Determination of Thyroxine-Binding Globulin

A Simplified Procedure Utilizing Dextran-Coated Charcoal

R. C. Roberts and T. F. Nikolai

A method for determining thyroxine-binding globulin (TBG) concentration as the total thyroxine-binding capacity, has been developed. Prior electrophoretic separation of the three serum thyroxine-binding proteins is not required. Serum samples are diluted with a barbital-salicylate buffer pH 8.6, which inhibits thyroxine-binding by prealbumin. After incubation with 100 µg/100 ml thyroxine containing 131I-thyroxine, dextran-coated charcoal is added to the sample, which binds all the thyroxine not bound to TBG. The radioactivity in the supernatant solution is directly related to the concentration of TBG present. The Pearson's correlation coefficient between the TBG results for this new assay and the polyacrylamide electrophoretic assay is 0.964. The mean TBG concentration and standard deviation for 80 normals was 19.2 ± 2.6 µg/100 ml. Pregnant women and women taking estrogens had a mean and standard deviation of 35.8 ± 4.8 µg/100 ml. Males from TBG-deficient families had TBG values of 5 µg/100 ml or less, and females from these families had values ranging from 8 to 12 µg/100 ml. The new assay is considerably simpler in equipment requirements and technic than the assays currently being used, and should be more practical for routine clinical laboratory use.

All the procedures available for the assay of thyroxine-binding globulin (TBG) concentrations require preliminary separation of TBG from the other two thyroxine-binding proteins in serum, albumin, and thyroxine-binding prealbumin (TBPA) (1-3). The electrophoretic step can be eliminated if the binding of these other two proteins can be inhibited.

The binding of TBPA can be inhibited by sodium salicylate and barbital salts (3, 4). Since albumin is the weakest binder of the three proteins, its binding may be eliminated by introducing a suitable binder,

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intermediate in binding capacity between albumin and TBG. We have found that dextran-coated charcoal can be used as such a binder.

The use of charcoal coated with macromolecules as micromolecular sieves to selectively bind low molecular weight nonprotein molecules has been used to measure intrinsic factor, gastric and serum unsaturated B$_{12}$-binding capacity (5), tri-iodothyronine (T$_{3}$) uptakes (6, 7), and unsaturated iron-binding capacity (8). Nugent and Mayes (9) used competitive binding between dextran-coated charcoal and cortisol-binding globulin to determine plasma corticosteroids. DeMoor and Heyns have used dextran-coated charcoal to determine cortisol-binding globulin concentrations. (10).

The total binding capacity of TBG can be determined by adding a sufficient amount of isotope-labeled thyroxine (T$_{4}$) to insure saturation, incubation which allows equilibration of labeled thyroxine on the binding sites, and then removal of the excess labeled thyroxine by absorption on dextran-coated charcoal. A new assay based on this concept for TBG is presented. It can be carried out quickly in any clinical laboratory having a refrigerated centrifuge and a gamma well counter. The serum sample is diluted with a barbital-salicylate buffer. After incubation with 100 µg $^{131}$I-T$_{4}$/100 ml, dextran-coated charcoal is added, incubated in the cold, and removed by centrifugation. The counts in the supernatant are a direct measure of the TBG concentration in terms of thyroxine-binding capacity.

**Materials and Methods**

Norite A charcoal (neutral) (Fisher Scientific Co.) was coated with Pharmacia Dextran T70 (wt av mol wt 70,000). Shipments of $^{131}$I-thyroxine were obtained* every two weeks. Polyacrylamide gels were prepared with Cyanogum 41 (Fisher Scientific Co.). L-thyroxine was obtained from Sigma Chemical Co. The barbital-salicylate buffer was prepared with sodium barbital, N.F. XI., and sodium salicylate, U.S.P. (Merek & Co., Inc.). All other chemicals were analytical reagent grade.

TBG determinations by electrophoresis on polyacrylamide were performed by the method of Nikolai and Seal (1). Protein-bound iodine (PBI) tests were performed on the AutoAnalyzer (11).

Normal serum samples were obtained from persons on routine physical examinations at our clinic and from laboratory personnel. All of these persons had normal PBI's and appeared to be euthyroid. High TBG serums were from women taking oral contraceptives containing

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*Abbott Laboratories kindly donated the $^{131}$I-L-thyroxine used in these studies.
estrogen or from pregnant women, and low or zero TBG samples were from members of families with TBG deficiencies (1).

Procedure

The procedure adopted for the routine assay of TBG is as follows:

**Barbital-salicylate Buffer** Barbital, 0.06 M (10.8 g sodium barbital; 1.5 g barbituric acid/liter), and sodium salicylate, 0.03 M (4.8 g sodium salicylate/liter), pH = 8.6.

**Dextran-coated Charcoal** Equal volumes of 0.6% (w/v) charcoal slurry in water and 0.06% (w/v) dextran in barbital-salicylate buffer are mixed at room temperature and stored in the cold room. This charcoal slurry is stable for at least 1 week. The final slurry contains 3 mg charcoal/ml.

**Assay Procedure**

An 0.20-ml sample of serum is added to a 12-ml conical centrifuge tube containing 1.8 ml of the barbital-salicylate buffer. Then 10 µl of tracer-containing thyroxine solution is added. This solution is made up by adding 0.5 ml of ¹³¹I-L-thyroxine to 1 ml of a cold 10 mg/100 ml solution of L-thyroxine and diluting to 5 ml with the barbital-salicylate buffer. This solution is equivalent to 100 µg thyroxine/100 ml of serum. The mixture is then incubated for 15 min at 37°. The samples are cooled to 4° and 1 ml of the cooled dextran-coated charcoal slurry is added. This slurry is mixed in its Erlenmeyer flask before each pipetting. After 45 min at 4°, the charcoal is sedimented in a refrigerated centrifuge at 2500 rpm for 15 min. An 0.5-ml aliquot of the supernatant is assayed in an auto-gamma counter. A measure of the total cpm of radioactivity per 0.5 ml of supernatant is obtained by assaying a control tube to which no charcoal has been added. Duplicate control tubes are run for each set of samples. Since the dextran-coated charcoal does not absorb the radioactivity completely, a duplicate set of samples are included in which 1 ml of charcoal slurry is added to 2.0 ml of salicylate-barbital buffer to determine the unabsorbed radioactivity.

**Calculation of µg T₄ bound/100 ml serum**

The calculation of the concentration of TBG in terms of µg T₄ bound/100 ml serum was carried out according to the following formula:

\[
\left( \frac{\text{cpm/0.5 ml supernate}}{\text{total cpm/0.5 ml}} \right) - \left( \frac{\text{cpm unabsorbed}}{\text{total cpm}} \right) \times \frac{\mu g \ T_4 \ added/100 \ ml}{\mu g \ TBG/100 \ ml}
\]

where:
- cpm = counts per minute
- total cpm = counts obtained in 0.5 ml when no charcoal was added
- cpm unabsorbed = counts in 0.5-ml supernatant solution obtained in control sample containing no serum
Routinely the equivalent of 100 \( \mu g \) of \( T_4/100 \) ml serum was added.
In the experiment where the amount of charcoal was varied, a slurry of coated charcoal containing 10 mg charcoal/ml was prepared. The amount of charcoal was varied by adding 0.1 ml to 1.0 ml of the slurry. The total volume 3.0 ml was completed by adding the necessary amounts of the buffer.
In the experiments where the amount of exogenous thyroxine was varied, the concentration of the cold thyroxine was varied while maintaining a constant amount of radioactive \( T_4 \).

**Results**

**Variation of Level of Exogenous Thyroxine**

The level of exogenous \( T_4 \) was varied to establish the level required to obtain complete saturation of the TBG binding sites. The results are shown in Fig 1. Each point on this graph is the average of ten replicate determinations. The serum sample was a pool from normal laboratory personnel. The curve shows a plateau from 100 \( \mu g/100 \) ml to 200 \( \mu g/100 \) ml.

**Fig 1.** Variation of \( \mu g \) thyroxine \( (T_4) \) bound/100 ml serum with amount of exogenous \( T_4 \) added to assay system.

**Influence of Amount of Coated Charcoal on the Percent Radioactivity Recovered in Supernatant**

Figure 2 illustrates the effect of varying the weight of charcoal on the radioactivity found in the supernatant; 100 \( \mu g/100 \) ml of thyroxine
was used. The sample was pooled serum drawn from 6 normal laboratory technicians (3 males and 3 females). Each point represents the mean of quadruplicate determinations. On the basis of these tests, a 3-mg aliquot of charcoal was selected for the routine assay.

![Graph](image)

**Fig 2.** Relationship between percent radioactivity due to $^{131}$I-thyroxine recovered in supernatant and quantity of charcoal added to assay system.

**Correlation with Polyacrylamide TBG Assay**

The correlation between the charcoal procedure and the polyacrylamide TBG assay of Nikolai and Seal (1) is shown in Fig 3 for 50 samples. The samples had a wide range of TBG values. The low TBG samples were selected from female heterozygotes and the zero TBG samples from male members of families having hereditary X-linked TBG deficiency (1, 11). The Pearson's correlation coefficient for this group of 50 samples is 0.964.

**Normal Range: Charcoal TBG Method**

The normal range for the charcoal TBG method was determined for 80 normal adults (37 females and 43 males). This normal range is compared with the normal range previously determined for the polyacrylamide TBG procedure (12) in Table 1. The mean and standard deviation for the charcoal assay is $19.2 \pm 2.6 \mu g/100 \text{ ml}$ as compared with $20.7 \pm 3.2 \mu g/100 \text{ ml}$ for the polyacrylamide electrophoretic procedure. The normal ranges for the two methods are similar; however, the charcoal method gives slightly lower results.
Table 1. COMPARISON OF NORMAL VALUES FOR TBG BY THE CHARCOAL AND POLYACRYLAMIDE ELECTROPHORETIC METHODS

<table>
<thead>
<tr>
<th>Method</th>
<th>Polycrylamide</th>
<th>Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of assays</td>
<td>315</td>
<td>80</td>
</tr>
<tr>
<td>TBG values:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>20.7</td>
<td>19.2</td>
</tr>
<tr>
<td>SD</td>
<td>± 3.2</td>
<td>± 2.6</td>
</tr>
<tr>
<td>Range</td>
<td>14-31</td>
<td>14-28</td>
</tr>
</tbody>
</table>

Charcoal TBG Results on Women Receiving Estrogen

The mean (± SD) in 13 women receiving estrogen (pregnant or taking birth control pills) was 35.8 ± 4.8 with a range of 26-45. This value compares favorably with the value of 36 ± 5.6 obtained previously (13) by the polyacrylamide electrophoretic method for women receiving estrogen.

Low TBG: Comparison of Results by Charcoal and Polyacrylamide Electrophoretic Methods

Table 2 compares the TBG results obtained for zero-TBG males and heterozygous females from families with hereditary X-linked TBG deficiency. For this group of samples, the two methods give similar results.
Table 2. Comparison of TBG Results for Family Members with Hereditary X-linked TBG Deficiency

<table>
<thead>
<tr>
<th>Subject</th>
<th>Charcoal (µg TBG/100 ml)</th>
<th>Polya crylamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.F.</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>D.W.</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>C.W.</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>R.C.</td>
<td>3</td>
<td>4</td>
</tr>
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<table>
<thead>
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<th>Heterozygous Females</th>
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<tbody>
<tr>
<td>H.F.</td>
</tr>
<tr>
<td>J.F.</td>
</tr>
<tr>
<td>J.H.</td>
</tr>
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<td>J.H.</td>
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</tbody>
</table>

Discussion

The essential requirements for a new assay for TBG have been met: the assay gives results which correlate closely with the results obtained by an established method. TBG binding appears to be the entity measured by the assay as evidenced by the values obtained for persons with zero TBG and elevated values obtained for persons on estrogen who have high TBG’s. The exogenous load of thyroxine and the amount of coated charcoal have been empirically selected to give results agreeing closely with assays requiring prior electrophoretic separation of the thyroxine-binding proteins.

The experiments with different loading levels of thyroxine (Fig 1) show that saturation of the TBG-binding sites have been achieved for normals and the elevated TBG samples at 100 µg thyroxine/100 ml. The rise in thyroxine binding found at thyroxine concentrations higher than 200 µg/100 ml is suspected to be due to albumin binding. Serum samples containing extremely high levels of TBG (male members of high TBG families) were not available. The possibility should be considered that 100 µg thyroxine/100 ml would not be sufficient to give total binding capacities in such cases, and higher concentrations of thyroxine should be tested where very high TBG’s are suspected.

The experiments in which the amount of added charcoal was varied (Fig 2) indicate that this parameter has a profound effect on the TBG determinations. The routine use of 3 mg charcoal/sample was selected on the basis of these experiments so that the normal pooled serum gave results close to the normal mean by the polyacrylamide electrophoresis assay. The slope of the line in Fig 2 in the region between 2 and 4 mg
charcoal indicates that ±10% variations in pipetting the charcoal slurry will lead to ±2 μg/100 ml in the TBG results. We have routinely used a 1-ml volumetric pipette with a large-bore opening to pipette the charcoal slurry. The precision is indicated by 25 replicate determinations on a normal pool which gave a standard deviation of ±0.85 μg TBG/100 ml. The slurry is prepared in an Erlenmeyer flask and is thoroughly shaken by swirling before each pipetting. These results indicate that even though there is an appreciable slope in the charcoal quantity versus the TBG curve, the slurry can be pipetted accurately enough to give precision similar to that reported for the polyacrylamide assay (13). We would advise the construction of a charcoal weight versus TBG curve by anyone setting up this assay in order to establish the performance of the coated charcoal.

The determination in the blank tube containing no serum is an indication that the charcoal does not quantitatively bind the radioactivity. This background varies between 5 and 12% of the total radioactivity. Repeated centrifugation of the supernatants does not lower this background, indicating that the charcoal has been thoroughly sedimented. Thin layer chromatography in a system which separates T₄, T₃, and iodide (15) from the supernatant highly enriched with radioactivity indicates that 66% of the unabsorbed radioactivity was in the iodide section of the chromatogram; 34% in the T₃ and T₄ region. These results indicate that the majority of this background arises from the iodide contaminant of the radioactive thyroxine which apparently is poorly bound by the charcoal. Similar unabsorbed radioactivity representing non-charcoal bound fractions have been reported by Herbert and coworkers in other assays using coated charcoal (5, 6).

The ease of this assay in detecting abnormal levels of TBG should make it suitable for intermittent use in clinical laboratories to aid in the evaluation of patients whose thyroid tests are ambiguous. A TBG test is indicated in any patient with a low or high PBI who appears clinically euthyroid. This test should be valuable in large scale screening studies for TBG abnormalities since 100 or more samples can be run daily.

References
5. Gottleib, C., Lau, K. S., Wasserman, L. R., and Herbert, V., Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated $B_1$ binding capacity, antibody to IF and serum unsaturated $B_1$ binding capacity. *Blood* 25, 875 (1965).


