BINDING OF THYROXINE BY SERUM PROTEINS EVALUATED BY EQUILIBRIUM DIALYSIS AND ELECTROPHORETIC TECHNIQUES. ALTERATIONS IN NON–THYROIDAL ILLNESS *

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Considerable evidence suggests that the concentration of free thyroxine, circulating unbound to serum proteins, is more closely related to a subject’s thyroidal status than is the concentration of total thyroxine (1). Because of the intense binding of thyroxine by the serum proteins, the concentration of free thyroxine is very small and not measurable by conventional chemical methods. Robbins and Rall (2), on the basis of electrophoretic data and the binding characteristics of bovine serum albumin, estimated that the concentration of free thyroxine in normal subjects was in the order of 6 × 10⁻¹¹ M. By equilibrium dialysis of whole serum, Sterling and Hegedus (3) arrived at a figure approximately twice this. Other techniques for assessing the relative concentrations of free thyroxine in various clinical and physiological states include measurement of the fractional rate of dialysis of I¹³¹-labeled thyroxine (T₄-I¹³¹) across a semipermeable membrane from one serum compartment to another (4, 5), and simultaneous determination of red-cell uptake of I¹³¹-labeled triiodothyronine (T₃-I¹³¹) and serum protein-bound iodine (PBI) (6).

In the present study, we used two independent methods to provide a relative measure of the fraction of thyroxine bound to serum proteins and the concentration of free thyroxine in serum. One method is based on the principle of equilibrium dialysis of diluted serum in an aqueous buffer at pH 7.4; the other involves determination of critical ratios by filter-paper electrophoresis of whole

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serum in a glycine-acetate buffer at pH 8.6. Such an electrophoretic system reveals three species of protein-binding sites: thyroxine-binding globulin (TBG), albumin, and thyroxine-binding prealbumin (TBPA). Applied to the study of sera of normal and pregnant subjects and patients with hypothyroidism and hyperthyroidism, these methods gave results that were consistent internally and also with the current view that concentrations of free thyroxine are elevated in hyperthyroidism, depressed in hypothyroidism, and normal in pregnancy. The investigation was therefore extended to a more intensive study of thyroxine-binding by the serum proteins of patients with nonthyroidal disease. Ingbar and Freinkel (7) have noted that in patients with a variety of diseases, a reduced quantity of thyroxine was associated with TBPA, and this finding suggested to us the possibility that such alterations could influence the level of free thyroxine in serum.

METHODS

Equilibrium dialysis

The method of these studies is partly based on the dialysis procedures used by Sterling and Tabachnick in their investigation of thyroxine-binding by serum albumin (8). Analogous techniques have been used to study the interaction of certain drugs with thyroxine-binding proteins (9-11). T₄-I¹³¹, tested for chromatographic purity as previously described (9), was added to the serum sample in quantities sufficient to increase the endogenous concentration of thyroxine by 1 μg per 100 ml. The mixture was allowed to equilibrate for 30 minutes at room temperature. One ml of serum enriched with T₄-I¹³¹ was added to 240 ml phosphate buffer (pH 7.4, ionic strength = 0.15), and a 5-ml sample of this dilution was pipetted into each of three specially prepared cellophane bags (8). These bags were suspended in 50-ml cellulose nitrate centrifuge tubes containing 25 ml of the same phosphate buffer used to dilute the serum.

1 Abbott Laboratories, North Chicago, Ill.
The stoppered tubes were inserted in a metal rack on a rotating platform in a bacterial incubator at 37° C. Equilibrium was attained in 20 hours; then, the concentration of T₄-I⁺⁺ inside and outside the bag was measured.

A major technical problem in dialysis of T₄-I⁺⁺ against an aqueous buffer is to distinguish the radioactivity originating from T₄-I⁺⁺ from the activity arising from the much more loosely protein-bound iodide-I⁺⁺. This problem was solved by the addition of outdated banked plasma to samples from the inside and the outside of the dialysis bag and the subsequent precipitation by trichloroacetic acid (TCA) of protein from the resultant mixtures. Two ml was withdrawn from inside and 5 ml from outside the bag. Equal volumes of plasma were added to both samples, and 3 ml of cold 20% TCA was added in steps. The precipitate was thoroughly mixed into a homogenous paste and centrifuged for 1 hour at 1,600 rpm in an International, size 2, model V centrifuge. The supernatant fluid was decanted, and the precipitate washed twice with 1% TCA. The final residue was assayed for radioactivity in a Packard automatic spectrometer until a statistical counting accuracy of at least 2% was achieved.

From the concentration of T₄-I⁺⁺ inside and outside the bag, the fraction of radioactivity existing in the system in the unbound form at equilibrium, or the dialyzable fraction (DF), was calculated as follows:

\[ DF = \frac{(V_s + V)SPI_{l^+}}{V_s \times SPI_{l^+} + V \times SPI_{l^+}} \]

where \( V_s \) = volume outside the bag, \( V \) = volume inside the bag, \( SPI_{l^+} \) = concentration of serum precipitable iodine⁺⁺ outside the bag, and \( SPI_{l^+} \) = concentration of serum precipitable iodine⁺⁺ inside the bag. The concentration of free thyroxine in the dialysis system (\( T_A \)) can then be calculated as:

\[ T_A = DF \times \text{concentration of total endogenous thyroxine in the system} \]

The concentration of total endogenous thyroxine can be estimated from the PBI.

To convert PBI, in micrograms iodine per 100 ml, into thyroxine concentration, in moles per liter, the PBI value is multiplied by the factor 1.968 × 10⁻⁸. Since 0.2 ml serum is in equilibrium with a volume of 30 ml, a dilution factor (1/150) is also introduced. Thus the expression for the free thyroxine concentration, (in moles per liter), will be:

\[ T_A = (1.31 \times 10^{-8}) \times DF \times PBI \]

In this study, \( T_A \) can be considered a simple empirical measure that is related to, but not necessarily equivalent to, the in vivo concentration of free thyroxine.

Analytical considerations

In three separate experiments, the sum of TCA-precipitable radioactivity inside and outside the dialysis bag accounted for more than 97% of the TCA-precipitable activity added to the system. Thus, less than 3% of T₄-I⁺⁺ could have adhered to the dialysis bag. Because of the serum's dilution, no significant changes in protein concentration inside the bag could be detected at the end of the dialysis, and possible oncocyt pressure effects on the volume of the compartments were considered to be negligible.

In order to test the effectiveness of the method of TCA precipitation in the discrimination between minute quantities of iodide-I⁺⁺ and T₄-I⁺⁺ in the dialyze, the following experiment was performed. A blank dialysis was carried out as usual except that nonradioactive thyroxine (1 μg per 100 ml) instead of T₄-I⁺⁺ was added to the original serum. To samples of the dialyze iodide-I⁺⁺ and T₄-I⁺⁺ were added separately and together in concentrations simulating those observed after routine dialysis with added T₄-I⁺⁺. The contaminating iodide-I⁺⁺ in the shipment of T₄-I⁺⁺, determined chromographically, was taken into consideration in the calculations. TCA precipitation after the addition of exogenous plasma was performed as usual. An excess of 97% T₄-I⁺⁺ was recovered, and more than 92% (generally 95%) of the iodide-I⁺⁺ was removed by the procedure.

The error due to coprecipitation of iodide-I⁺⁺ with T₄-I⁺⁺ can be calculated as follows. Assume that 5% of the radioactivity of the stock shipment of T₄-I⁺⁺ is in the form of iodide-I⁺⁺ and that iodide is freely diffusible and not protein bound. Since 5% of iodide-I⁺⁺ remains with the residue after TCA precipitation, iodide-I⁺⁺ could account for an apparent DF of 0.05 × 0.05 = 0.0025. This would introduce a 6% error in a normal serum with a DF of 0.0416 and a lesser error in sera with higher DF values.

The average of the results of three simultaneous determinations of DF was employed for all calculations. In a series of ten dialysis experiments conducted simultaneously, the mean DF was found to be 0.0461 ± 0.0024 (1 SD). Sera were analyzed immediately after separation from red cells, or after storage in frozen form for not over 2 weeks. In general, sera yielded similar results after prolonged storage, but a few specimens showed increased values of DF after repeated freezing and thawing. In the latter stages of this study, every precaution was taken to dialyze the serum as soon as possible after separation from red cells.

Electrophoretic studies

Methods for determining the distribution of T₄-I⁺⁺ (3 μg per 100 ml) among serum proteins separated by paper electrophoresis in a glycine-acetate buffer (pH 8.6, ionic strength, 0.15) have been described (10). We used an electronically integrating recorder to measure the areas of radioactive peaks. Vertical starch-gel electrophoresis was performed by a modification (12) of the method of Smithies (13). Radioactive distribution of T₄-I⁺⁺ in starch gel was determined as previously described (12).

Maximal binding capacity of TBPA and TBG. All sera were subjected to paper electrophoresis in duplicate after the addition of 0, 200, and 800 μg nonradioactive thyroxine per 100 ml. For this purpose, stock solutions of thyroxine were prepared in 4% propylene glycol dissolved in 2 M KOH (200, 600, and 800 μg per ml), and

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² Bio-Science Laboratories, Los Angeles, Calif.

³ Texas Instruments Inc, Houston, Texas.
## TABLE I

**Summary and statistical analysis of binding studies**

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal</th>
<th>Pregnancy</th>
<th>Hypothyroidism</th>
<th>Hyperthyroidism</th>
<th>Nonthyroidal illness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Dialysis data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBI, µg/100 ml</td>
<td>5.57</td>
<td>0.69</td>
<td>22</td>
<td>8.53</td>
<td>.73</td>
</tr>
<tr>
<td>DF</td>
<td>.0416</td>
<td>.0044</td>
<td>22</td>
<td>.0277</td>
<td>.0039</td>
</tr>
<tr>
<td>T4 × 10^{-6} moles/L</td>
<td>3.02</td>
<td>0.37</td>
<td>22</td>
<td>3.11</td>
<td>.61</td>
</tr>
<tr>
<td>Electrophoretic ratios</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapp × 10^6</td>
<td>10.63</td>
<td>2.32</td>
<td>21</td>
<td>9.76</td>
<td>2.51</td>
</tr>
<tr>
<td>Rapp/T4 × 10^6 µg</td>
<td>12.58</td>
<td>2.05</td>
<td>21</td>
<td>7.64</td>
<td>2.54</td>
</tr>
<tr>
<td>Distribution, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBPA</td>
<td>32.00</td>
<td>5.40</td>
<td>21</td>
<td>14.42</td>
<td>4.21</td>
</tr>
<tr>
<td>TBA</td>
<td>10.60</td>
<td>1.33</td>
<td>21</td>
<td>6.70</td>
<td>0.96</td>
</tr>
<tr>
<td>TBG</td>
<td>57.41</td>
<td>6.68</td>
<td>21</td>
<td>79.08</td>
<td>4.32</td>
</tr>
<tr>
<td>Max. binding capacity, µg/100 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBPA</td>
<td>256.3</td>
<td>37.6</td>
<td>21</td>
<td>193.8</td>
<td>22.81</td>
</tr>
<tr>
<td>TBG</td>
<td>24.54</td>
<td>2.61</td>
<td>21</td>
<td>52.14</td>
<td>5.71</td>
</tr>
<tr>
<td>Serum proteins, g/100 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>4.43</td>
<td>0.28</td>
<td>20</td>
<td>3.60</td>
<td>0.22</td>
</tr>
<tr>
<td>Globulin</td>
<td>2.61</td>
<td>0.39</td>
<td>20</td>
<td>2.76</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*SD = standard deviation, n = number of patients, PBI = protein-bound iodine, DF = dialyzable fraction, T4 = free thyroxine in dialysis system, TBPA = thyroxine-binding prealbumin, TBA = thyroxine-binding albumin, and TBG = thyroxine-binding globulin. For explanation of DF, T4, Rapp, and Rapp/T4 see Methods. p = probability that the value in the test group is identical to the corresponding value in the normal group. NS = not significant (p > .05).
with digitoxin, that than with TBG of capacity as Ag thyroxine concentration solution. albumin of diluent and tive, roxine tained with stock 100 ml, and

$1772$

No. Patient Age Sex PBI DF $T_d$ $R_{oa}/T_d$ $R_{oa}$ TBG TBPA Diagnosis

<table>
<thead>
<tr>
<th>No.</th>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>PBI</th>
<th>DF</th>
<th>$T_d$</th>
<th>$R_{oa}/T_d$</th>
<th>$R_{oa}$</th>
<th>TBG</th>
<th>TBPA</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I.K.</td>
<td>59</td>
<td>M</td>
<td>6.4</td>
<td>.0622</td>
<td>5.22</td>
<td>18.2</td>
<td>17.8</td>
<td>25.9</td>
<td>115</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>2</td>
<td>E.W.</td>
<td>57</td>
<td>M</td>
<td>4.1</td>
<td>.0588</td>
<td>3.16</td>
<td>14.2</td>
<td>11.1</td>
<td>21.2</td>
<td>102</td>
<td>Hodgkin’s disease</td>
</tr>
<tr>
<td>3</td>
<td>L.W.</td>
<td>18</td>
<td>M</td>
<td>5.7</td>
<td>.0526</td>
<td>3.94</td>
<td>13.5</td>
<td>11.8</td>
<td>27.4</td>
<td>192</td>
<td>Collagen disease</td>
</tr>
<tr>
<td>4</td>
<td>B.W.</td>
<td>57</td>
<td>M</td>
<td>4.6</td>
<td>.0709</td>
<td>4.27</td>
<td>26.1</td>
<td>18.4</td>
<td>25.4</td>
<td>64.6</td>
<td>Rectal abscess</td>
</tr>
<tr>
<td>5</td>
<td>J.T.</td>
<td>60</td>
<td>M</td>
<td>6.5</td>
<td>.0709</td>
<td>6.04</td>
<td>27.2</td>
<td>27.0</td>
<td>32.0</td>
<td>56.7</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>6</td>
<td>E.L.</td>
<td>76</td>
<td>M</td>
<td>4.2</td>
<td>.0517</td>
<td>2.85</td>
<td>21.0</td>
<td>13.5</td>
<td>25.7</td>
<td>98.9</td>
<td>Cancer of esophagus</td>
</tr>
<tr>
<td>7</td>
<td>R.S.</td>
<td>62</td>
<td>F</td>
<td>5.6</td>
<td>.0463</td>
<td>3.40</td>
<td>17.6</td>
<td>14.3</td>
<td>30.0</td>
<td>100</td>
<td>Typhoid fever</td>
</tr>
<tr>
<td>8</td>
<td>M.G.</td>
<td>64</td>
<td>M</td>
<td>4.6</td>
<td>.0506</td>
<td>3.05</td>
<td>18.0</td>
<td>12.7</td>
<td>22.8</td>
<td>169</td>
<td>Obstructed duodenal ulcer</td>
</tr>
<tr>
<td>9</td>
<td>J.F.</td>
<td>67</td>
<td>M</td>
<td>5.4</td>
<td>.0398</td>
<td>2.82</td>
<td>12.8</td>
<td>10.6</td>
<td>23.9</td>
<td>263</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>10</td>
<td>M.V.</td>
<td>75</td>
<td>F</td>
<td>4.4</td>
<td>.0649</td>
<td>3.75</td>
<td>18.4</td>
<td>12.4</td>
<td>23.0</td>
<td>70</td>
<td>Myeloproliferative disease</td>
</tr>
<tr>
<td>11</td>
<td>P.S.</td>
<td>48</td>
<td>F</td>
<td>5.2</td>
<td>.0520</td>
<td>3.55</td>
<td>15.5</td>
<td>12.3</td>
<td>31.3</td>
<td>73.5</td>
<td>Infectious mononucleosis</td>
</tr>
<tr>
<td>12</td>
<td>B.S.</td>
<td>52</td>
<td>F</td>
<td>4.8</td>
<td>.0630</td>
<td>3.96</td>
<td>23.7</td>
<td>17.4</td>
<td>26.7</td>
<td>67.2</td>
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</tr>
<tr>
<td>13</td>
<td>J.T.</td>
<td>76</td>
<td>M</td>
<td>5.1</td>
<td>.0552</td>
<td>3.69</td>
<td>17.6</td>
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<td>25.4</td>
<td>155</td>
<td>Common bile obstruction</td>
</tr>
<tr>
<td>14</td>
<td>E.S.</td>
<td>63</td>
<td>M</td>
<td>4.1</td>
<td>.0687</td>
<td>3.69</td>
<td>25.5</td>
<td>16.0</td>
<td>14.4</td>
<td>123</td>
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<tr>
<td>15</td>
<td>H.S.</td>
<td>69</td>
<td>M</td>
<td>5.4</td>
<td>.0803</td>
<td>5.69</td>
<td>24.0</td>
<td>19.8</td>
<td>23.0</td>
<td>128</td>
<td>Amyloidosis</td>
</tr>
<tr>
<td>16</td>
<td>H.N.</td>
<td>78</td>
<td>M</td>
<td>2.5</td>
<td>.0933</td>
<td>3.06</td>
<td>33.2</td>
<td>12.7</td>
<td>18.6</td>
<td>60.2</td>
<td>Cancer of lung, duodenal ulcer</td>
</tr>
<tr>
<td>17</td>
<td>L.K.</td>
<td>76</td>
<td>M</td>
<td>4.7</td>
<td>.0683</td>
<td>4.21</td>
<td>28.1</td>
<td>20.2</td>
<td>26.2</td>
<td>21.8</td>
<td>Cancer of stomach with metastases</td>
</tr>
<tr>
<td>18</td>
<td>D.M.</td>
<td>70</td>
<td>M</td>
<td>4.5</td>
<td>.0972</td>
<td>5.73</td>
<td>59.1</td>
<td>39.1</td>
<td>24.7</td>
<td>20.5</td>
<td>Cancer of lung</td>
</tr>
<tr>
<td>19</td>
<td>C.A.</td>
<td>72</td>
<td>M</td>
<td>5.8</td>
<td>.0525</td>
<td>4.00</td>
<td>22.2</td>
<td>19.7</td>
<td>35.0</td>
<td>40.5</td>
<td>Cancer of lung with metastases</td>
</tr>
</tbody>
</table>

*No patient received any medications at the time of this study except for Patients 15 and 17, who were treated with digitoxin, and Patient 10, who was taking erythromycin and tetracycline.*

TABLE II

Results of binding studies in patients with nonthyroidal illness

$R_{fa} = \frac{T_P}{k_i(M_i - T_P)}$  \hspace{1cm} \text{Equation [1]}

where $T$ is the concentration of free (nonprotein-bound) thyroxine; $T_P$, the concentration of the complex formed by thyroxine and the binding site $P_i$; $M_i$, the total number of binding sites $P_i$, both free and occupied; and $k_i$, the apparent association constant governing this relationship. A ratio $R_{fa}$ may be defined as $T_P/(M_i - T_P)$ and Equation 1 rewritten as

$R_{fa} = \frac{T}{k_i}$ \hspace{1cm} \text{Equation [2]}

If $k_i$ is unchanged in a variety of clinical situations, then $R_{fa}$ may be considered proportional to the level of free thyroxine. Evidence justifying this assumption will be presented. Another useful ratio is $R_{fa}/T_d$. If $T_d$ de-
Terminated by dialysis is considered to be directly proportional to $T_k$, then

$$R_k = K \times DF \times T_k, \quad \text{Equation [3]}$$

where $K$ is a constant and $T_k$ the concentration of total thyroxine. Dividing both sides of Equation 3 by $T_k$ gives

$$R_k/T_k = K \times DF. \quad \text{Equation [4]}$$

Thus, $R_k/T_k$ may be considered analogous to $DF$ in the dialysis system.

The expressions $R_k$ and $R_k/T_k$ can be evaluated for TBG and TBPA, and the results compared with the analogous functions in the dialytic system. $TP_k$ is determined by multiplying the fraction of tracer $T_k$ bound to a particular binding site, $F_k$, by the concentration of endogenous thyroxine, $T_k$ as determined from the PBI. $M_k$ is the maximal binding capacity of the binding species under consideration and is determined as described above.

In the following studies, electrophoretic ratios based on prealbumin, $R_{PA}$ and $R_{PA}/T_k$, were preferred to those based on TBG for the following reasons. 1) Determinations of TBPA were thought to be technically more accurate than those of maximal binding capacity of TBG for two reasons. For one, since TBPA is the most rapidly moving electrophoretic component in our system, we were not faced with the problem of small quantities of trailing albumin contaminating TBG. Also, since maximal binding capacity of TBPA (normal range, 189 to 308 $\mu g$ per 100 ml) is approximately 10 times that of TBG, its determination for TBPA depends only minimally on an accurate determination of serum PBI. 2) Since the function $DF$ is based entirely on measurement of radioactivity and does not involve determination of serum PBI, it is desirable that the analogous electrophoretic expression $R_k/T_k$ also be based primarily on radioactive data. Since maximal binding capacity of TBPA ($M_{PA}$) greatly exceeds $TP_{PA}$,

$$R_{PA} = \frac{TP_{PA}/T_k}{M_{PA} - TP_{PA}} = \frac{(F_{PA} \times T_k)/T_k}{M_{PA} - TP_{PA}}$$

$$= \frac{F_{PA}}{M_{PA} - TP_{PA}} = \frac{F_{PA}}{M_{PA}}, \quad [5]$$

where $F_{PA}$ is the fraction of tracer $T_k$ bound by TBPA. It is thus apparent that the function $R_{PA}/T_k$ is largely independent of measurements of the concentration of nonradioactive thyroxine. Because of the more limited binding capacity of TBG, $R_{PB}/T_k$ cannot be approximated without introducing a separate determination of the nonradioactive thyroxine concentration (serum PBI). Changes in $R_{PA}$ and $R_{PA}/T_k$, are not contingent on changes of TBPA alone, and will respond appropriately to changes in the binding capacity of TBG.

Other procedures and patients. Concentrations of albumin and globulin in serum were determined by the precipitation techniques of Saiter and Zymaris (15), and the statistical significance of difference of means was evaluated by the $t$ test (16). Normal subjects were selected largely from laboratory personnel and house staff. Sera were obtained from women in their last trimester of uncomplicated pregnancy. The hypo- and hyperthyroidal patients chosen manifested the classical clinical and laboratory features of these diseases. With the exceptions noted in Table II, no patients with nonthyroidal disease were receiving drugs of any kind, and none received any drug known to interfere with thyroxine-binding. In general, the patients with nonthyroidal illness had marked systemic manifestations and were sufficiently ill to require hospitalization. Diagnoses of individual patients are indicated in Table II.

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FIG. 1. SERUM PROTEIN-BOUND IODINE IN VARIOUS CLINICAL STATES. Solid lines indicate mean values and broken lines indicate normal limits (mean ± 2 SD of normal group), as also in Figures 2, 3, 5, 6, 8, and 9.

4 We wish to acknowledge the co-operation of Dr. Norman Herzig, of the Department of Gynecology and Obstetrics of the Montefiore Hospital Medical Group, for allowing us to study the pregnant women under his care.
RESULTS

Table I gives a summary and statistical analysis of results. Binding studies on sera of patients with nonthyroidal illness are separately listed in Table II.

Dialysis studies (Figures 1–3)

In the group of normal subjects, the mean $T_d (\times 10^{-11} \text{ M})$ was $3.02 \pm 0.35$ (SD). No statistical difference was found between normal men and women, and there was no significant correlation between individual values and age.

In the group of hyperthyroidal patients, PBI, DF, and $T_d$ were markedly elevated. The greatest separation from the normal group was effected by use of $T_d$ as the discriminant. Among hypothyroidal subjects, PBI, DF, and $T_d$ were all significantly lower than in normal subjects, although for DF the depression below normal was min-

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Fig. 2. Dialyzable fraction in various clinical states.

Fig. 3. Free thyroxine concentration ($T_d$) in various clinical states.
thyroidal patients (0.0661). Since, however, there was a marked discrepancy between the levels of serum PBI, the mean Td in the group of patients with nonthyroidal illness (4.00 x 10^{-11} M) was considerably less than that of the hyperthyroidal patients (12.07 x 10^{-11} M).

Figure 4 summarizes the distinguishing features of the groups studied. The group of patients with hyperthyroidism are segregated by their high PBI and high DF, the group of hypothyroid patients, principally by their low PBI and low to normal DF, the pregnant women, by their high PBI and low DF, and the patients with nonthyroidal illness, both by their low to normal PBI and their elevated DF. Td of a given point can be judged by its distance from the isobar Td = 3.02 x 10^{-11} M, the mean Td of the normal group. If the metabolic activity of thyroxine is determined by the concentration of free thyroxine, then all subjects exhibiting the same thyroidal status should have values of Td falling along a single isobar.

**Electrophoretic studies**

Distribution of tracer T4-I^{131} and determination of maximal binding capacity of TBG and TBPA (Figures 5-7; Tables I and II). An increased percentage of radioactivity is associated with TBG in pregnancy, hypothyroidism, and nonthyroidal disease when low concentrations of T4-I^{131} are
added to serum (3 μg per 100 ml). Conversely, there is a fall in the amount of $T_4^{131}$ associated with TBPA in these clinical states.

Maximal binding capacity of TBG is known to be elevated in pregnancy (2, 17). A lesser but nonetheless significant elevation was encountered in hypothyroidism, in agreement with earlier findings (2). In confirmation of previous results (1, 18), no significant changes in maximal binding capacity of TBG occurred in hyperthyroidism, or in nonthyroidal illness. Maximal binding capacity of TBPA in normal subjects was approximately twice that determined by Ingbar and Freinkel (7), but its depression in patients with nonthyroidal illness and hyperthyroidism agrees with their findings. Modest decreases were also noted in pregnancy and hyperthyroidism.

An over-all correlation ($r = 0.84$) between the level of serum albumin as determined by salting

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3 The reason for this discrepancy does not lie in our use of glycine-acetate buffer, since identical values for maximal binding capacity and TBPA were found when Tris-maleate buffer, as employed by Ingbar, was substituted in our system. Furthermore, no changes were noted when the stock solutions of thyroxine were dissolved in a serum albumin solution according to Ingbar's instructions (19).
out techniques and maximal binding capacity of TBPA was noted (Figure 7). Depression of serum albumin was most marked in the patients with nonthyroidal illnesses.

Electrophoretic ratios \( R_{pa}/T_4 \) and \( R_{ps} \) (Figures 8 and 9; Tables I and II). By analogy with the directional shifts of DF, \( R_{pa}/T_4 \) was elevated in hyperthyroidism and nonthyroidal illness and reduced in pregnancy and hypothyroidism. In the last, the reduction was marginal, although significant at the 5% probability level, and individual values overlapped considerably with the normal range. By definition, a similar correspondence must exist between \( R_{ps} \) and \( T_4 \). Thus, \( R_{ps} \) was normal in pregnancy, elevated in hyperthyroidism, and depressed in hypothyroidism. The elevation of \( R_{pa} \) in the group of patients with nonthyroidal illness was statistically highly significant.

Correlation between dialytic and electrophoretic data. Figure 10 illustrates the generally linear relationship between \( R_{ps}/T_4 \) and DF \((r = 0.88)\). This proportionality thus satisfies the expectation expressed in Equation 4. Furthermore, it implies that the association constant of TBPA in the various clinical states studied does not vary widely from the normal value, and thus justifies the use of the ratio \( R_{ps} \) as a measure of free thyroxine concentration.

Studies using vertical starch-gel electrophoresis. Previous studies (12, 20) have indicated that TBPA in paper electrophoresis corresponds qualitatively to prealbumin I as demonstrated by starch-gel electrophoresis. A limited number of experiments have also demonstrated that the percentage of radioactivity associated with prealbumin 1 is diminished in patients with nonthyroidal illness. The percentage of \( T_4-I^{131} \) carried by prealbumin 1 in two normal subjects was 15.9 and 19.7, whereas in four patients with nonthyroidal illness and a low maximal binding capacity of TBPA, it was 5, 6, 2.2, 1.2, and 1.7%. At the same time, the protein stain of prealbumin 1 was strikingly reduced and frequently invisible in 5 “sick” patients examined who had a low maximal binding capacity of TBPA (Figure 11). One patient (P.S.) showed an increased intensity of the pre-
Estimating Equation: 
\[ \frac{R_{pa}}{T_4} = -6.07 + 46.5(DF) \]
Coefficient of Correlation 0.88

Fig. 10. Correlation between dialyzable fraction and \( \frac{R_{pa}}{T_4} \). 95% confidence limits of estimating equation are indicated by dashed lines.

Fig. 11. Starch-gel protein patterns in three subjects. Nomenclature is after Smithies (13). Note absence of PA 1 in D.M. and diminution of stain in P.S. with subsequent restoration of TBPA-binding capacity and protein stain to near normal limits.
albumin 1 stain together with a return of maximal binding capacity of TBPA toward normal levels 4 weeks after recovery from infectious mononucleosis. These results indicate that the reduction of thyroxine-binding by prealbumin is due to a lower concentration of circulating prealbumin rather than to reduced binding by normal concentrations of this protein.

**DISCUSSION**

Both methods employed in this study provide only a relative estimate of the serum concentration of free thyroxine (T₄ and Rₚₐ) and the fraction of thyroxine bound to serum proteins (DF and Rₚₐ/T₄). Even if the equilibrium concentration of free thyroxine in separated serum were precisely determined, it is not immediately apparent that such a value would be identical with that of in vivo plasma. First, thyroxine in separated serum has been removed from the competing cellular and extravascular binding sites, and second, a state of stable equilibrium cannot axiomatically be assumed to exist in vivo (1). Nevertheless, the concept of free thyroxine as a determinant of hormonal activity remains operationally valuable, as previously cited studies show.

Evidence for the validity of our electrophoretic and dialytic methods lies in the linear correlation of the results they give (DF against Rₚₐ/T₄, and hence T₄ against Rₚₐ, Figure 10). Furthermore, these results are consonant with the currently accepted concepts that the levels of free thyroxine are elevated in hyperthyroidism, depressed in hypothyroidism, and normal in pregnancy. In hyperthyroidism, the level of serum free thyroxine is elevated because of both an increase in total circulating thyroxine and a net decrease in serum protein-binding, owing not only to the diminution of unoccupied binding sites on TBG, but also to the fall in maximal binding capacity of TBPA (7). In hypothyroidism, the level of free thyroxine is diminished primarily because of the fall in serum PBI. In pregnancy, the concentration of total thyroxine is elevated, but because of the primary increase in the maximal binding capacity of TBG, net serum protein-binding is increased and the resultant level of free thyroxine falls into the normal range.

The finding of increased levels of Rₚₐ/T₄ and DF in the sera of patients hospitalized for a variety of nonthyroidal illnesses suggested major changes in thyroxine-binding proteins and led us to study these changes more intensively. The diminished net binding of thyroxine appeared to be largely a consequence of the fall in maximal binding capacity of TBPA (7, 18), although the decreased albumin concentration could also have made a minor contribution to this effect. No over-all change in maximal binding capacity of TBG was noted in these patients.

The modest reduction of the mean serum PBI in the group with nonthyroidal illness was responsible for the less consistent alteration of the level of free thyroxine as gauged by Rₚₐ and T₄ than of Rₚₐ/T₄, DF, and maximal binding capacity of TBPA. Nevertheless, the increase of both Rₚₐ and T₄ was statistically highly significant, and 9 of 19 patients had values of Rₚₐ and T₄ exceeding the corresponding normal mean ± 2 SD. The highest individual values of T₄ and Rₚₐ in this group, however, only approached the lower range of values observed in hyperthyroidism.

A wide variety of nonthyroidal diseases is represented (Table II). In general, the most pronounced abnormalities in thyroxine-binding were found in patients with the most marked systemic manifestations, including fever, weight loss, and malnutrition. Alterations of thyroxine-binding were encountered both in patients with malignant disease and in those with self-limiting viral and bacterial processes. Previous studies of net thyroxine-binding by the serum proteins of such patients have been conflicting. Richards, Dowling, and Ingbar (21) indicated that the red-cell uptake of ¹³¹I-labeled hormones is increased in certain patients with nonthyroidal disease. Also, Hamolsky, Golodetz, and Freedberg (22) have reported an increased T₃-I¹³¹I red-cell uptake in patients with metastatic cancer. On the other hand, Sterling and Hegedus (3) observed no increase in dialysis of thyroxine in sera of twelve patients with nonthyroidal illness. Since these patients were for the most part ambulatory (3, 23), they may not have been so "sick" as ours. This difference could explain differences in our results.

Because insufficient prealbumin is present in serum to yield a protein stain on ordinary paper
electrophoretic strips, it is impossible to decide on the basis of this technique alone whether the diminished binding of thyroxine in illness is due to an inhibition of thyroxine-binding by TBPA, or to an actual decrease in the concentration of this protein. In starch-gel electrophoresis, a visible protein band, prealbumin I, corresponds to the position of the thyroxine-protein complex migrating most rapidly toward the anode. The parallel variation in the intensity of the stained protein band, the amount of radioactivity associated with it, and the maximal binding capacity of TBPA as determined by paper electrophoresis indicate that the reduction in prealbumin-binding is due to an actual fall in the level of protein and suggests that prealbumin I may be specifically concerned with the transport of thyroxine.

The significance of the correlation between concentrations of TBPA and albumin remains unclear. When crystallized human albumin was added to serum deficient both in albumin and TBPA, however, no change in the concentration of thyroxine associated with TBPA, or in the maximal binding capacity of TBPA was noted, despite correction of the albumin deficiency. The possibility remains that the synthesis of albumin and prealbumin is synthetically linked.

Variations in the concentration of both TBG and TBPA as measured electrophoretically at pH 8.6 are reflected in a corresponding variation in the dialysis of thyroxine at pH 7.4. The partition of thyroxine at a physiological pH would therefore appear to be in reasonable agreement with the distribution of thyroxine as determined by paper electrophoresis at pH 8.6. On the other hand, if filter-paper electrophoresis is carried out at pH 7.4, no thyroxine will be found in association with prealbumin (24, 25). Thus, one is led to the apparently paradoxical conclusion that the physiological distribution of thyroxine is more accurately mirrored by the results of paper electrophoresis at pH 8.6 than at pH 7.4. The following considerations, however, suggest that this conclusion is in fact acceptable. Ingbar (26) has proposed that the failure to demonstrate TBPA-binding at pH 7.4 is due to the relatively increased thyroxine-binding by paper at this pH. Furthermore, Hollander, Odak, Prout, and Asper (27) have shown that the fraction of T4-131 associated with TBPA in agar-gel electrophoresis is the same at pH 8.6 and 7.4. Our results thus add to the accumulating evidence that TBPA plays a significant physiological role in the peripheral transport of thyroxine (26, 28).

The biological significance of diminished thyroxine-binding by serum proteins in such a large variety of diseases is not at all clear. It is tempting to speculate on the possibility of a causal relationship between an elevation of free thyroxine and the catabolic process characteristic of these disease states. Nevertheless, not all patients with severe wasting disease had elevated Rpa and Td, principally because the binding alterations were offset by a fall in serum PBI. Low PBI concentrations in such clinical states have been noted previously (29). Evaluation of free thyroxine in these patients clearly depends on the accuracy with which serum PBI reflects the circulating concentration of thyroxine. Systematic difference in the iodinated constituents of PBI between the normal group and patients with nonspecific illness could obscure basic physiological relationships. Further attempts to clarify the clinical significance of these alterations in binding are currently in progress.

**SUMMARY**

The thyroxine-binding characteristics of serum proteins in a variety of clinical states were evaluated by both equilibrium dialysis and electrophoresis. Equilibrium dialysis of diluted serum at pH 7.4 determined the fraction (DF) of 131I-labeled thyroxine (T4-I131) not bound to protein in the dialysis system, as well as the concentration of free thyroxine in the system (T4). Paper electrophoresis with glycine-acetate at pH 8.6 determined the maximal binding capacity of thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA) and two ratios Rpa and Rpa/T4, theoretically proportional to Td and DF, respectively. Both functions Td and Rpa were considered to be relative indexes of the free thyroxine concentration in vivo, whereas DF and Rpa/T4 were considered to be an inverse measure of the net intensity of thyroxine-binding by the serum proteins. There was excellent linear correlation between the dialytic and electrophoretic functions.

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6 Pentex Corporation, Kankakee, Ill.
Levels of \( T_d \) and \( R_{pa} \) were elevated in hyperthyroidism, depressed in hypothyroidism, and normal in pregnancy, in agreement with previous findings. In a group of 19 patients with a variety of nonthyroidal illnesses, average \( DF, R_{pa}/T_4, T_d, \) and \( R_{pa} \) were significantly elevated above normal limits. These changes were ascribed to a marked fall in maximal binding capacity of TBPA. Starch-gel electrophoresis indicated that the intensity of the protein stain of prealbumin 1 and the fraction of \( T_d \)-\( ^{131}I \) associated with this protein paralleled the variation in the maximal binding capacity of TBPA as determined by paper electrophoresis. The fall in the maximal binding capacity of TBPA in patients with non-thyroidal disease thus appeared to be secondary to an actual decrease in the concentration of the binding protein, rather than to inhibition of thyroxine-binding by TBPA. The effect of the diminished maximal binding capacity of TBPA in enhancing the dialysis of thyroxine at \( pH \) 7.4 adds to the evidence that TBPA is physiologically important in the peripheral transport of thyroxine.

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REFERENCES

23. Sterling, K. Personal communication.