THE VALUE OF GLOBULIN-BOUND IODINE DETERMINATION IN THE DIFFERENTIAL DIAGNOSIS OF THYROID DISEASE

By

Dora Winikoff

The nature of the circulating thyroid hormone has become a subject of intensive study. It was established by Trevorow (1939), Taurog & Chaikoff (1948), Laidlaw (1949) and Rosenberg (1951) that it appears in the plasma mostly in the form of thyroxine, extractable by butanol without hydrolysis, and it is also generally agreed that it is in some way associated with plasma proteins.

Experiments with paper electrophoresis provided the basis for the concept of a specific »thyroxine-binding protein«, although the nature of this protein still remains in doubt.

At pH 8.6 in barbital buffer and also at pH 6 and 7.6, »thyroxine-binding protein« has an electrophoretic mobility intermediate between $a_1$ and $a_2$ globulins (Gordon et al., 1952, Deiss et al., 1952, Winzler & Notrica, 1952, Robbins & Rall, 1952, Horst & Rossler, 1953, Larson et al., 1954). Furthermore, this protein appears to be closely related to $a_2$-glycoproteins having a similar electrophoretic mobility, isoelectric point and sedimentation constant in the ultracentrifuge (Schmidt, 1953, Peterman et al., 1954, Robbins et al., 1955). Experiments with radioactive thyroxine and tri-iodothyronine, performed in vivo as well as in vitro, showed that the radioactivity in both cases was mostly confined to the $a_1$ – $a_2$-globulin fractions, but that thyroxine, having greater affinity, can displace tri-iodothyronine from its protein complex (Deiss et al., 1952, 1953, Gordon et al., 1952, Albright et al., 1955). However, the binding capacity of this specific »thyroxine-binding protein« is strictly limited and, once the saturation point is exceeded, radioactivity can be found in other plasma proteins with albumin as the major component (Albright et al., 1955, Robbins & Rall, 1955).

This distribution, however, is only characteristic for the electrophoretic tech-
nique. Many earlier workers using salting-out fractionation methods and, recently, Salter (1947), Taurog & Chaikoff (1948) and Klein (1955) found the major part of protein-bound iodine (PBI) in the albumin fraction of plasma or serum. Moreover, Klein concluded that the butanol-extractable iodine of the plasma albumin is easily diffusible and is raised in thyrotoxicosis, while the globulin-bound iodine (GBI) is almost constant, regardless of the functional state of the gland.

It was decided to re-examine the distribution of iodine among the plasma proteins in the hope that such studies might prove helpful in borderline cases or when the PBI values are in disagreement with obvious clinical symptoms. As the electrophoretic technique would not permit quantitative measurements on patients without administering large doses of $^{131}$I, a salting-out procedure had to be selected.

MATERIALS AND METHODS

The work presented in this paper has been carried out on the blood of normal control subjects and patients from public hospitals, suspected of having thyroid disorders. Their clinical conditions were established after a prolonged period of observation and by PBI estimations. In some cases, radioiodine (uptake and 48 hr. PBI$^{131}$) studies were performed concurrently with the fractionation experiments. Total PBI was estimated by using either of the two precipitants – (1) zinc sulphate-sodium hydroxide or (2) trichloracetic acid – and, at times, both of them concurrently. Results of fractionation were accepted if the combined albumin + globulin iodine value did not differ by more than 2 $\mu$g.$/\%$ from the total PBI value. As 1 $\mu$g.$/\%$ is usually regarded as the maximum difference allowed between duplicate estimations, it was considered that, by performing fractional analyses, requiring two separate lots of reagents, 2 $\mu$g.$/\%$ represented only the two-fold permissible error. In Table 1 comparison of PBI values obtained by using the two precipitants is presented.

As far as possible, the patients selected had no medication, which could alter their thyroid function, for a period considered safe as specified by Winihoff (1954) although, as previously, it was noticed that marked individual variations occurred in the after-effects of treatment. In some cases, this was reflected in a considerable difference between total PBI and the combined (albumin + globulin) iodine values. This was very pronounced following administration of Lugol’s solution.

Originally it was intended to employ more than one method of salt fractionation and also Cohn’s alcohol-electrolyte procedure. After preliminary trials, it proved impractical to fractionate plasma using sodium sulphite or sulphate, due to unusually high blanks. Cohn’s method, although having the advantage of
Table 1.
Comparison of PBI values using zinc sulphate and trichloracetic acid as precipitating agents.

<table>
<thead>
<tr>
<th></th>
<th>Normal controls</th>
<th>Euthyroid group</th>
<th>Hyperthyroid group</th>
<th>Hypothyroid group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>zinc-sulphate</td>
<td>trichloro-acetic acid</td>
<td>zinc-sulphate</td>
<td>trichloro-acetic acid</td>
</tr>
<tr>
<td>4.0</td>
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<td>4.1</td>
<td>5.5</td>
<td>12.7</td>
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<tr>
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<td>5.6</td>
<td>6.1</td>
<td>8.7</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Table 2.
Iodine distribution after Cohn's fractionation of normal plasma.

<table>
<thead>
<tr>
<th>Total PBI (µg./%)</th>
<th>µ-g. %</th>
<th>α-Globulin</th>
<th>µ-g. %</th>
<th>β-Globulin</th>
<th>µ-g. %</th>
<th>Albumin</th>
<th>µ-g. %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iodine</td>
<td>%</td>
<td>Iodine</td>
<td>%</td>
<td>Iodine</td>
<td>%</td>
<td>Iodine</td>
</tr>
<tr>
<td>3.9</td>
<td>1.95</td>
<td>30</td>
<td>1.10</td>
<td>17.0</td>
<td>3.45</td>
<td>55</td>
<td></td>
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<tr>
<td>5.2</td>
<td>1.98</td>
<td>32</td>
<td>1.13</td>
<td>18.6</td>
<td>2.78</td>
<td>46</td>
<td></td>
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<tr>
<td>7.2</td>
<td>3.56</td>
<td>47</td>
<td>0.79</td>
<td>9.9</td>
<td>3.27</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>1.54</td>
<td>32</td>
<td>0.99</td>
<td>22.0</td>
<td>2.20</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>36.0</td>
<td>17.0</td>
<td>47.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

separating the various globulin fractions, was far too complicated for a large series of tests. The few results obtained on normal plasma are presented in Table 2. It was also feared that the alcohol used might disrupt the thyroxine-protein bond and, in addition, the contamination caused by the use of various reagents might progressively increase in the final fractions, giving artifactual
values. The ammonium sulphate precipitation technique as worked out by Klein (1955) was finally chosen, with some modifications.

By precooling all reagents to 2°C and centrifuging the precipitate obtained by the addition of half-saturated ammonium sulphate to plasma in the cold room, it was possible to separate the globulin fraction, leaving the albumin in the supernatant. This eliminated the tedious process of filtering, and, by packing the precipitate tightly, diminished the admixture of albumin trapped in the precipitate. It also allowed for the assay of both albumin-bound and globulin-bound iodine (ABI and GBI) instead of calculating the latter as a difference between total PBI and ABI.

Reagents

The reagents used were of the highest purity and were recrystallized where necessary. For iodine assays these were as specified by Winikoff (1954).
The ammonium sulphate and propylene glycol were of A. R. quality.
The trichloracetic acid was B. P. quality (Kaylene Ltd., London).
The thyroxine (monosodium salt, B. D. H.) was recrystallized.

Albumin- and globulin-bound iodine (ABI and GBI) assays

Two ml. of oxalated plasma is pipetted into a 50 ml. pyrex round-bottom centrifuge tube of 50 ml. capacity with a lip. Four ml. of distilled water are added followed by 6 ml. of saturated ammonium sulphate solution, slowly with constant stirring. The stirring rod is carefully withdrawn and kept for the washing operation. The tube is left in the cold room for not less than 6–8 hours (usually overnight) and centrifuged in the cold room at 3500 r. p. m. (International S B-1) for 45 minutes. The supernatant is carefully poured off into a second centrifuge tube over the lip and, while held at an angle of 45°, the walls of the original tube are washed three times with 1 ml. of distilled water and drained as completely as possible, allowing the washing fluid to flow directly into the second tube. The volume at this stage is about 15 ml. Introducing a new rod, 1.5 ml. of 70% trichloracetic acid is carefully added to the albumin solution and the precipitated protein centrifuged at room temperature. Both globulin and albumin precipitates are then washed three times with 6 ml. of 5% trichloracetic acid in order to bring the reagent blanks to the same value and to remove the ammonium sulphate. Ten ml. of 70% sulphuric acid are added to both fractions and the tubes placed in a boiling water bath until the precipitates dissolve. Each solution is transferred into a 500 ml. digestion flask by the use of three 5 ml. portions of sulphuric acid, followed by 5 ml. of water. The rest of the assay has been previously described (Winikoff, 1954), Barker’s (1948) distillation method with minor modification being used. All assays are performed in triplicate. This is particularly important for GBI because of the small amounts of iodine present.

Total PBI assay (trichloracetic acid precipitation)

Two ml. of oxalated plasma and 4 ml. of water are pipetted into a centrifuge tube at room temperature, followed by 6 ml. of saturated ammonium sulphate and 1.5 ml. of trichloracetic acid (70%). After standing, the precipitate is centrifuged down and the supernatant discarded. After triple washing with 6 ml. of 5% trichloracetic acid, the precipitate is dissolved and treated as above.
"Thyroxine loading" experiments

Four mg. of l-thyroxine are dissolved in 100 ml. 0.1 N NaOH containing 20% propylene glycol v/v. One ml. of this solution, equivalent to 40 µg. of thyroxine is further diluted with the same solution to the required concentration.

To 19 ml. of plasma, 1 ml. of thyroxine solution is added and left at room temperature for an hour. To another 19 ml. of plasma, 1 ml. of 0.1 N NaOH-propylene glycol mixture is also added.

Two ml. portions, in triplicate, of both solutions are then fractionated as above. A further three 2 ml. portions are assayed for total PBI (using trichloracetic acid as the precipitating agent).

As the loading experiments involved a large number of estimations and extended over a period of time, three different normal control plasmas had to be used. Their PBI values varied from 4.7 to 6.0 µg.%o. The amounts of thyroxine added to the plasma ranged from 4.0 to 100 µg.%o.

Reagent blanks

In order to check the reagents used in the fractionation procedure, 0.1 gm. of freeze-dried haemoglobin was suspended in 6 ml. of water and 6 ml. of saturated ammonium sulphate followed by 1.5 ml. of 70% trichloracetic acid added with stirring. After centrifuging and triple washing with 6 ml. of 5% trichloracetic acid and, finally, 5 ml. of water, the assay was performed as for the total PBI (trichloracetic acid procedure).

A second 0.1 gm. of freeze-dried haemoglobin was introduced directly to a digestion flask and used for a blank estimation (with the reagents only).

The difference in "iodine" value between the ammonium sulphate - trichloracetic acid – haemoglobin and the reagent "blank" did not usually exceed 0.5 µg.%o.

Thyroxine control

To check the thyroxine recovery 2 ml. of the thyroxine solution in 0.005 N NaOH containing 1% propylene glycol was pipetted into a digestion flask containing 2 ml. of 5% haemoglobin solution as a protein carrier. The thyroxine-iodine value for this assay did not differ by more than 10% from the figure obtained by subtracting the total plasma PBI from the total plasma + thyroxine-bound iodine. In view of the large amount of thyroxine iodine added, which required considerable dilution in order to bring values within the usual colorimetric range, and the difficulty in avoiding losses during analysis, this recovery seems satisfactory.

It was realized that the ammonium sulphate procedure did not separate quantitatively the globulin fraction from the albumin; moreover, a small amount of albumin was left trapped in the globulin precipitate after separation. However, all attempts to resolve this admixture resulted in losses during the process of washing and had to be abandoned.

To clarify this point, the globulin precipitate was dissolved in a small volume of veronal buffer, pH 8.6, and dialysed with stirring against the same buffer for several hours. A sample was subjected to paper electrophoresis according to the method of Flynn & de Mayo (1951) and run for 18 hours. After drying and staining with bromphenol blue, the result proved that only a very small amount of albumin was trapped on the precipitate. Similarly, the albumin solution after dialysis against the same buffer gave only a faint α-globulin band. This could be interpreted that the fractionation procedure was satisfactory for the purpose of separating the iodine bound to albumin from the combined globulin-bound fractions. Excepting high PBI values or a considerable shift in albumin/globulin ratio, the error was probably not significant for different
groups of patients. It is intended to study this point further by investigating the distribution of iodine in renal and hepatic disorders.

RESULTS

From the preliminary results it became apparent that the distribution of iodine bound to plasma proteins followed the general pattern found by other investigators using salt fractionation, regardless of the particular technique used. The major part of the total PBI (in our case 71–75 %) was linked with the albumin fraction, the remainder being globulin-bound. The question arose whether the rise and fall of any of these values could be correlated with a particular thyroid function. According to Klein, only the ABI fluctuates in thyroid dysfunction, the GBI being relatively constant. Moreover, he claims that the increase in the total butanol-extractable iodine in thyrotoxicosis, which represents the increase in the circulating hormone, is only reflected in the albumin fraction. Furthermore, if the ABI exceeds 6 µg.%(-), this is an indication of thyroid hyperactivity resulting in hyperthyroidism. Our results, however, confirming the consistent rise in ABI above the normal values in hyperthyroidism, revealed in addition a small but significant difference in GBI values between groups of patients with various thyroid disorders. (See statistical analysis).

Total PBI estimations and fractionation studies were performed on the following three groups of patients.

The Euthyroid Group

This group was comprised of 6 healthy controls and 23 euthyroid subjects suffering from a variety of diseases. Two of them were post-thyroidectomy cases who remained euthyroid for long periods. The iodine values ranged from 2.9–6.6 µg.%(-) (mean 4.7 µg.%(-)) for ABI, and 0.5–1.7 µg.%(-) (mean 1.2 µg.%(-)) for the GBI fraction, the total PBI being 3.3–7.4 µg.%(-) (mean 5.3 µg.%(-)). (See Figs. 1 and 2).

The Hyperthyroid Group

This group consisted of two series of cases. These were:

(a) 37 patients who were seen for the first time or were previously treated with antithyroid drugs. However, all medication administered to them had been stopped for at least four weeks preceding the test. They were diagnosed toxic on clinical grounds and their PBI values were, with the exception of one, within the toxic range 8.0–21.6 µg.%(-) (mean 12.1 µg.%(-)). ABI was 6.1–14.8 µg.%(-) (mean 10.0 µg.%(-)). GBI was 2.0–5.0 µg.%(-) (mean 2.8 µg.%(-)). (See Figs. 1 and 2).

(b) 16 patients who had had unsuccessful treatment with one or more therapeutic doses of I(131). The degree of their persistent toxicity was being
investigated for the purpose of further treatment. Their PBI values ranged from 6.2–16.0 μg.% (mean 10.1 μg.%), ABI 4.6–11.2 μg.% (mean 8.3 μg.%), while GBI was 1.9–3.2 μg.% (mean 2.5 μg.%). These are not presented in Figs. 1 and 2 but are included in the statistical analysis.

The Hypothyroid Group

Due to a scarcity of cases of authentic untreated myxedema, this group consisted of 5 cases of secondary (pituitary) hypothyroidism and 9 cases of primary myxedema, developing spontaneously or resulting from surgery or therapeutic doses of radiiodine. In Sheehan’s disease (post partum pituitary necrosis) of short duration, PBI does not necessarily fall to a very low level,
but remains for a long time in the low normal range. This accounts for the relatively broad range of iodine values in our series, total PBI 0.3–3.3 µg./°/o (mean 2.6 µg./°/o), ABI 1.1–3.4 µg./°/o (mean 2.3 µg./°/o), and GBI 0.3–1.4 µg./°/o (mean 0.7 µg./°/o). (See Figs 1 and 2).

Two additional cases, one of primary and one of secondary myxedema, whose clinical condition required a prolonged period of observation, were also included in the statistical analysis. Their respective values were – total PBI 2.2 and 1.3 µg./°/o, ABI 2.7 and 1.5 µg./°/o, and GBI 0.6 and 0.8 µg./°/o.

A comparison of the ABI and GBI levels alongside the total PBI values for all these groups of patients is presented in Table 3. From Figs. 1 and 2 it is clear that for the GBI there exists a line of demarcation dividing the hyperthyroid from the euthyroid patients. If one accepts all the euthyroid patients as being below, and the hyperthyroid as being above 1.9 µg./°/o, there is no overlap in either of these groups. On the other hand, in the ABI group there is a definite overlap in the range of value between 6.0 and 7.0 µg./°/o. This would make the definition of thyroid activity based only on ABI more difficult, even in this series of specially selected patients where the diagnosis was never in doubt.

As the hypothyroid series could not be chosen better owing to lack of clinical material, the failure to establish an equally clear line of demarcation is not surprising. In spite of this, however, as 100 °/o of cases have a GBI below 1.5 µg./°/o and 71 °/o of them below 1.0 µg./°/o, it could be a help in diagnosis when the total PBI is in the borderline zone. The ABI values for the euthyroid and hypothyroid groups overlap between 2.5 and 4.0 µg./°/o. It is probable that in the cases where the iodine level is low and the experimental error is likely

<table>
<thead>
<tr>
<th>Type of patient</th>
<th>No. of cases</th>
<th>PBI (µg./°/o)</th>
<th>ABI (µg./°/o)</th>
<th>GBI (µg./°/o)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>29</td>
<td>3.3– 7.4</td>
<td>5.3</td>
<td>2.9– 6.6</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>37</td>
<td>6.4–21.6</td>
<td>12.1</td>
<td>6.1–14.8</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>6.2–16.0</td>
<td>10.1</td>
<td>4.6–11.2</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>14</td>
<td>0.5– 3.3</td>
<td>2.6</td>
<td>1.1– 3.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.3– 2.2</td>
<td>1.8</td>
<td>1.5– 2.7</td>
</tr>
</tbody>
</table>

* Group which had previously been treated with I¹³¹.
** Additional cases mentioned in text.
to be considerable. all these three values, PBI, ABI and GBI, in combination, could be of use.

By comparison, in the series presented by Klein (1955), the ABI range has an overlap between 5.0 and 6.0 µg.% for the euthyroid-hyperthyroid borderline zone, while the GBI values do not permit any line of demarcation. The reason for this can be explained on the grounds of deriving these latter values by calculation (PBI – ABI = GBI) instead of by direct assay.

It appears that the GBI fraction which constitutes only 21–25% of the total PBI is an equally reliable and, in many instances, a much better index of thyroid activity than either total PBI or ABI as it reflects the changes brought about by therapy or medication often ahead of the other two. A series of twelve cases is listed in Table 7 which had either a PBI in the »borderline« (overlap) range of values or where the PBI values were in disagreement with clinical symptoms. ABI did not contribute towards the correct diagnosis except in cases 4, 6 and 12. GBI, on the other hand, indicated in all instances the true state of thyroid function which was later confirmed by the patient’s condition.

It seems that, although the fractional analysis on the basis of a single test cannot supersede the total PBI in the majority of uncomplicated cases, it can be most helpful in many uncertain cases of borderline hyper- or hypothyroidism. As an illustration, one case with definite clinical symptoms of post-thyroidectomy myxedema showed a PBI of 3.3 µg.% (within the normal range), ABI 3.4 µg.%, but GBI only 0.3 µg.%, this last value being strong evidence in favour of hypothyroidism. After being placed on 1 grain of desiccated thyroid per day, the patient promptly experienced relief from all her symptoms (see Table 7, Case 5).

Although the butanol-extractable iodine estimation (Man et al., 1951) has proved an excellent aid in these doubtful cases and is widely used in some laboratories, the salt fractionation procedure is technically easier to perform in a busy institution and it could be attempted first.

Influence of Medication and I¹³¹ Therapy

During treatment of thyrotoxicosis with antithyroid drugs, GBI and ABI values drop alongside total PBI to euthyroid levels. In some cases, however, after the withdrawal of the drug, GBI precedes the other indices in returning to the previous toxic range of values, indicating an escape from control. This was particularly striking in a case where, after prolonged use of Neomercazol ‘cover’, following the administration of 6 mc. of I¹³¹ four weeks after cessation of the drug, the PBI value rose to 4.6 µg.% from a previous level of 3.4 µg.%. The ABI remained at the same level, 3.6 and 3.3 µg.%, while the GBI rose to 2.1 µg.% from a value of 0.7 µg.%, indicating clearly the return of toxicity. This was clinically confirmed (Table 7, Case 11).

A similar trend was also sometimes noticed after I¹³¹ administration for
therapeutic purposes and the impression remains that a series of GBI estima-
tions throughout a period of time might prove valuable in the management of
these patients. There is often a considerable discrepancy between laboratory
findings (e.g. I$^{131}$ uptake and similar indices) and the clinical picture. Conse-
sequently, when further treatment is contemplated, there is a particular need
for additional information.

The Administration of Contrast X-Ray Media

The administration of iodide- and iodine-containing compounds causes a
spurious elevation in PBI values (Danowski et al., 1950 a, b). This index, how-
ever, returns to normal in a matter of weeks after the withdrawal of medication.
The estimation of butanol-extractable iodine usually gives an indication of the
external source of such high values. X-ray contrast media, however, particularly
iodised oils used for cholecystography, will elevate both PBI and butanol-
extractable iodine for a considerable period of time.

Having at our disposal the blood of several healthy individuals, who received
Neohydriol intramuscularly (the account of this investigation will be published
elsewhere) as a source of continuous supply of iodine to the thyroid, we per-
formed fractional analyses on these patients. The results are presented in
Table 4. It can be observed that originally both the ABI and GBI were con-
sistently elevated. After a period of four months, however, while the PBI was
still in the toxic range 9.9–17.8 µg/%, the GBI was already returning to the
normal range 0.8–1.7 µg/%. If further confirmed, this observation would be of
great value in testing patients whose diagnosis, after the administration of such
contrast media, cannot be clearly established.

Iodine Distribution after the Administration of I$^{131}$

Iodine distribution after the administration of I$^{131}$ was investigated in a few
cases following tracer or therapeutic doses of the radioactive isotope. The
results are presented in Table 5 which also shows the distribution of I$^{127}$. In-
dividual albumin and globulin precipitates with known amount of added
radioactivity served as separate controls, to equalise the differences caused by
absorption of I$^{131}$ in these protein fractions. Similarly to the distribution of
stable iodine, most of the radioactivity appears in the albumin fraction. This
trend has not been observed in electrophoretic fractionation, which indicates
that the pattern of iodine distribution is dependent on the technique employed.

Effect of Thyrotrophic Hormone Administration

The increase in PBI values is used as an indicator in diagnosing secondary
hypothyroidism (Jefferies et al., 1953; Skanse, 1953; Schneeberg et al., 1954). The
method employed is the measurement of the PBI level (or I$^{131}$ uptake)
before and after the administration of thyrotrophic hormone by injection. This

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Table 4.
Protein bound iodine and globulin bound iodine values in normal subjects after neohydriol injected intramuscularly.

<table>
<thead>
<tr>
<th>Time interval after injection (weeks)</th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Subject 5</th>
<th>Subject 6</th>
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<tr>
<td></td>
<td>PBI</td>
<td>GBI</td>
<td>PBI</td>
<td>GBI</td>
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<td>GBI</td>
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<tr>
<td>12</td>
<td>17.6</td>
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<td>17</td>
<td>11.8</td>
<td>1.6</td>
<td>9.1</td>
<td>–</td>
<td>10.6</td>
<td>0.8</td>
</tr>
<tr>
<td>19</td>
<td>13.8</td>
<td>1.7</td>
<td>9.9</td>
<td>0.8</td>
<td>13.7</td>
<td>1.4</td>
</tr>
<tr>
<td>57</td>
<td>10.9</td>
<td>–</td>
<td>5.8</td>
<td>1.3</td>
<td>9.7</td>
<td>–</td>
</tr>
</tbody>
</table>

Subjects 1, 2 and 3 received 0.5 ml. neohydriol intramuscularly. Subjects 4, 5 and 6 received 1.5 ml. neohydriol intramuscularly.
Table 5.
Distribution of I\textsuperscript{127} and I\textsuperscript{131} in plasma fractions following the administration of (a) a tracer dose and (b) a therapeutic dose of I\textsuperscript{131} to seven hyperthyroid patients.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>I\textsuperscript{127} (µg.\textsuperscript{g}V\textsuperscript{o})</th>
<th>I\textsuperscript{131} (% dose/litre)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total PBI</td>
<td>ABI + GBI</td>
</tr>
<tr>
<td>1*</td>
<td>6.8</td>
<td>7.2</td>
</tr>
<tr>
<td>2*</td>
<td>3.4</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>7.7</td>
<td>8.1</td>
</tr>
<tr>
<td>4**</td>
<td>13.6</td>
<td>13.8</td>
</tr>
<tr>
<td>5</td>
<td>8.8</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>16.4</td>
<td>13.8</td>
</tr>
<tr>
<td>7</td>
<td>8.4</td>
<td>8.9</td>
</tr>
</tbody>
</table>

With Patients Nos. 1, 2 and 3, the test was carried out 48 hr. after the administration of a tracer dose of I\textsuperscript{131}.
With Patients Nos. 4, 5, 6 and 7, the test was carried out 3 weeks after administration of a therapeutic dose of I\textsuperscript{131}.
* On thiouracil.
** Ten days off neomercazole.
*** On neomercazole.

Table 6.
Increase in iodine values in albumin and globulin fractions following the administration of thyrotrophic hormone to five cases of Sheehan's disease.

<table>
<thead>
<tr>
<th>PBI (µg.\textsuperscript{g}V\textsuperscript{o})</th>
<th>ABI (µg.\textsuperscript{g}V\textsuperscript{o})</th>
<th>GBI (µg.\textsuperscript{g}V\textsuperscript{o})</th>
<th>Increase in ABI (µg.\textsuperscript{g}V\textsuperscript{o})</th>
<th>Increase in GBI (µg.\textsuperscript{g}V\textsuperscript{o})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before thyrotrophic hormone 48 hrs. after thyrotrophic hormone</td>
<td>Before thyrotrophic hormone 48 hrs. after thyrotrophic hormone</td>
<td>Before thyrotrophic hormone 48 hrs. after thyrotrophic hormone</td>
<td>Before thyrotrophic hormone 48 hrs. after thyrotrophic hormone</td>
<td>Before thyrotrophic hormone 48 hrs. after thyrotrophic hormone</td>
</tr>
<tr>
<td>0.5</td>
<td>3.7</td>
<td>-</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>2.8</td>
<td>5.2</td>
<td>3.9</td>
<td>5.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1.8</td>
<td>5.2</td>
<td>3.0</td>
<td>6.0</td>
<td>1.2</td>
</tr>
<tr>
<td>1.6</td>
<td>3.9</td>
<td>1.7</td>
<td>2.3</td>
<td>0.4</td>
</tr>
<tr>
<td>3.0</td>
<td>5.3</td>
<td>2.9</td>
<td>3.7</td>
<td>0.6</td>
</tr>
<tr>
<td>4.2*</td>
<td>3.5*</td>
<td>1.5*</td>
<td>0.6*</td>
<td>0.9*</td>
</tr>
</tbody>
</table>

* 72 hrs. after thyrotrophic hormone.
### Table 7.
Comparative diagnostic value of total protein bound iodine, albumin bound iodine and globulin bound iodine in doubtful cases.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Total PBI (µg.%)</th>
<th>ABI + GBI (µg.%)</th>
<th>ABI (µg.%)</th>
<th>GBI (µg.%)</th>
<th>Clinical Assessment</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>At time of test</td>
<td>Subsequently</td>
</tr>
<tr>
<td>1</td>
<td>7.7</td>
<td>7.9</td>
<td>6.2</td>
<td>1.7</td>
<td>toxic → euthyroid</td>
<td>6 weeks after I\textsuperscript{131} therapeutic dose</td>
</tr>
<tr>
<td>2</td>
<td>7.3</td>
<td>9.6</td>
<td>6.4</td>
<td>3.2</td>
<td>non-toxic → toxic</td>
<td>3 weeks after I\textsuperscript{131} therapeutic dose</td>
</tr>
<tr>
<td>3a</td>
<td>7.2</td>
<td>8.9</td>
<td>7.7</td>
<td>1.4</td>
<td>?non-toxic→ euthyroid</td>
<td>7 months after I\textsuperscript{131} therapy. At time of remission</td>
</tr>
<tr>
<td>3b</td>
<td>6.7</td>
<td>7.1</td>
<td>4.6</td>
<td>2.5</td>
<td>toxic</td>
<td>10 months after I\textsuperscript{131} therapeutic dose. Becoming toxic again</td>
</tr>
<tr>
<td>4</td>
<td>7.5</td>
<td>7.2</td>
<td>5.5</td>
<td>1.7</td>
<td>euthyroid</td>
<td>12 months after I\textsuperscript{131} therapeutic dose</td>
</tr>
<tr>
<td>5</td>
<td>3.3</td>
<td>3.7</td>
<td>3.4</td>
<td>0.3</td>
<td>?hypothyroid myxoedema</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3.3</td>
<td>2.5</td>
<td>2.0</td>
<td>0.5</td>
<td>?hypothyroid myxoedema</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7.7</td>
<td>9.5</td>
<td>6.5</td>
<td>3.0</td>
<td>non-toxic → toxic</td>
<td>5 months after I\textsuperscript{131} therapeutic dose. 1 week off thiouracil</td>
</tr>
<tr>
<td>8</td>
<td>7.2</td>
<td>8.5</td>
<td>5.8</td>
<td>2.7</td>
<td>toxic</td>
<td>On neomercazole</td>
</tr>
<tr>
<td>9</td>
<td>6.2</td>
<td>7.8</td>
<td>5.8</td>
<td>2.0</td>
<td>toxic</td>
<td>6 months after I\textsuperscript{131} therapeutic dose</td>
</tr>
<tr>
<td>10</td>
<td>6.4</td>
<td>8.4</td>
<td>6.1</td>
<td>2.5</td>
<td>(?)toxic</td>
<td>7 months after I\textsuperscript{131} therapeutic dose. 4 weeks off thiouracil</td>
</tr>
<tr>
<td>11</td>
<td>4.6</td>
<td>5.4</td>
<td>3.3</td>
<td>2.1</td>
<td>?toxic</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3.0</td>
<td>3.5</td>
<td>2.9</td>
<td>0.6</td>
<td>hypothyroid</td>
<td></td>
</tr>
</tbody>
</table>

Normal range for PBI in this laboratory -3.0–7.5 µg.%; 2.8–3.0 and 7.5–8.0 µg.% overlap zones for the hypothyroid-euthyroid and euthyroid-hyperthyroid patients, respectively.
Fig. 3.
Total PBI, GBI and ABI levels following the "loading" of normal plasma with thyroxine.

Fig. 4.
Increase in GBI and ABI values following the "loading" of normal plasma with thyroxine.
augmentation of organically bound iodine in the blood level is supposed to reflect the response of the thyroid gland to trophic stimulation by increased production of the hormone, as well as its release into the blood stream. In Table 6 are the results of the fractionation procedure performed on the blood of 5 cases of Sheehan's disease before and after the administration of 'Ambinon' over a period of 48 hr. The increase in total PBI values was within the range 2.3–3.4 μg%/o. This increase was distributed between both of the plasma fractions.

**Thyroxine Binding Capacity of Albumin and Globulin Fractions**

The binding capacity of both of these components of plasma was investigated by the addition of thyroxine to the plasma of normal controls as well as patients with thyroid disease. The amounts of thyroxine added ranged from 4.0 μg%/o

![Graph](image)

**Fig. 5.** Increase in the GBI fraction in the plasma of euthyroid and thyrotoxic patients (following «loading» with 40 μg%/o of thyroxine) as compared with the original PBI and GBI levels.

Patient (1) Normal control.

- (2) Euthyroid.
- (3) Euthyroid (three days off mercurial diuretics).
- (4) Thyrotoxic maintained euthyroid with methyl thiouracil (three weeks off medication).
- (5) Thyrotoxic.
- (6) Thyrotoxic (three days off Neomercazol).
- (7) Thyrotoxic (three weeks off methyl thiouracil).
- (8) Thyrotoxic (five months after I131 therapy dose, ten weeks off Neomercazol).
Increase in the GBI fraction in the plasma of euthyroid, hypothyroid and thyrotoxic patients (following "loading" with 80 µg.% of thyroxine) as compared with the original PBI and GBI levels.

Patient (1) Normal control.
(2) Hypothyroid (two weeks off desiccated thyroid).
(3) Hypothyroid (on desiccated thyroid).
(4) Thyrotoxic.
(5) Thyrotoxic.

To 100 µg.% Results are presented in Table 7 and Figs. 3 and 4. By performing parallel analyses of the total PBI, ABI and GBI on the original plasma and the plasma containing the added thyroxine, it was proved that the ABI rises steeply, almost parallel to the total PBI levels, while the binding capacity of the globulin fractions, being relatively limited, the magnitude of increase is gradually diminished and follows a flat course. With the amounts of added thyroxine rising from 4.0 to 20, 40 and 100 µg.% respectively, the content of GBI falls from 28 % to 20 %, 13 % and 12 % of the total PBI. Nevertheless, the capacity of binding thyroxine is definitely demonstrated even at the highest level (corresponding to 75 µg.% of total iodine), as at this point the original GBI has been increased five-fold from its normal level.

On the basis of these experiments it is believed that, since the PBI level in thyrotoxicosis seldom exceeds 50 µg.% (unless the patient is receiving Lugol's solution), the small but significant increase in GBI is due to the presence of true hormonal iodine. It is intended to confirm this point by employing butanol extractions.

The next series of experiments (Fig. 5) with thyroxine (at two different
levels) added to the plasma of patients, in the absence or in various stages of treatment, plainly demonstrates, however, that no simple correlation exists between the clinical state, total PBI and the binding capacity of the globulin fraction for additional thyroxine.

DISCUSSION

During the progress of the present investigation, it became apparent that the salted-out combined globulin fraction contained regularly a small proportion of the total PBI. The question arose as to whether this fraction could have any points of similarity with the thyroxine-binding protein identified by electrophoresis and, secondly, whether the purely empirical but highly significant difference between the GBI level of the euthyroid and hyperthyroid individuals could be accepted on theoretical grounds as an index of thyroid activity.

The existence of a well-defined specific thyroxine-binding protein has been confirmed by a series of investigators. Its binding capacity for thyroxine, however, seems to be limited and varies inversely with thyroid function (Horst, 1954; Albright et al., 1955). The saturation level has been defined by Albright et al. (1955) at approximately 0.4 mg. thyroxine per ml. in athyreotic individuals, while Robbins & Rall (1955) observed, instead of a plateau, a continuous increase of thyroxine present in the α-globulin fraction throughout the whole range employed, and they attributed this to the binding of the hormone to the secondary sites on the thyroxine-binding protein. Maurer & Müller (1953) and others, however, made the important observation that the accumulation of radioactivity in the respective proteins does not remain constant and a definite shift in peaks occurs with time and dose. In view of all this, Horst (1954) and Freinkel et al. (1955) came to the conclusion that, in serum, free thyroxine remains in ready exchange and reversible equilibrium with thyroxine-binding protein and secondary carriers, principally albumin. Displaced thyroxine can be reversibly recovered from albumin onto thyroxine-binding protein by decreasing the concentration of thyroxine or increasing the availability of the binding protein.

This concept of a dynamic equilibrium could explain the basic difference between the distribution of iodine following electrophoresis and salting-out methods. The various factors of ionic strengths, differences in pH or even the presence of the electric field could influence the nature of the bonds and linkages formed between the proteins and the functional groups on the thyroxine molecule. In addition, the 'globulin' and 'albumin' fractions separated by various techniques will contain a different proportion of these proteins with a wide range of molecular weights, resulting in different levels of iodine linked to them.
During salting-out experiments, it could be argued, the small quantity of GBI would represent the limit of its binding capacity under the existing conditions and the large fraction of ABI an overflow. During the »loading« experiments at every increased level of thyroxine a new equilibrium would come into existence, which would allow an additional amount of thyroxine to be incorporated into the GBI fraction. In thyrotoxicosis, where the elevated PBI represents, at least in part, the increase in the amount of circulating thyroid hormone, the small but significant rise in GBI could be utilized. Therefore, as an index of thyroid activity.

The findings that this fraction has been definitely increased after thyrotrophic hormone stimulation in cases of pituitary hypothyroidism (where the thyroid gland is still capable of response) and that, according to Klein, GBI is fully butanol-extractable, are further confirmatory points.

The actual magnitude of this increase does not, however, seem to be a simple function of the total amount of hormone in the blood at a given moment and the level of circulating proteins. The »loading« experiments performed on several patients subjected to various forms of therapy give the impression that there are unknown factors which determine the nature and extent of the thyroxine binding above physiological levels. The existence of the state of equilibrium when an increase in GBI fraction takes place in the presence of a normal or borderline PBI cannot be easily explained; but this question also arises in cases of normal PBI occurring in thyrotoxicosis as observed by many workers. Nevertheless, as the increased GBI often indicates an altered thyroid function, it can be utilized in diagnostic work.

Although the necessity of carrying out multiple tests and the strict attention to detail make the procedure of fractional analysis more tedious than ordinary PBI, the value of more precise information during the management of difficult cases should compensate for the additional work involved.

STATISTICAL ANALYSIS

Statistical analysis was performed on the GBI values from 29 euthyroid, 16 hypothyroid and 53 hyperthyroid patients mentioned in the text (see Table 3).

The direct GBI values were not suitable for statistical analysis but it was found that the log10 of the data was sufficiently close to normal distribution to justify the application of the ordinary standard statistical tests of significance.

The following means (on log10 GBI) were obtained:
- For the hyperthyroid group 0.423 on 53 observations
- For the euthyroid group 0.066 on 29 observations
- For the hypothyroid group 1.832 on 16 observations

and the pooled standard deviation of a single observation was 0.180.
An analysis of variances showed that differences between the treatments as a whole were highly significant and, in fact, that the GBI values for the hyperthyroid group exceeded the euthyroid \( t = 11.8 \) on 95 degrees of freedom) and that the euthyroid group exceeded the hypothyroid \( t = 5.77 \) on 95 degrees of freedom).

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I wish to express my thanks to the doctors and staff of the Royal Melbourne and Alfred Hospitals and Miss J. Milne of the Peter MacCallum Clinic for supplying the clinical material. I am indebted to Miss E. V. Sherriff of the Commonwealth X-Ray and Radium Laboratory for performing the PBI\( ^{131} \) determinations, to Dr. J. Bornstein of the Biochemistry Department, University of Melbourne, for carrying out the Cohn fractionations and to Miss M. J. Hughes for technical assistance. Mr. R. T. Leslie and the Department of Statistics, University of Melbourne, kindly performed the statistical analysis.

I also wish to thank Professor V. M. Trikojus for his criticism and advice and interest in this work.

This investigation was carried out during the tenure of a grant from the National Health and Medical Research Council, Commonwealth of Australia.

SUMMARY

(1) Fractional iodine assays on the plasma of 98 euthyroid, hyperthyroid and hypothyroid patients, following fractionation with ammonium sulphate, have been carried out. Albumin-bound iodine (ABI) and globulin-bound iodine (GBI) levels have been compared with the total protein-bound iodine in order to evaluate them as possible indices of thyroid function.

(2) GBI has proved to be the best single index distinguishing between the hyperthyroid and euthyroid groups, while total PBI gave the best indication of hypothyroidism. ABI values did not contribute to a better separation in any of these groups.

(3) After the administration of therapeutic doses of radioiodine or after treatment with antithyroid drugs, ABI and GBI follow the downward trend of total PBI. In many instances, however, GBI values indicated the true functional activity of the gland ahead of the other two indices.

(4) Increase in PBI following the administration of thyrotrophic hormone to five cases of Sheehan's disease was reflected in both protein fractions.

(5) The distribution of radioactivity in albumin and globulin fractions after the administration of tracer or therapeutic doses of radioiodine was similar to that of the stable isotope.

(6) »Loading« experiments with thyroxine added to normal plasma in vitro covering a range of 4 \( \mu \)g. – 100 \( \mu \)g. per 100 ml. revealed a definite in-
crease in the GBI and ABI fractions throughout the whole range. The GBI level, however, fell gradually from 28% to 12% of the total plasma iodine, the remainder being albumin-bound.

(7) The difference in iodine distribution between plasma fractions separated by paper electrophoresis as compared with salting-out methods is discussed and a possible explanation suggested.

REFERENCES