OBSERVATIONS OF THE EFFECT OF ORGANIC DYES AND DIPHENYLHYDANTOIN ON THE IN VITRO BINDING OF RADIOACTIVE $^{131}$I LABELED L-THYROXINE, L-TRIIODOTHYRONINE AND TETRAIODOTHYROACETIC ACID BY HUMAN ERYTHROCYTES AND SERUM PROTEINS

By

M. J. Olichney, K. R. Crispell and J. D. Harrah

ABSTRACT

The human erythrocyte is used as a model to study the in vitro binding of thyroid hormones to tissue proteins. Studies on the in vitro binding of $^{131}$I labeled l-thyroxine, l-triiodothyronine and tetraiodothyroacetic acid to human erythrocytes in buffered saline are reported. The effect of human serum, human serum albumin, the organic dyes, Evans Blue and Trypan Blue and diphenylhydantoin sodium on the binding of these labeled hormones to human erythrocytes has been studied. Evans Blue dye will block the in vitro uptake by the erythrocyte of l-thyroxine and l-triiodothyronine but not tetraiodothyroacetic acid. This suggests that the binding of the latter to the erythrocyte is qualitatively different than that of l-thyroxine or l-triiodothyronine. Trypan Blue decreased the serum binding of l-thyroxine and tetraiodothyroacetic acid while 5,5-diphenylhydantoin reduced the serum binding of l-thyroxine and l-triiodothyronine. These results are in accord with the binding properties of human serum proteins for thyroid hormones as demonstrated by other techniques.

The binding of thyroid hormones by serum proteins has been evaluated by the use of compounds which displace thyroxine and its analogues from their various binding sites (Crispell et al. 1957; Robbin & Rall 1955; Larson & Albright 1955; Oppenheimer & Taubernetti 1962; Wolff et al. 1961; Ingbar

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1960). Previous work from this laboratory using an in vitro technique has shown that the organic dye, Evans Blue, would compete with l-thyroxine for binding sites on the human red cell. Likewise, Trypan Blue was shown to compete with l-thyroxine for binding sites on human plasma (Crispell et al. 1957). Yamata (1960) has recently demonstrated that Trypan Blue has an in vivo effect on thyroid function similar to thyroxine, i.e., it will inhibit the secretion of thyrotrophin by the rat adenohypophysis. D'Addabbo et al. (1961) have also shown that certain organic dyes, including Trypan Blue will reduce the radioiodine uptake of the rat thyroid.

This paper extends previous studies to include the effect of these organic dyes and 5,5-diphenylhydantoin sodium (DPH) on the red blood cell and serum protein binding of $^{131}$I labeled thyroid hormones using an in vitro method.

**MATERIAL AND METHODS**

Human red blood cells were washed five times in buffered saline$^1$ by the technique previously described (Crispell & Coleman 1956). In some instances, oxalated blood rather than heparinized blood was used. No difference in hormone binding could be demonstrated between the two groups of washed red blood cells. Fresh pooled serum obtained daily from several healthy volunteers was diluted to a 1:10 solution with buffered saline$^1$ (final proteins concentration $= 7.6$ mg/ml). Human serum albumin was suspended in buffered saline to yield a solution containing 7.6 mg/ml albumin. $^{131}$I labeled l-thyroxine, l-triiodothyronine and tetraiodothyroacetic acid solutions$^2$ were diluted with buffered saline containing 0.1 mg/ml albumin so that 1 ml registered approximately $2.5 \times 10^4$ counts per minute. Solutions of Trypan Blue, Evans Blue, 5,5-diphenylhydantoin sodium$^3$ or stable tetraiodothyroacetic acid$^4$ were prepared with buffered saline in the following concentrations: Trypan Blue, 5 mg/ml and 10 mg/ml$^5$; Evans Blue, 5 mg/ml, 10 mg/ml, and 20 mg/ml; 5,5-diphenylhydantoin sodium, 0.04 mg$^5$; stable tetraiodothyroacetic acid, 0.1 mg/ml.

For red blood cell uptake of hormones, 1 ml sample of radioactive labeled hormones were pipetted into 15 ml pyrex test tubes and counted in a well counter. Two ml of red blood cells were added to each tube and incubated for thirty minutes in a 37°C water bath. The cells were then washed by adding 8 ml buffered saline, shaking and centrifuging. The supernate was discarded and the red blood cells washed again with 8 ml buffered saline by re-suspending and re-centrifuging. The radioactivity remaining with the red blood cell was counted in a well counter. This count divided by the count of the original radioactivity of the labeled hormone times 100, represents the percentage of labeled hormone remaining with the red cell.

Where serum or albumin solutions were used, the procedure was as above except...

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1 Sørensen’s Buffer (Difco 512-33) $pH$ 7.3.
2 Obtained from Abbott Laboratories, Oak Ridge, Tennessee.
3 Supplied by Parke Davis and Company.
4 Supplied by Warner Lambert Research Institute.
5 Represents a saturated solution.
that 1 ml of the protein solution was added to the counted radioactive compound and permitted to incubate for fifteen minutes before the red blood cells were added. When Trypan Blue, Evans Blue, 5,5-diphenylhydantoin sodium or stable tetraiodothyroacetic acid were used, they were mixed in appropriate concentrations before being added to the counted radioactive compound.

Conventional electrophoresis was performed on cellulose acetate strips in a Shandon Universal Electrophoresis apparatus using Trismaleate buffer (pH 8.6). When Trypan Blue (0.5%) was used in electrophoresis, the suggestion of Braverman & Ingbar (1961) was followed and the Trypan Blue was added to the buffer as well as to the serum mixture of labeled tetraiodothyroacetic acid. The radioactive tetraiodothyroacetic acid was added to 1 ml of undiluted serum in 15–20 µl volumes resulting in a final concentration of approximately 0.3-0.4 µg per ml of labeled tetraiodothyroacetic acid.

It should be noted that the albumin that was used was free of thyroxine binding globulin on electrophoresis with 1-thyroxine but contained small amounts of thyroxine binding pre-albumin as demonstrated on electrophoresis with tetraiodothyroacetic acid.

RESULTS

Studies Involving Red Blood Cells without Additional Serum or Albumin

The results of our studies with red blood cells are summarized in Table 1. The uptakes by red blood cells of radioactive 1-thyroxine, 1-triiodothyronine and tetraiodothyroacetic acid were 72%, 78% and 45%, respectively.

Addition of Evans Blue (1%) reduced the red blood cell uptake of

<table>
<thead>
<tr>
<th></th>
<th>RBC</th>
<th>+ EB 1%</th>
<th>+ TB 1%</th>
<th>+ DIL 4 mg/100 ml</th>
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<tr>
<td>T₄</td>
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<td>27</td>
<td>66</td>
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</tr>
<tr>
<td>TA₄</td>
<td>45</td>
<td>43</td>
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</table>

All values are expressed as % uptake by RBC of Radioactive T₄, T₃, or TA₄ present. (Indicated at left). The values in italic are significantly different from RBC uptake to the left. (P = 0.025 by runs test).

* For the sake of simplicity, Evans Blue and Trypan Blue solutions will be discussed in the 1% concentration. In all cases when significant changes were noted 0.5% solutions were intermediate in effect. Two percent solutions were also tested and produced no significantly greater effect than the 1% solution.
l-thyroxine from 72% to 27% and the red blood cell uptake of l-triiodothyronine from 78% to 26%. In contrast to the red blood cell binding of l-thyroxine and l-triiodothyronine, binding of tetraiodothyroacetic acid by red blood cells was not changed when Evans Blue was added.

Trypan Blue (1%) had only slight effect on the various red blood cell uptakes. Red blood cell uptake of l-thyroxine was reduced from 72% to 66%, l-triiodothyronine was reduced from 78% to 63%, and the uptake of tetraiodothyroacetic acid was increased from 45% to 54%. These values were not statistically significant. The addition of stable tetraiodothyroacetic acid had no effect on the red blood cell uptake of l-thyroxine. Similarly, 5, 5-diphenylhydantoin had no significant effect on the red blood cell uptake of l-thyroxine, l-triiodothyronine or tetraiodothyroacetic acid.

Studies Involving Red Blood Cell with Serum or Albumin

The results are summarized in Table 2. In the presence of diluted human serum, uptake by red blood cells of l-thyroxine, l-triiodothyronine and tetraiodothyroacetic acid were 11%, 42% and 4%, respectively; in the presence of diluted human albumin, corresponding red blood cell uptake was 27%, 64% and 6% (Table 2).

When serum was used, Trypan Blue increased the red blood cell uptake of

| Table 2. |

The in vitro uptake of 131I labeled thyroid hormones by human RBC from serum or albumin diluted with buffered saline.

<table>
<thead>
<tr>
<th></th>
<th>Serum + RBC</th>
<th>+ TB 1%</th>
<th>+ DIL 4 mg/100 ml</th>
</tr>
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<tbody>
<tr>
<td>T4</td>
<td>11</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>T3</td>
<td>42</td>
<td>28</td>
<td>61</td>
</tr>
<tr>
<td>TA4</td>
<td>4</td>
<td>10</td>
<td>7</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Albumin + RBC</th>
<th>+ TB 1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4</td>
<td>27</td>
<td>46</td>
</tr>
<tr>
<td>T3</td>
<td>64</td>
<td>59</td>
</tr>
<tr>
<td>TA4</td>
<td>6</td>
<td>18</td>
</tr>
</tbody>
</table>

All values are expressed as % uptake by RBC of Radioactive T4, T3, or TA4 present. (Indicated at left). The values in italic are significantly different from RBC uptake to the left. (P = 0.025 by runs test).
Fig. 1.
Upper portion – The electrophoretic pattern of $^{131}$I labeled tetraiodothyroacetic acid (TA$^{4}$) in Tris-maleate buffer.
Lower portion – The same except Trypan Blue 0.5 % has been added causing displacement of TA$^{4}$ from TBPA to albumin.

L-thyroxine from 11 % to 32 %. Red blood cell uptake of tetraiodothyroacetic acid was similarly increased from 4 % to 10 %. In contrast, l-triiodothyronine uptake was significantly decreased from 42 % to 28 % with Trypan Blue. When albumin was used, l-thyroxine red blood cell uptake was increased by Trypan Blue from 27 % to 46 % and that of tetraiodothyroacetic acid from 6 % to 13 %. L-triiodothyronine uptake by red blood cells, which had been decreased in the serum solutions, was not significantly changed by Trypan Blue in the albumin solution.

When unlabeled tetraiodothyroacetic acid (10 mg per 100 ml) and labeled l-thyroxine were combined in the presence of serum, the red blood cell uptake of l-thyroxine was increased from 11 % to 25 %, a two-fold increase over the l-thyroxine uptake without unlabeled tetraiodothyroacetic acid. The 5, 5-diphenylhydantoin sodium (4 mg per 100 ml) also increased l-thyroxine as well as l-triiodothyronine red blood cell uptake from 11 % to 25 % and from
42% to 61% respectively, but 5,5-diphenylhydantoin did not significantly alter the red blood cell uptake of tetraiodothyroacetic acid in the presence of serum.

Electrophoretic serum binding studies of labeled tetraiodothyroacetic acid are shown in Fig. 1. In the upper determination without Trypan Blue, serum binding of tetraiodothyroacetic acid is almost exclusively to the thyroxine binding pre-albumin fraction. The lower determination, where Trypan Blue was used, shows the radioactive tetraiodothyroacetic acid appeared mainly with the albumin fraction. (It should be noted that Trypan Blue has caused a cathodal migration of serum. This may represent an osmophoretic effect by the addition of Trypan Blue to the buffer).

DISCUSSION

Studies Involving Only the Red Blood Cell

The percentage uptake of tetraiodothyroacetic acid by the red blood cell from a buffered saline solution is much less than either 1-thyroxine or 1-triiodothyronine, i.e., 45% versus 78% and 72%, respectively (Table 1). The presence of the dye, Evans Blue, markedly inhibits the uptake of 1-triiodothyronine and 1-thyroxine but has no effect on the uptake of tetraiodothyroacetic acid (Fig. 1). This suggests that a qualitative difference in red cell binding exists; one type of site binds 1-triiodothyronine and 1-thyroxine and is altered by the dye. The other type binds tetraiodothyroacetic acid and is not altered by the presence of the dye.

The presence of Trypan Blue or 5,5-diphenylhydantoin sodium in the incubation mixture did not change the degree of uptake by the cell of either 1-triiodothyronine, 1-thyroxine or tetraiodothyroacetic acid. Minor changes were recorded but they were not statistically significant (Table 1).

Studies Involving Red Blood Cell and Serum or Albumin

Previous work by others suggest that 1-thyroxine binds principally to thyroxine binding globulin and thyroxine binding pre-albumin, tetraiodothyroacetic acid to thyroxine binding pre-albumin and, 1-triiodothyronine to thyroxine binding globulin. All will bind to albumin to some degree but thyroxine binding globulin and thyroxine binding pre-albumin serve as primary binding sites (Ingbar 1960, 1963).

The technique used in this study allows one to study the equilibrium of the labeled hormones between red blood cell and serum. It is also possible to study changes in equilibrium between the cell and serum for the hormones by introducing substances which effect only the binding sites on the serum. Trypan Blue and 5, 5-diphenylhydantoin were chosen as they had little effect on the red cell binding of the hormones incubated with buffered saline alone.
As reported previously, the presence of serum in the incubating mixture markedly reduces the red blood cell uptake of 1-triiodothyronine and 1-thyroxine (Larson & Albright 1955). This is also true for tetraiodothyroacetic acid (Table 2). Since red blood cell uptake of 1-thyroxine, 1-triiodothyronine and tetraiodothyroacetic acid differ, the ratio of the red blood cell uptake in the presence of serum to the red blood cell uptake without serum, better represents the relative binding affinities between red blood cell and serum. Thus a compound which was not bound at all by serum would have a ratio of one and the compound bound most strongly would have the lowest ratio. The ratio was 0.09 for tetraiodothyroacetic acid, 0.15 for 1-thyroxine and 0.54 for 1-triiodothyronine. This pattern of binding was also suggested by the work of Christensen (1960) using red blood cells and serum with the technique of dialysis.

Previous work, which was repeated and is again reported, shows that Trypan Blue shifts equilibrium for 1-thyroxine in favour of the red blood cell, i.e., a greater red blood cell uptake than with serum alone (Braverman & Ingbar 1961). A similar shift occurs also for tetraiodothyroacetic acid (Table 2). These shifts probably result from the ability of Trypan Blue to compete with or displace 1-thyroxine and tetraiodothyroacetic acid from binding sites on thyroxine binding pre-albumin. This hypothesis is supported by the electrophoretic pattern shown in Fig. 1 for tetraiodothyroacetic acid and the electrophoretic data of Braverman & Ingbar (1961) for 1-thyroxine. No explanation or even speculation can be offered at this time for the finding that the equilibrium for 1-triiodothyronine is shifted further away from the red blood cell by the presence of Trypan Blue. The recent report of Ingbar (1963) stating «the inhibition of thyroxine-binding by thyroxine binding pre-albumin induced by Trypan Blue was accompanied by an unexplained increase in thyroxine binding by protein migrating in the a-globulin zone» is of interest. This could conceivably explain the results with 1-triiodothyronine and Trypan Blue but we have no data at the present time to support this concept. Further work is in progress in an attempt to explain this interesting finding.

When human serum albumin (containing a small amount of thyroxine binding pre-albumin by electrophoretic analysis) is substituted for serum, the same pattern was obtained for 1-thyroxine and tetraiodothyroacetic acid. However, there was no change in the equilibrium for 1-triiodothyronine when albumin replaced serum, as the red cell uptake remained the same (Table 2). This would be the expected result if Trypan Blue effects only the thyroxine binding pre-albumin sites as 1-triiodothyronine has very little affinity for thyroxine binding pre-albumin (Hollander et al. 1962).

The presence of 5, 5-diphenylhydantoin sodium (DPH) in the red blood cell serum mixture shifts the equilibrium for 1-triiodothyronine and 1-thyroxine
in favour of the red blood cell as evidenced by the increased red cell uptake. 5,5-diphenylhydantoin sodium (DPH) had no effect on tetraiodothyroacetic acid in a similar system. The results for l-triiodothyronine and l-thyroxine confirm the results of Oppenheimer et al. (1961) and Oppenheimer & Tavernetti (1962 a, b). The pattern which we have observed is in line with electrophoretic data of Wolff et al. (1961) showing that 5,5-diphenylhydantoin sodium blocks l-thyroxine binding by serum protein at the thyroxine binding globulin site but not at the thyroxine binding pre-albumin site (D'Addabbo et al. 1961).

The red blood cell uptake of labeled l-thyroxine in the presence of serum enriched with stable tetraiodothyroacetic acid fit with previous electrophoretic data that tetraiodothyroacetic acid can block l-thyroxine binding to thyroxine binding pre-albumin (Ingbar 1960). Moreover, the values for the red blood cell uptake of l-thyroxine when blocking thyroxine binding pre-albumin by stable tetraiodothyroacetic acid approximate those obtained when blocking thyroxine binding pre-albumin by Trypan Blue, Table 2).

These results are in accord with those of Hollander et al. (1962) and point to a significant effect of thyroxine binding pre-albumin at physiological $p_H$.

REFERENCES


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