-bound T4 per gram of albumin.

Figure 4 summarizes the distribution of T4 in binding proteins divided by the mass of the protein in various clinical disorders.

In Tg deficiency and Tg excess, the results were within normal limits when expressed per unit mass of Tg. In contrast, results for binding protein variants, FDH, Tg variant, and the transthyretin variant were abnormal when so expressed (Figure 4). Hence, this method of analysis can differentiate abnormalities in protein binding affinity from altered binding-protein concentrations.

Abnormal results were also seen in the euthyroid hyperthyroxinemia of illness. They were consistent with diminished affinity of Tg for T4 in 20 of 20 patients, diminished affinity of transthyretin in 16 of 20, and diminished affinity of albumin in six of 20.

Values for T4-binding expressed per mass of binding protein for samples from patients with hypothyroidism and hyperthyroidism are similar to such values for samples with molecular variant proteins (Figure 4). These samples could be differentiated because the changes in hypothyroidism and hyperthyroidism occur in all three binding proteins and are associated with abnormal concentrations of free T4 and TSH.
Discussion

The use of radiolabeled T₄ and serum protein electrophoresis to assess the distribution of T₄ in various binding proteins is well documented (10, 11, 21, 22). Our electrophoresis procedure is specific and reproducible, TBG, transthyretin, and albumin being well separated (Figure 1A). T₄-binding antibodies can also be identified easily with this procedure (Figure 1B). The distribution of T₄ among binding proteins can be determined in various clinical conditions (Figure 2). It is possible to quantify concentrations of binding protein and diagnose patients with abnormally high or low concentrations of T₄-binding proteins (Figure 3) by using sensitive immunoassays.

Several methods have been described to characterize molecular variants of albumin, including Scatchard plot (4, 20) and saturation with nonradioactive T₄ (11). Preparation of Scatchard plots is tedious, and they have been applied to only one binding protein at a time. Saturation with nonradioactive T₄ with electrophoresis produces variable results, because constant amounts of T₄ are used even though the concentration of binding proteins is variable. The present procedure is simple and can be used to assess all three of the T₄-binding proteins. When the concentration of T₄ bound to each protein is expressed per unit mass of protein, only proteins with altered affinities show abnormal distributions (Figure 4). This method can be used for the diagnosis of all the described T₄-binding protein variants, including the TBG variant, the dysalbuminemic variant, and the transthyretin variant.

When the T₄ distribution is abnormal and involves all of the binding proteins, hyperthyroidism or hypothyroidism is to be suspected (Figure 4), but measurements of free T₄ and TSH are necessary to distinguish the euthyroid hyperthyroxinemia of illness from hypothyroidism.

In clinical practice, diagnosis and follow-up of patients with thyroid disorders is commonly accomplished by applying T₄ RIA procedures, and T₄-binding abnormalities can cause factitious results for T₄. Recent developments in TSH assay sensitivity minimize diagnostic errors and help in the management of thyroid disorders. However, when results for thyroxin or the free-thyroxin index are not consistent with the clinical situation or with the TSH concentrations, it is important to consider the possibility of T₄-binding-protein abnormalities. The method described here allows, for the first time, rapid and reliable determination of the various causes of euthyroid hyperthyroxinemia and euthyroid hyperthyroxinemia. In addition, it may be helpful and important to distinguish genetic molecular variants of the T₄-binding proteins from disease-related abnormalities in protein concentrations. In the latter, the discovery of abnormal thyroid-hormone-binding protein concentrations may be the first evidence of previously unrecognized organic disease. Applying the presently described method, one can now characterize the type of T₄-binding abnormality in individual patients encountered in clinical practice.

References
2. Borst GC, El, C, Burman KD. Euthyroid hyperthyroxinemia.