METABOLIC EFFECTS OF THYROID HORMONES IN VITRO

II. INFLUENCE OF THYROXINE AND TRIIODOTHYRONINE ON OXIDATIVE PHOSPHORYLATION*

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Because of the profound effect of thyroxine on the metabolic rates of animals, several groups of investigators have sought a relationship between this hormone and oxidative phosphorylation (1-4). The work in this laboratory was designed to test the hypothesis that the thyroid hormone may exert its salutary effects by uncoupling an oxidative phosphorylation just prior to a rate-limiting step in the electron transport sequence (5). The toxic manifestations of the hormone would result from the same action carried beyond the point of benefit. In support of the hypothesis, enzyme preparations from livers of hyperthyroid rats were found to be less efficient in coupling phosphorylation with oxidation than were preparations from normal rats (1). Under specified conditions, thyroxine added to normal tissue preparations in vitro was found to depress P:O ratios, although it also depressed respiration (1). Martius and Hess (4) have reported that thyroxine applied in vivo or in vitro inhibits uptake of radioactive orthophosphate by rat liver mitochondria. However, they reported no data for oxygen consumption. Since thyroxine inhibits respiration in some systems (1, 2), it is essential to measure both respiration and phosphorylation in experiments of this type.

Triiodothyronine has recently been identified by Gross and Pitt-Rivers (6) as a plasma-borne, iodine-containing compound which may be produced from thyroxine even in thyroidectomized animals (7). Triiodothyronine has been synthesized (6, 8), and Gross and Pitt-Rivers (9) have demonstrated that it is 3 to 4 times as active as thyroxine in preventing thyroid enlargement in rats fed thiouracil. Its influence on myxedematous patients is manifested by an increased basal metabolic rate, decreased body weight, and decreased cholesterol of the blood (10).

In this paper data are presented which demonstrate that thyroxine and triiodothyronine added in vitro depress oxidative phosphorylation by rat

* This work was supported by grants from the Nutrition Foundation, Inc., and from the Office of Naval Research. For the first paper in this series see Lardy and Feldott (1).
kidney mitochondria with some substrates and prolong the oxidation and phosphorylating abilities of mitochondria with other substrates.

**EXPERIMENTAL**

The enzyme preparation consisted of twice washed rat kidney mitochondria prepared in isotonic sucrose by the method of Schneider (11) and taken up in 1 ml. of isotonic sucrose per gm. of wet weight of original tissue. The system contained 40 μM of phosphate buffer, pH 7.2, 15 μM of MgSO₄, 0.03 μM of cytochrome c, 6 μM of adenosinetriphosphate, and 0.5 ml. of mitochondrial suspension in the main compartment of the flask. The quantity of substrate is indicated for each experiment with the tabular data. When malonate was added, the concentration was 10 μM per flask, unless otherwise indicated. Isotonic KCl (0.15 M) was added to make the final volume 3.0 ml.

For oxidative phosphorylation experiments the side arm contained 50 μM of glucose and 0.05 ml. of yeast hexokinase (12) purified to Stage 3a. 0.013 to 0.026 μM of L-thyroxine (as a 4 × 10⁻⁴ M solution of the potassium salt) was added at the expense of isotonic KCl. Respiration was measured by the conventional Warburg technique at 37°. After a 5 minute equilibration period, initial readings were taken and the contents of the side arm were tipped into the main compartment. A zero time flask was removed and enzymatic action stopped by deproteinization with cold 10 per cent perchloric acid. Oxidation was allowed to proceed for 20 minutes. The final readings were taken and the contents of the flasks were deproteinized as above. The deproteinized samples were analyzed for inorganic phosphate by the method of Lowry and Lopez (13). P:O ratios (micromoles of orthophosphate fixed per microatom of oxygen consumed) were calculated from the phosphate disappearance and the concurrent oxygen consumption. The P:O values with various substrates were in close agreement with those previously reported from this laboratory for rat liver mitochondria (14).

**Results**

**Effects of Thyroid Hormones on Phosphorylation during Glutamate Oxidation**—We have previously reported (1) that thyroxine, added to the washed residue of rat kidney homogenized in isotonic KCl, did not influence P:O ratios in systems which allow the substrate to be oxidized to completion. However, if the oxidation of glutamate was blocked at the succinate level by malonate, added thyroxine depressed phosphate uptake more strikingly than it depressed respiration, thus decreasing the P:O ratios.

Experiment 1 of Table I demonstrates that these phenomena occur also
with kidney mitochondria. As shown in the subsequent experiments of Table I, thyroxine rarely uncoupled phosphorylation from oxidation unless it significantly inhibited oxidation. Analogues of thyroxine which possess hormonal activity in vivo (15) uncouple oxidative phosphorylation, but, again, depression of oxygen consumption was observed (Table II).

**Table I**

Effect of Thyroxine on Phosphorylation Associated with Glutamate Oxidation by Rat Kidney Mitochondria

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Molarity of thyroxine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2.64*</td>
</tr>
<tr>
<td>2</td>
<td>2.75</td>
</tr>
<tr>
<td>3</td>
<td>2.68</td>
</tr>
<tr>
<td>4</td>
<td>3.02</td>
</tr>
<tr>
<td>5</td>
<td>3.16</td>
</tr>
<tr>
<td>6</td>
<td>2.52</td>
</tr>
<tr>
<td>7</td>
<td>2.83</td>
</tr>
<tr>
<td>8</td>
<td>2.75</td>
</tr>
<tr>
<td>9</td>
<td>2.55</td>
</tr>
<tr>
<td>10</td>
<td>2.44</td>
</tr>
<tr>
<td>11</td>
<td>2.74</td>
</tr>
<tr>
<td>Average</td>
<td>2.71</td>
</tr>
</tbody>
</table>

The numbers in parentheses refer to the percentage inhibition of oxidation. Each flask contained, in addition to the standard components, 60 μM of glutamate. Respiration in the presence of hexokinase and glucose was measured for 20 minutes at 37°C; 20 to 30 μM of phosphate were fixed during this period in the control experiments.

* No malonate added; all other flasks contained 0.033 M malonate and the averages are calculated from the latter.

Levels of thyroxine which consistently produced effects with glutamate as substrate did not uncouple phosphorylation during oxidation of α-ketoglutarate to succinate (Table III). In two experiments in which the highest level of thyroxine depressed respiration a decreased P:O ratio was observed.

The results of a typical experiment comparing the effects of thyroxine

1 All data reported here are for L-thyroxine. The D and L isomers are equally effective in influencing oxidative phosphorylation by rat kidney mitochondria.
### Table II

**Effect of Thyroxine Analogues on Phosphorylation Associated with Glutamate Oxidation by Rat Kidney Mitochondria**

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Experiment No</th>
<th>( \mu \text{M} ) P fixed per microatom</th>
<th>( \text{O consumed} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1.3 ( \times 10^{-5} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroxine</td>
<td>1</td>
<td>2.95</td>
<td>2.40 (24)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.75</td>
<td>1.95 (19)</td>
</tr>
<tr>
<td>3,5-Diiodo-4-(3',5'-diiodo-4'-hydroxy-phenoxy)benzoic acid</td>
<td>1</td>
<td>2.95</td>
<td>2.13 (12)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.75</td>
<td>1.77 (24)</td>
</tr>
<tr>
<td>3,5-Diiodo-4-(3',5'-dibromo-4'-hydroxyphenoxy)benzoic acid</td>
<td>1</td>
<td>2.95</td>
<td>2.32 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.75</td>
<td>2.84 (21)</td>
</tr>
<tr>
<td>3,5-Diiodo-4-(4'-hydroxyphenoxy)benzoic acid</td>
<td>1</td>
<td>2.95</td>
<td>2.35 (11)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.75</td>
<td>2.27 (10)</td>
</tr>
</tbody>
</table>

The numbers in parentheses refer to the percentage inhibition of oxidation. Malonate (0.033 M) was present in all the flasks; other experimental conditions as described in Table I.

The analogues of thyroxine were kindly supplied by Professor R. J. Winzler and Professor E. Frieden.

### Table III

**Effect of Thyroxine on Phosphorylation Associated with \( \alpha \)-Ketoglutarate Oxidation by Rat Kidney Mitochondria**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>( \mu \text{M} ) P fixed per microatom</th>
<th>( \text{O consumed} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1.3 ( \times 10^{-5} )</td>
</tr>
<tr>
<td>1</td>
<td>3.52</td>
<td>3.66</td>
</tr>
<tr>
<td>2</td>
<td>4.25</td>
<td>4.31</td>
</tr>
<tr>
<td>3</td>
<td>4.25</td>
<td>4.30</td>
</tr>
<tr>
<td>4</td>
<td>3.82</td>
<td>3.88</td>
</tr>
<tr>
<td>5</td>
<td>4.05</td>
<td>4.26</td>
</tr>
<tr>
<td>6</td>
<td>3.77</td>
<td>3.77</td>
</tr>
<tr>
<td>Average........</td>
<td>3.94</td>
<td>4.03</td>
</tr>
</tbody>
</table>

In addition to the standard components, each flask contained 20 \( \mu \text{M} \) of \( \alpha \)-ketoglutarate and 30 \( \mu \text{M} \) of malonate. Respiration in the presence of hexokinase and glucose was measured for 20 minutes at 37°C.

* In Experiments 2 and 3 respiration was inhibited 23 and 35 per cent, respectively, by the highest concentration of thyroxine.
and triiodothyronine\(^2\) on oxidation and phosphorylation are shown in Table IV. In twelve similar experiments triiodothyronine uncoupled phosphorylation and differed from thyroxine in the important fact that it did not inhibit respiration. In a few experiments triiodothyronine slightly inhibited the oxidation of glutamate, but this inhibition was much less than that produced by an equivalent concentration of thyroxine. The effect of triiodothyronine on oxidative phosphorylation was most readily detected when oxidation of glutamate was blocked at the stage of succinate by malonate.

### Table IV

**Comparative Effects of Triiodothyronine and Thyroxine on Oxidation of Glutamate and on Phosphorylation by Rat Kidney Mitochondria**

<table>
<thead>
<tr>
<th>Additions</th>
<th>No malonate</th>
<th>0.033 M malonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O(_2) consumed</td>
<td>P esterified</td>
</tr>
<tr>
<td></td>
<td>micro-atoms</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>None (control)</td>
<td>9.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Thyroxine 0.65 (\times 10^{-4}) M</td>
<td>9.0</td>
<td>25.0</td>
</tr>
<tr>
<td>&quot; 1.3 (\times 10^{-5}) &quot;</td>
<td>8.7</td>
<td>20.9</td>
</tr>
<tr>
<td>&quot; 2.0 (\times 10^{-5}) &quot;</td>
<td>8.9</td>
<td>23.4</td>
</tr>
<tr>
<td>Triiodothyronine 0.65 (\times 10^{-4}) M</td>
<td>9.8</td>
<td>23.2</td>
</tr>
<tr>
<td>&quot; 1.3 (\times 10^{-5}) &quot;</td>
<td>9.6</td>
<td>23.6</td>
</tr>
<tr>
<td>&quot; 2.6 (\times 10^{-5}) &quot;</td>
<td>9.5</td>
<td>24.1</td>
</tr>
</tbody>
</table>

Flasks without malonate were incubated 10 minutes at 37° in the presence of hexokinase; those containing malonate were incubated 20 minutes. Other experimental conditions as in Table I.

In agreement with previous results obtained with washed tissue residues (1), thyroxine (and also triiodothyronine) added in vitro did not detectably influence P:O ratios when other tricarboxylic acid cycle intermediates were oxidized by rat liver or kidney mitochondria.

Although thyroxine and triiodothyronine act as uncoupling agents under specified conditions, neither of these compounds enhances respiration in systems which contain no phosphate acceptors and which are capable of marked stimulation by either dinitrophenol or phosphate acceptors (16).

**Effects of Thyroxine and Triiodothyronine on Fatty Acid Oxidation—Data**

\(^2\) We are indebted to Dr. Gross and Dr. Pitt-Rivers for providing us, very early in their work, with valuable samples of triiodothyronine. More recently, triiodothyronine has been obtained through the courtesy of Dr. A. E. Heming, Smith, Kline and French Laboratories, who obtained it from the Glaxo Laboratories, Ltd., where it was synthesized.
have been presented previously (1) which demonstrate that thyroxine inhibited the oxidation of β-hydroxybutyrate and certain fatty acids by washed residues of rat kidney homogenates when low concentrations of tissue preparation were used, but enhanced the oxidation of these substrates when the reactions were catalyzed by higher levels of the washed residue. The effects of thyroxine were usually manifested during the 2nd and succeeding hours of the experimental period.

These experiments have been repeated with rat kidney mitochondrial suspensions as the enzyme source. As shown in Fig. 1, thyroxine had little effect on the oxidation of β-hydroxybutyrate by mitochondria during the 1st and 2nd hours of incubation. However, during the 3rd and 4th hours of incubation, when oxidation in the control vessels was rapidly decreasing, thyroxine appeared to maintain the oxidation of β-hydroxybutyrate at or near the initial rate. Comparable results were obtained with higher (0.67 mg. of N per flask) and lower (0.40 mg. of N) enzyme concentrations than that recorded in Fig. 1. In these systems, with kidney mitochondria in the presence of catalytic quantities of C4-dicarboxylic acids, both caprylate and β-hydroxybutyrate are oxidized completely to CO₂.

Measurements of phosphorylating capacity were also made during these extended respiratory experiments. Data for β-hydroxybutyrate and caprylate as substrates are presented in Table V. Hexokinase and glucose were kept in the side arm of the flask until 30, 60, 120, or 180 minutes after the respiratory measurements had begun. After dumping the contents of the side arm into the main compartment, one flask of each set was removed for phosphate analysis. In the others, respiration was measured during

![Graph showing the influence of thyroxine on the oxidation of β-hydroxybutyrate by rat kidney mitochondria.](http://www.jbc.org/DownloadedFrom)

**Fig. 1.** Influence of thyroxine on the oxidation of β-hydroxybutyrate by rat kidney mitochondria. Enzyme concentration, 0.54 mg. of N per flask. 15 μM of DL-β-hydroxybutyrate and 1 μM of succinate per flask.
the following 10 minute period, after which they were deproteinized for phosphate analysis. The P:O ratios reported in Table V were calculated for this 10 minute period. With β-hydroxybutyrate as substrate, phosphorylating efficiency had decreased markedly after 2 hours and no net phosphate uptake occurred when hexokinase and glucose were added after 3 hours. Thyroxine at a concentration of $1.3 \times 10^{-5}$ M improved phos-

### Table V

**Influence of Thyroxine on Phosphorylation Associated with Oxidation of β-Hydroxybutyrate and Caprylate by Rat Kidney Mitochondria**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration of thyroxine</th>
<th>Experiment No.</th>
<th>μM P fixed per microatom O consumed measured after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>0</td>
<td>1</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2.46</td>
</tr>
<tr>
<td></td>
<td>$1.3 \times 10^{-5}$</td>
<td>1</td>
<td>2.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2.45</td>
</tr>
<tr>
<td></td>
<td>$2.6 \times 10^{-5}$</td>
<td>1</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>2.68</td>
</tr>
<tr>
<td>Caprylate</td>
<td>0</td>
<td>3</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td>$1.3 \times 10^{-5}$</td>
<td>3</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>$2.6 \times 10^{-5}$</td>
<td>3</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2.49</td>
</tr>
</tbody>
</table>

The substrates were 15 μM of L-β-hydroxybutyrate per flask in Experiments 1 and 2 and 6 μM of caprylate in Experiments 3 and 4; 1 μM of succinate was added to all flasks. Approximately 1 mg. of mitochondrial N per flask in each experiment. Other experimental conditions as in Table I, except that glucose and hexokinase were added from the side arms at the time indicated. Oxidative phosphorylation was measured during the following 10 minute period.

L-β-Hydroxybutyrate was generously provided by Professor M. Lemoigne of the Pasteur Institute, Paris.

phorylating ability after prolonged incubation and, when added to give a final concentration of $2.6 \times 10^{-5}$ M, high P:O ratios were maintained even after 3 hours at 37°C. Thyroxine exerted a similar, but quantitatively somewhat lesser, beneficial effect on phosphorylating efficiency when caprylate was the substrate. The maintenance of vigorous oxidizing activity in the presence of thyroxine (Fig. 1) undoubtedly is dependent upon the better maintenance of phosphorylating ability (17) in the presence of the hormone. The effect of thyroxine on maintenance of phosphorylating ability of mitochondria during prolonged incubation is reminiscent of its ability to counteract the "uncoupling" action of cysteine (18).
Like thyroxine, triiodothyronine also prolongs respiration of kidney mitochondria with β-hydroxybutyrate as substrate, as is shown in Table VI. In this experiment, respiration was well maintained even in the control flasks, but the beneficial effects of thyroxine and triiodothyronine were readily apparent during the 4th and 5th hours of the incubation. Triiodothyronine also prolongs phosphorylating capacity of the kidney mitochondria (Table VII).

**Table VI**

*Comparative Effects of Thyroxine and Triiodothyronine on Oxidation of β-Hydroxybutyrate by Rat Kidney Mitochondria*

<table>
<thead>
<tr>
<th>Additions</th>
<th>Q_{O2} (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st hr.</td>
</tr>
<tr>
<td>None (control)</td>
<td>322</td>
</tr>
<tr>
<td>2.6 × 10^{-6} M thyroxine</td>
<td>377</td>
</tr>
<tr>
<td>2.6 × 10^{-6} M triiodothyronine</td>
<td>416</td>
</tr>
</tbody>
</table>

Each flask contained 15 μM of L-β-hydroxybutyrate, 1 μM of succinate, and 0.74 mg. of mitochondrial N.

**Table VII**

*Influence of Triiodothyronine on Phosphorylation Associated with Oxidation of β-Hydroxybutyrate by Rat Kidney Mitochondria*

<table>
<thead>
<tr>
<th>Concentration of triiodothyronine</th>
<th>μM P fixed per microatom 0 consumed measured after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>0</td>
<td>2.46</td>
</tr>
<tr>
<td>1.3 × 10^{-5} M</td>
<td>2.51</td>
</tr>
<tr>
<td>2.6 × 10^{-6} M</td>
<td>2.42</td>
</tr>
</tbody>
</table>

Experimental conditions as in Table V; 0.73 mg. of mitochondrial N per flask.

**DISCUSSION**

These studies are aimed at a better understanding of the mechanism by which the thyroid hormone exerts its physiological effects. The effects of the compounds tested *in vitro* do not correspond to the characteristic effects *in vivo*. For example, neither thyroxine nor triiodothyronine added *in vitro* stimulates oxygen consumption of liver or kidney mitochondria, although this is the most characteristic response of the intact animal given an excess of thyroid hormone. Furthermore, to obtain effects *in vitro* it has been necessary to use concentrations of thyroxine (10^{-8} M) which are
considerably in excess of the thyroxine concentration in the whole animal (19). On the other hand, it may be unreasonable to expect thyroxine or triiodothyronine to produce effects analogous to those obtainable in vivo, for it is not known whether either of these compounds is the metabolically active form of the thyroid hormone. There is reason to believe that triiodothyronine more nearly represents the true hormone than does thyroxine (9), but it is possible that triiodothyronine undergoes further transformations before acquiring full activity.

Another seeming lack of correlation between effects in vitro and in vivo is observed in a comparison of the activity of the optical isomers of thyroxine. The D, L, and DL compounds are all equally effective in uncoupling phosphorylation during glutamate oxidation and in maintenance of oxidative and phosphorylating abilities of mitochondria during prolonged incubation. In contrast, the L isomer is approximately 6 times more effective than the D isomer in vivo. However, the presence of an asymmetric carbon atom is not essential for thyroxine-like activity. Analogues of thyroxine in which, among other changes, the side chain has been replaced by carboxyl (15), amino (20), or acrylic or propionic acid (21) are active. Therefore, it is possible that the relatively poor activity of D-thyroxine in the intact animal reflects a greater rate of destruction and excretion (22) or a slower rate of cell penetration of this isomer than of the naturally occurring L form.

Recognizing the limitations of the in vitro approach, we may interpret the results obtained as, at least, providing some leads to the mode of action of the hormones. The fact that thyroxine or triiodothyronine influences oxidative phosphorylation (Tables I, II, IV, V, VII), induces phosphate liberation from adenosinetriphosphate (23), and prevents the uncoupling effect of cysteine (18) indicates that these compounds have affinity for enzymes participating in the phosphate-transferring reactions of the mitochondria.

The ability of thyroxine and especially of triiodothyronine to uncouple oxidative phosphorylation, the decreased P:O ratios obtained with tissue preparations from hyperthyroid animals (1, 24), and the decreased efficiency of work performance of hyperthyroid patients (25) may have a common biochemical basis (5).

**Summary**

Thyroxine, at \(10^{-6} \text{ M}\), depressed the oxygen consumption of rat kidney mitochondria with glutamate as the substrate when the tricarboxylic acid cycle was blocked with malonate. Under these conditions thyroxine decreased the efficiency with which phosphorylation is coupled to respiration. Thyroxine at this concentration did not significantly inhibit respiration or
phosphorylation when other tricarboxylic acid cycle intermediates were oxidized by liver or kidney mitochondria.

Triiodothyronine (10^-5 M) uncoupled phosphorylation associated with glutamate oxidation; it did not significantly depress respiration.

Both thyroxine and triiodothyronine prevent the rapid decline in mitochondrial respiration and oxidative phosphorylation which occurred during the 3rd and 4th hours of incubation at 37° with β-hydroxybutyrate or caprylate as the substrate.

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