Heart Mitochondrial Function in Acute and in Chronic Hyperthyroidism in Rats

By Dorothy A. Piotnek-Leunissen, Ph.D., and Reinhard L. A. Leunissen, M.D.

ABSTRACT

The effects of acute versus chronic hyperthyroidism on rat heart mitochondria were explored. Acute, severe hyperthyroidism with an 18% loss of body weight was induced by injecting subcutaneously 10 μg/100g/day of triiodothyronine for 6 to 14 days, and chronic, anabolic, moderate hyperthyroidism was induced by placing triiodothyronine in the drinking water at a dose of 25 or 50 μg/100 ml for 15 to 60 days. Mitochondrial function was assessed polarographically using α-ketoglutarate as the oxidizing substrate, and mitochondrial structure was assessed indirectly from changes in rates of swelling in decimolar alkaline salt solutions in vitro. The P-O ratios of heart mitochondria isolated with Nagarse incubation from both types of hyperthyroid rats decreased slightly (15%) but significantly (P = 0.05). Simple dilution of the hyperthyroid mitochondrial suspensions effected a 75% increase in the P-O ratio of the acutely treated rats but only a 19% increase in that of the chronically treated rats. Significantly increased susceptibility to swelling in vitro was exhibited only by the mitochondria of the chronically triiodothyronine-treated rats. On the other hand, only those of the acutely treated rats showed significant increases in the activity of Mg2+-stimulated mitochondrial ATPase. These data suggest that the mechanisms whereby excess thyroid hormone in vivo affects the function and structure of isolated rat heart mitochondria vary with the mode of induction and the duration of the hyperthyroid state.

ADDITIONAL KEY WORDS thyrotoxicosis triiodothyronine Nagarse incubation oxidative phosphorylation phosphorylative capacity swelling in vitro mitochondrial ATPase and azide mitochondrial free fatty acid

The etiology of cardiac failure observed in the hyperthyroid patient (1, 2) and demonstrated in the chronic hyperthyroid dog (3) is as yet undefined. Studies to date suggest that a biochemical lesion in the area of oxidative phosphorylation is not involved in the heart as in other tissues. Neither sarcosomal function (3-5) nor myocardial levels of the end products of oxidative phosphorylation, phosphocreatine and adenosine triphosphate (ATP) (3, 4, 6) were decreased in acute or chronic hyperthyroid animals. Nevertheless, the reduced myocardial resistance to anoxia (7) and the increased oxygen consumption (3, 8) in the hyperthyroid heart would appear to reflect involvement of energy production in the mitochondria. The question arises, therefore, as to whether the indices and the species used to assess mitochondrial integrity were sufficiently sensitive to demonstrate thyroid-induced changes in this remarkably resistant organ.

This study was designed to explore the effects of a prolonged, chronic anabolic hyperthyroid state on the functional and structural integrity of heart mitochondria of the more susceptible species, the rat, and to compare these effects with those produced by...
the acute, severe hyperthyroid state more frequently employed by investigators. Functional integrity of the isolated mitochondria was evaluated polarographically. In addition, the structural integrity, a possibly more sensitive index, was assessed as the susceptibility of the mitochondria to swell in vitro in alkaline salt solutions.

Materials and Methods

Preparation of animals.—An acute, severe hyperthyroid state, as indicated by 16% to 20% loss of body weight, was induced in 200-g male, Wistar rats by injecting subcutaneously 10 μg triiodothyronine1/100g/day for 6 to 14 days. A chronic, anabolic, moderate hyperthyroid state was induced by administering triiodothyronine in the drinking water at a dose of 25 or 50 μg/100 ml for periods of 15 to 60 days. As noted by Gemmill (9), this latter mode of induction evokes signs of hyperthyroidism, such as an increased rate of metabolism, increased heart rate, increased systolic pressure, and increased heart weight without the complicating effect of a marked weight loss. The animals were maintained on ordinary lab chow and housed in air-conditioned rooms.

Isolation of Mitochondria.—At various time intervals fasted rats were killed in groups of 4 to 6 after they were anesthetized with injections of sodium pentobarbital (Nembutal, Abbott Laboratories) intraperitoneally at a dose of 10 mg/100 g body weight. The chest was quickly opened and the beating heart immediately transferred to a beaker of chilled 0.32M sucrose:0.001M EDTA for isolation of heart mitochondria essentially according to the method of Slater and Cleland (10). Only the ventricles of the pooled hearts were used. To evaluate both oxidative phosphorylation and swelling of mitochondria from the same tissue pool, the homogenate was divided into two portions: two-thirds of the total volume to provide mitochondria for assays of oxidative phosphorylation and one-third for swelling studies. The schedule of centrifugal force and time employed was as follows: (a) homogenate, 700g for 10 minutes; (b) supernatant fluid, 7,000g for 10 minutes; (c) recovered mitochondria were resuspended and recovered again at 7,000g for 10 minutes. In later studies, to increase the yield of mitochondria the tissue mince was first homogenized 3 × 15 seconds with 3 ml/g of the isolation medium and 2 mg/g of crystalline bacterial proteinase, Nagarse.3 After a 10-minute incubation at 4°C, 7 ml/g of isolation medium was added, and the mixture was again homogenized for 15 seconds and the isolation procedure continued as described above. The final suspensions of mitochondria were made as follows: for oxidative studies, 1 ml of 0.32M sucrose:0.001M EDTA per 2 g of original tissue mince; for swelling studies, 2 ml of 0.40M sucrose (no EDTA) per 1 g of tissue mince. Mitochondrial protein was assayed by the nitrogen method of Miller and Miller (11) or by the biuret method of Weichselbaum (12).

Functional Assays.—The efficiency with which the mitochondria performed oxidative phosphorylation was measured polarographically according to Packer (13) using a stationary platinum electrode system in an open, rotating cuvette of 2-ml capacity at room temperature (24 to 25°C). The reaction mixture of 1 ml contained mitochondria (2 to 3 mg protein) in a sucrose (0.32M), EDTA (0.001M), phosphate buffer (0.016M) pH 7.4, sodium malonate (0.026M), KCl (0.008M) medium with alpha-ketoglutarate (0.028M) as the oxidizable substrate. Three to four polarographic assays were performed on each preparation, and the following mean values were obtained: Oxygen uptake (μmoles/min/g mitochondrial protein) before, with, and after the addition of 200 to 500 μM of the phosphate acceptor adenosine diphosphate (ADP); the phosphorylative capacity as the rate of esterification of the added ADP (μmoles/min/g mitochondrial protein); the respiratory control no. 1 (oxygen uptake with ADP/oxygen uptake before ADP) as defined by Lardy and Wellman (14) and no. 2 (oxygen uptake with ADP/oxygen uptake after ADP) as defined by Chance and Balscheffsky (15).

Structural Assays.—The structural integrity of the mitochondria was assessed indirectly by measuring the rate of swelling in vitro; that is, as the increase in permeability to water resulting in a decrease in optical density. As described by Lehninger and Remmert (16), aliquots of the mitochondrial suspensions in 0.4M sucrose (no EDTA) corresponding to 0.3 to 0.5 mg mitochondrial protein were added to 2.5 ml of 0.125M KCl in 0.02M Tris-HCl buffer, pH 7.4, to give an initial optical density of 0.300 to 0.500 at 520 μm in a 3.0-ml cuvette of 1-cm light path. Readings were obtained at 15 seconds and every 5 minutes over a 30-minute period. The amount of mitochondria added corresponded to a mean of 0.48 ± 0.05 (SEM) mg protein for all four

1Triiodothyronine (Cytomel) was kindly supplied by Smith, Kline and French Laboratories, Philadelphia, Pa.
2Ethylenediaminetetraacetic acid, at pH 7.4.
3Biddle Sawyer Corp., 64 Wall Street, New York, N. Y.
HYPERTHYROIDISM AND HEART MITOCHONDRIA

groups. The rate of swelling was calculated from duplicate or triplicate assays as the mean percent decrease in optical density from the initial reading at 15 seconds to the final reading at 30 minutes.

Results

As described elsewhere (17), 10 μg/100g/day of triiodothyronine injected subcutaneously for 6 to 14 days produced a severe hyperthyroid state with an excessive rate of catabolism and a survival rate of 78%. A loss of body weight began on the fourth day of treatment and continued to a mean total loss of 18% by the time the animals were killed. On the other hand, the ad libitum administration of similar doses as 25 or 50 μg triiodothyronine/100 ml drinking water, estimated to be 8 to 10 μg/200g/day, for as long as 60 days produced a well tolerated hyperthyroid state with only a slightly reduced rate of growth and a survival rate of 97%. Cardiovascular effects were apparent in the chronically treated rats as evidenced by increases in heart rate from a normal mean of 350 beats/min for this size rat to a mean of 425 beats/min, as measured in several groups using the electrocardiogram and needle electrodes.

FUNCTIONAL STATUS OF HEART MITOCHONDRIA

Phosphorylative Capacity and P-O Ratios.

As illustrated in Tables 1 to 4, excess thyroid hormone in vivo can result in a decreased efficiency of oxidative phosphorylation in isolated rat heart mitochondria in vitro. The mechanisms for effecting this decrease appear to vary with the mode of induction and the duration of the hyperthyroid state.

In Table 1 are presented the polarographic data obtained from acute and chronic hyperthyroid rat heart mitochondria isolated without a preincubation of the tissue mince with the bacterial proteinase, Nagarse. The mean data of three or four polarographic assays of each mitochondrial preparation, representing 4 to 6 rats, have been compiled to give a mean and standard error of the mean for each of the control and experimental groups. The significance of the difference between the normal and experimental group means was evaluated using Student's t-test.

These early studies without preincubation

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of Acute and of Chronic Triiodothyronine Treatment on Rat Heart Mitochondria Isolated without Nagarse Incubation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of groups*</th>
<th>Oxygen uptake (μmoles/min/g)</th>
<th>Respiratory control</th>
<th>Phosphate esterification (μmoles/min/g)</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>Before</td>
<td>With</td>
<td>After</td>
<td>no. 1</td>
</tr>
<tr>
<td>Acute (8 days)</td>
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<td>0.8</td>
<td>0.7</td>
<td>0.9</td>
<td>2.28</td>
</tr>
<tr>
<td>Chronic I (15 to 20 days)</td>
<td>3</td>
<td>9.9</td>
<td>12.1</td>
<td>2.54</td>
<td>2.32</td>
</tr>
<tr>
<td>Chronic II (39 to 47 days)</td>
<td>3</td>
<td>10.0</td>
<td>17.3</td>
<td>2.83</td>
<td>2.03</td>
</tr>
</tbody>
</table>

*Four to six rats per group. †Before, with, and after addition of ADP. ‡Probability that the difference between the group means of the normal and experimental groups could occur by chance as determined by Student's t-test.
TABLE 2

Effect of Dilution of Mitochondria Isolated without Nagarse Incubation from Hearts of Triiodothyronine-Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (mg/assay)</th>
<th>Oxygen uptake (μmoles/min/g)*</th>
<th>Phosphate esterification (μmoles/min/g)</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>With</td>
<td>After</td>
<td></td>
</tr>
<tr>
<td>Acute (N = 1)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(8 days)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated</td>
<td>2.56</td>
<td>12.5</td>
<td>21.4</td>
<td>19.2</td>
</tr>
<tr>
<td>Dilute</td>
<td>2.28</td>
<td>21.4</td>
<td>19.2</td>
<td>14.9</td>
</tr>
<tr>
<td>Chronic II (N = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(39 to 47 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated</td>
<td>2.54</td>
<td>10.0</td>
<td>27.8</td>
<td>17.3</td>
</tr>
<tr>
<td>Dilute</td>
<td>1.26</td>
<td>16.7</td>
<td>31.3</td>
<td>24.3</td>
</tr>
</tbody>
</table>

* Before, with, and after addition of ADP.

with Nagarse revealed markedly depressed rates of phosphorylation and P-O ratios ($P = 0.001$) in heart mitochondria from the acute, severe, and the long-term chronic (chronic II), moderate hyperthyroid rats. Interestingly, respiratory control was decreased in only the acute, severe hyperthyroid preparations. The depressed phosphorylative function appeared to be a product of both mode and time of administration, because "acute" (15 to 20 days) treatment (chronic I) with the oral doses of triiodothyronine in the drinking water increased, rather than decreased, the rate of phosphate esterification. With a concomitant increase in the rate of oxygen uptake with acceptor ADP, however, there was no net change in the P-O ratio. Only with more prolonged treatment with these oral doses of triiodothyronine did depressed phosphorylative efficiency result.

During the course of these early polarographic assays the resting $Q_{O_2}$ (oxygen uptake before ADP) of the triiodothyronine-treated rat heart mitochondria did not appear to be increased. To rule out the possibility that the suspension was too concentrated, thus depressing the sensitivity of this uncoated electrode assembly, assays were immediately repeated on a 1:1 (vol/vol) dilution of the original suspension when possible. The dilution was made using the sucrose:EDTA isolation medium. The results of several such dilution studies are presented in Table 2.

The dilution of these triiodothyronine-treated rat heart mitochondria resulted in an increase in the resting $Q_{O_2}$ of the chronic II preparations, but not of the acute. Since subsequent protein analyses indicated that the two were of similar concentration, the lower $Q_{O_2}$ of the concentrated chronic II suspensions and the diluted acute suspensions would not appear to be a result of electrode insensitivity. However, in addition to these effects on oxygen uptake, dilution produced a marked increase in the rate of phosphate esterification and in the P-O ratios of both types of hyperthyroid heart mitochondria.

When preincubation of the tissue mince with Nagarse was introduced into the isolation procedure, both the quantity and quality of mitochondria obtained from the triiodothyronine-treated, but not the normal, rats improved significantly. The rates of phosphate esterification and the P-O ratios of both acute and chronic II triiodothyronine-treated heart mitochondria increased (Table 3). No statistically significant differences were observed in mitochondria from normal rats; therefore, data from the normal groups (with and without Nagarse) have been combined.

It was first noted that the acute triiodothyronine-treated rat heart mitochondria obtained by this isolation procedure demonstrated better respiratory control than did those described in Table 1, without Nagarse. The $Q_{O_2}$ in state 3, with ADP, was three times greater than that in state 4, before and after ADP, whereas the increase observed previous-
TABLE 3

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of groups*</th>
<th>Oxygen uptake (μmoles/min/g)</th>
<th>Respiratory control (μmoles/min/g)</th>
<th>Phosphate esterification (μmoles/min/g)</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>With</td>
<td>After</td>
<td>no. 1</td>
</tr>
<tr>
<td>Normal†</td>
<td>11</td>
<td>10.0</td>
<td>26.8</td>
<td>13.9</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>±SEM</td>
<td>0.5</td>
<td>1.6</td>
<td>1.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Acute (6 to 12 days)</td>
<td>5</td>
<td>11.6</td>
<td>30.7</td>
<td>12.1</td>
<td>2.91</td>
</tr>
<tr>
<td></td>
<td>±SEM</td>
<td>1.2</td>
<td>3.9</td>
<td>2.0</td>
<td>0.30</td>
</tr>
<tr>
<td>Chronic II (42 to 56 days)</td>
<td>11</td>
<td>0.8</td>
<td>24.1</td>
<td>11.0</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>±SEM</td>
<td>0.5</td>
<td>1.3</td>
<td>0.5</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Four to six rats per group. † Before, with, and after addition of ADP. ‡ Normals with and without Nagarse have been combined because the difference between the group means was not statistically significant.

Effect of Acute Versus Chronic Triiodothyronine Treatment on Rat Heart Mitochondria Isolated with Nagarse Incubation

Hypothyroidism and Heart Mitochondria

Effectively was less than twofold. As mentioned above, the rates of phosphate esterification were significantly increased, although that of the chronic II groups still remained below normal \((P = 0.01)\). This appeared to account for the still significantly lower than normal P-O ratio \((P = 0.05)\) in this group. In the acute preparations the concomitant increase in both \(Q_o^2\) with ADP and the \(Q_v\), rate of phosphate esterification, netted a still significantly lower than normal P-O ratio \((P = 0.05)\).

In view of the previous findings of improved \(Q_v\) with dilution of the suspension, it was considered whether such a procedure might further improve the still significantly lowered \(Q_v\) of these chronic preparations. Polarographic data compiled from such "dilution" studies in normal, acute, and chronic II hyperthyroid heart mitochondria isolated with Nagarse preincubation are presented in Table 4. This procedure did increase the \(Q_v\) of the chronic II preparations by 29%, but it also resulted again in marked increases in the \(Q_o^2\), before, with, and after ADP. Thus, the net increase in the P-O ratio with dilution was only 19%, and the P-O ratio still remained significantly lower than normal. Conversely, this dilution procedure with the acute preparations resulted in a 29% decrease in the \(Q_o^2\), before and with ADP, and a 10% increase in the \(Q_v\). The net effect on the P-O ratio was an increase of 75%, raising it to the normal mean of 3.58.

Mitochondrial \(Mg^{2+}\)-Stimulated ATPase.— These observations of the changes in rates of phosphate esterification suggested the influence of an increased activity of ATPase in the mitochondrial fraction. In polarographic assays even in the absence of added \(Mg^{2+}\), as performed here, some activity of mitochondrial ATPase utilizing endogenous \(Mg^{2+}\) has been suggested by Packer (13). The levels of \(Mg^{2+}\)-stimulated mitochondrial ATPase were therefore measured according to the method of Kielley (18) in six normal, ten acute, and nine chronic II triiodothyronine-treated rat heart mitochondrial fractions obtained with Nagarse preincubation. The hydrolysis of ATP was assayed over a 10-minute period at 28°C in a 2-ml reaction volume containing histidine buffer, 0.05M at a pH 7.4, with 0.005M ATP, 0.005M \(MgCl_2\), 1 to 2 mg mitochondrial protein and with or without 0.005M sodium azide. The inorganic phosphate formed was
TABLE 4
Effect of Dilution of Mitochondria Isolated with Nagarse from Hearts of Triiodothyronine-
Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of groups</th>
<th>Protein (mg/assay)</th>
<th>Oxygen uptake (μmoles/min/g/mg)</th>
<th>Phosphate esterification (μmoles/min/g/mg)</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated</td>
<td>3.67</td>
<td>8.7</td>
<td>30.2</td>
<td>15.6</td>
<td>204</td>
</tr>
<tr>
<td>Dilute</td>
<td>1.94</td>
<td>9.4</td>
<td>27.6</td>
<td>10.1</td>
<td>206</td>
</tr>
<tr>
<td>Acute (10 to 12 days)</td>
<td>2</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated</td>
<td>3.64</td>
<td>14.4</td>
<td>27.8</td>
<td>11.3</td>
<td>121</td>
</tr>
<tr>
<td>Dilute</td>
<td>1.82</td>
<td>9.2</td>
<td>20.0</td>
<td>10.7</td>
<td>133</td>
</tr>
<tr>
<td>Chronic II (20 to 40 days)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated</td>
<td>4.53</td>
<td>9.5</td>
<td>27.2</td>
<td>11.1</td>
<td>154</td>
</tr>
<tr>
<td>Dilute</td>
<td>2.39</td>
<td>13.2</td>
<td>32.1</td>
<td>16.7</td>
<td>213</td>
</tr>
</tbody>
</table>

* Four to six rats per group.
† Before, with, and after addition of ADP.

measured by the method of Eneror and Stocken (19). As illustrated in Table 5 a significant ($P = 0.01$) increase of 24% was observed in the activity of this enzyme in fractions from acute triiodothyronine-treated rats. A similar (24%) but variable and statistically insignificant increase was also observed in the chronic II preparations. It would appear that this activity was essentially all mitochondrial ATPase in the chronic II preparations as suggested by the 90% inhibition with azide observed in two preparations. Only 68% inhibition was observed with azide, however, in two acute preparations.

Other than the thyroid hormone (triiodothyronine) itself acting as the stimulator to increase the ATPase activity, the possibility was considered that an endogenous metabolite present under the influence of the excess triiodothyronine was the direct stimulator. Free fatty acids are metabolites known to be both stimulators of mitochondrial ATPase (20) and to be taken up by the hyperthyroid heart in greater amounts (3, 21). Direct

TABLE 5
Mg²⁺-Stimulated Heart Mitochondrial ATPase in Triiodothyronine-Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of groups</th>
<th>ATPase activity (μmoles P/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without azide</td>
</tr>
<tr>
<td>Normal</td>
<td>5</td>
<td>198</td>
</tr>
<tr>
<td>Acute (6 to 13 days)</td>
<td>10</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>±SEM</td>
<td>±SEM</td>
</tr>
<tr>
<td>Chronic II (20 to 60 days)</td>
<td>9</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>±SEM</td>
<td>±SEM</td>
</tr>
</tbody>
</table>

* Mitochondria isolated with Nagarse incubation. † n = number of experiments with azide. ‡ Probability that the difference between the means of the normal and acute groups could occur by chance as determined by Student's t-test.
measurements of the free fatty acid content of several of the various heart mitochondrial fractions were made by a modified extraction and titration according to the method of Kelley (22). Of the three normal, four acute, and six chronic II triiodothyronine-treated heart mitochondrial fractions examined, no significant change in the concentration of free fatty acid was found, being 0.028 ± 0.001 μmoles/mg protein in the normal, 0.024 ± 0.004 in the acute, and 0.030 ± 0.007 in the chronic II groups.

**Structural Status of Heart Mitochondria.**

Concomitant with these measurements of the functional integrity of the rat heart mitochondria, the structural integrity of the same preparations was assessed as the degree of swelling in vitro in a decimolar alkaline salt solution. There were no differences in the susceptibility to such swelling in any of the groups between mitochondria isolated with or without Nagarse preincubation. The data were therefore compiled as single groups: normal, four; acute, six; chronic I (15 to 20 days), three; and chronic II (29 to 54 days), seven. The mean percent changes in optical density at 520 μm over the 30-minute observation period are presented graphically in Figure 1.

The isolated mitochondria of the acute T₃-treated rats did not demonstrate an increased susceptibility to swell in vitro in this assay system, decreasing in optical density only 19.6 ± 1.9% over 30 minutes, as compared to 17.3 ± 1.1% in normal rat heart mitochondria. Interestingly, three preparations isolated from rats treated for only 15 to 20 days with the oral doses of triiodothyronine (chronic I) demonstrated a 22.6 ± 2.3% decrease in optical density over 30 minutes, a rate of swelling 30% greater than normal. It may be recalled that these same chronic I preparations evidenced no impairment of oxidative phosphorylation.

With continued feeding of triiodothyronine in the drinking water for 29 to 54 days (chronic II), the rate of swelling of isolated heart mitochondria in vitro increased further, showing a decrease in optical density of 30.6 ± 3.9%, twice that of normal rat heart mitochondria and statistically significant with a P value of 0.05.

**Discussion**

This study has demonstrated that treatment with excess thyroid hormone (triiodothyronine) in vivo can result in a decrease in the efficiency of oxidative phosphorylation in heart mitochondria as in other tissues. Demonstration of such a change appears to depend on the species used and on the mode and duration of thyroid treatment. The rat is a more susceptible species than the dog. In the latter no decreases in efficiency of oxidative phosphorylation have been observed following treatment for as long as 6 months to 2 years (3, 5).

Even in the susceptible rat, however, the mode of treatment used to induce the hyperthyroid state determines the mechanism of the observed mitochondrial change. In this study comparison of two types of hyperthyroid states suggests that the decreased efficiency of oxidative phosphorylation found in heart mitochondrial fractions of the acutely

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induced, severely hyperthyroid rat may be due largely to "extra"-mitochondrial factors while that in the prolonged chronic, moderately hyperthyroid rat may result from direct changes in the mitochondria per se.

Not only the mode but also the duration of treatment is an important factor in demonstrating mitochondrial changes in the rat heart. As noted previously (4) and in this study, short-term (7 to 20 days) treatment with oral doses of thyroid hormone does not invoke changes at the mitochondrial level. Also, the severity of the clinical state observed, assessed in the rat as weight loss, was not directly related to the degree of change observed at the mitochondrial level. In both the acute studies here and when desiccated thyroid powder was administered in the diet (4), loss of body weight ensued but there were no demonstrable changes directly in the heart mitochondria. On the other hand, the regime used here to induce a chronic but moderate hyperthyroid state without weight loss did evoke mitochondrial changes following prolonged treatment. It would seem, therefore, that a long-term chronic, moderately hyperthyroid state may be a better experimental model to simulate the more often observed chronic clinical hyperthyroid state than the animal made acutely hyperthyroid by injecting large amounts of hormone subcutaneously. This was first suggested by Shelley et al. (23).

The decreased efficiency of oxidative phosphorylation observed in this study was characterized in both types of hyperthyroid heart mitochondria by a decreased rate of phosphorylation. In the acute state this may be explained by the significant increase in ATPase activity of the mitochondrial fraction. This could effect a net imbalance in the rates of hydrolysis and synthesis in the polarographic assay system. Whether this increased enzymatic activity is solely mitochondrial, however, is not clear. Fairhurst et al. (24) in their studies with the function of thyrotoxic rat liver mitochondria suggested that the decreased phosphorylative function was due to extramitochondrial factors. Indirect evidence in support of such factors in acute, thyrotoxic heart mitochondrial fractions was suggested here by the following.

First, by diluting the acute mitochondrial suspensions, an increase in the rate of phosphate esterification and a decrease in the rate of oxygen uptake were effected. The net result was a return of the P-O ratio to normal. Although this could be explained by a diluting out of the stimulating effect of triiodothyronine on both the oxidative and phosphorylative systems, it could also be explained in part by a diminution of an extramitochondrial ATPase. The finding of only partial suppression by azide of the increased ATPase activity of these acute fractions would tend to support the idea that an extramitochondrial factor may be important here. It does not seem likely that the factor is microsomal because recovery of microsomes requires prolonged centrifugation (90 to 100,000g for 1 hour versus the 7,000 to 8,000g for 10 minutes employed here to recover the mitochondria, 25). Also, it would have to be postulated that the contaminating microsomes were of very high ATPase activity to produce a significant increase in that of the bulk mitochondria observed here. The known metabolic stimulator of mitochondrial ATPase, free fatty acid (20), was likewise not responsible for the increased activity noted here because no increase in levels of free fatty acid either in these or the chronic II mitochondrial fractions was found.

The mechanism of the reduced phosphorylative efficiency of the chronic hyperthyroid heart mitochondria would appear to be different. Although a 24% increase in activity of mitochondrial ATPase was also observed, there were marked variations in the nine preparations examined, so that no statistically significant increase could be demonstrated. It may be noted, however, that in two preparations with increased ATPase activity, the activity could be completely suppressed by azide, suggesting that in this case it was essentially of mitochondrial origin as compared to that of the acute, thyrotoxic heart fractions.

The response of the chronic preparations to
dilution was also markedly different from that of the acute. Although dilution of the chronic preparations increased the rate of phosphate esterification, the increase in the rate of oxygen uptake was proportionately greater. The net result was no increase in the P-O ratio. This "unmasking" of the effect of excess triiodothyronine on the oxidative system by dilution of the suspension may suggest mitochondrial aggregation or that structural alterations in the mitochondria of chronically hyperthyroid animals had rendered the essential intramitochondrial sites inaccessible to substrates, oxygen and ADP, in the previously "concentrated" form. Gustaffson et al. (26) have presented electronmicroscopic evidence of increases in both size and number of skeletal muscle mitochondria isolated from rats in an anabolic hyperthyroid state for 3 weeks. Whether such increases in mitochondrial size may affect rates of accessibility of substrates into and out of the mitochondrion remains to be explored.

Anomalous behavior of four chronic preparations in the polarographic assay system may also suggest alterations in accessibility of substrates in chronic hyperthyroid rat heart mitochondria. Additions of 400 or 500 µM amounts of phosphate acceptor ADP in the polarographic assay systems of these preparations was accompanied by abnormally high P-O ratios of 4.19 to 6.75. These artificially high P-O ratios appeared to be due to premature cessation of the state-3 rate of respiration with ADP. When lower amounts (200 µM) of ADP acceptor were used, near normal P-O ratios were obtained. The cause of this abnormal behavior is as yet unexplained. Similar behavior has been observed in this laboratory in heart mitochondria isolated from dogs subjected to systemic hypoxia (27). Tarjan and Von Korff (28) have occasionally observed such anomalies with normal rabbit heart mitochondria and have suggested that the cessation of the rapid rate of respiration may reflect a brief time of high intramitochondrial ratios of ATP/ADP. Further study of the decreased oxidative phosphorylative capacity of long-term chronic hyperthyroid heart mitochondria with regard to the intra- and extramitochondrial nucleotide pools may be of importance.

In addition to the functional alteration of heart mitochondria accompanying prolonged treatment with small amounts of triiodothyronine, structural "fragility" in vitro was also demonstrated. The twofold increase in susceptibility of these mitochondria to swell in decimolar alkaline salt solutions was significant. The insignificant increase in swelling in the acute, thyrotoxic heart mitochondria would not seem to be due to an already swollen state on isolation. The more sensitive liver mitochondria isolated from these same rats did show increased rates of swelling in vitro (17). Electronmicrographs of heart mitochondria isolated from these two hyperthyroid states, acute and chronic, may aid in resolving the question of their structural status in vivo and in vitro.

It is possible that susceptibility to swelling in vitro may be a more sensitive index of thyroid-induced changes directly in the mitochondrion per se as first suggested by Tapley (29). As noted above, the impaired phosphorylative efficiency of the heart mitochondria of the acutely treated rats would appear to be due to extramitochondrial factors, and these same fractions showed no increased susceptibility to swell in vitro. On the other hand, the chronic regime appears to affect the function of mitochondria by affecting the mitochondrion directly. Likewise, the early onset of the chronic state (chronic I) was accompanied by increases in the rates of swelling when no functional impairment was noted. With more prolonged treatment the rate of swelling increased further and significantly impaired phosphorylative efficiency became apparent.

Whether the swelling of the chronic triiodothyronine-treated rat heart mitochondria represents a basic change in composition of the inner mitochondrial membrane, with which it has been associated (30), or an indirect change via an enzyme system that could affect the structure remains to be defined. It is of interest that Galindo et al. (31) have reported
changes in the fatty acid profile of phospholipids of rat liver mitochondrial membranes following prolonged treatment with small amounts of thyroxine. On the other hand, no such changes have been found in acute, thyrotoxic rats (32), nor have we observed as marked an increase in swelling in acute as in chronically triiodothyronine-treated rat liver mitochondrial fractions (17).

A final note may be made of the functional improvement in heart mitochondrial fractions isolated with Nagarse, as compared to those without Nagarse, from hyperthyroid but not from normal rats. Since the yield of mitochondria is increased in both cases, it may be considered whether Nagarse treatment yields a more heterogeneously affected mitochondrial population in the triiodothyronine-treated animals. The effectiveness of triiodothyronine in vivo at the mitochondrial level, therefore, may be dependent on the number of mitochondria affected within the time of a given course of treatment. Heterogeneity of thyrotoxic rat liver mitochondria has been demonstrated by Grief et al. (33) by the use of sucrose density gradient centrifugation. Whether such may be demonstrated in the heart should be of interest.

In conclusion, prolonged chronic treatment with triiodothyronine can produce detrimental effects on both structure and function of isolated rat heart mitochondria. Although further study is needed to determine the significance of these mitochondrial changes on the work capacity of the chronic hyperthyroid rat heart in vivo, it is suggested that this area of metabolism should not yet be excluded from considerations in the etiology of the cardiac failure observed in hyperthyroidism.

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