Early Effect of Thyroxine in Vivo on Rapidly Labeled Mitochondrial Protein Fractions and Respiratory Control*

(Received for publication, May 1, 1969)

PIERRE VOLFIN,‡ SURESH S. KAPLAI, AND D. RAO SANADI§

From the Department of Bioenergetics Research, Retina Foundation, Boston, Massachusetts 02114

SUMMARY

Incorporation of ¹⁴C-leucine, injected intraperitoneally into rats, reached a plateau in 30 min in the liver mitochondrial fractions. Part of the mitochondrial soluble protein, amounting to about 6% of the total protein, was labeled more rapidly by ¹⁴C-L-leucine than the rest of the protein. Thyroidectomy produced a decrease in the rate of labeling of all the fractions. A single dose of L-thyroxine (0.52 µg per g) stimulated the incorporation in the above rapidly labeled fractions of soluble protein distinctly within 60 min without appreciably affecting the other protein fractions. Respiratory rate during active phosphorylation of ADP (active or State 3 respiration) and after ADP was completely converted to ATP (controlled or State 4 respiration) were proportionately depressed in thyroidectomy, but only controlled respiration was increased after thyroxine treatment.

Thyroxine affects mitochondrial respiration and amino acid incorporation into protein. One hypothesis considers that the respiratory stimulation results from an increased production of mitochondrial respiratory assemblies under the stimulus of thyroid hormones (1-3) whereas another proposes an initial effect on mitochondrial function followed by mitochondrial proliferation (4-6). The earliest effect, observed within 2 min after administration of the hormone to thyroidectomized rats, was an increase in controlled respiration (State 4) of the isolated liver mitochondria. This stimulation was overcome by the addition of bovine serum albumin to the reaction medium, suggesting that the hormone or its metabolic derivative remained bound to the mitochondria and was responsible for the respiratory increase (5, 7). The stimulation of incorporation of ¹⁴C-leucine in vitro in the mitochondria was also observed within minutes under the influence of trifluorothyroxine and thyroxine in a system containing oxidizable substrate (8). Another early effect was the increased uptake in vitro of the labeled amino acid into microsomal protein, the first time point in these studies being 2 hours (9). This effect proceeded RNA synthesis and was presumably expressed through translational control. Subsequent phenomena, apparent after increasing periods of lag, included increase in nuclear RNA synthesis, RNA polymerase activity, and microsomal RNA content (2). Finally, an increase in basal metabolic rate indicative of a net increase in mitochondria or respiratory assemblies in the cells was observed (1, 3). The present experiments were designed to explore the relationship between the early effects of thyroxine on mitochondrial protein synthesis and respiratory changes. A preliminary report of these findings has been presented (10).

METHODS

Normal and thyroidectomized male albino rats weighing 50 to 100 g were obtained from Charles River Breeding Laboratories. Thyroidectomized animals received 0.25 mCi of ¹²⁵I 1 to 2 weeks after surgery and were maintained on a vitamin-enriched low iodine test diet (Nutritional Biochemicals). The basal metabolic rate was determined by measuring oxygen uptake by the resting rat in a closed chamber during an experimental period of 15 to 30 min. Only those thyroidectomized rats which showed depression in basal metabolic rate of 30 to 40% were selected. The animals were always fasted 10 to 16 hours before sacrifice.

All the reagents were of the highest purity grade. L-Thyroxine (free acid), purchased from Sigma, was dissolved freshly in 0.1 N NaOH and diluted in 0.85% saline. L-¹⁴C-Leucine uniformly labeled was obtained from New England Nuclear and DEAE-cellulose, microgranular DE-52, from Whatman. Fasted animals in groups of three were injected intraperitoneally with 5 µCi of ¹⁴C-leucine per 100 g of body weight and sacrificed at 15-, 30-, 60-, and 180-min intervals.

Preparation of Mitochondria—The rat liver was homogenized in a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. The medium contained 250 mM sucrose, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4. The homogenate was centrifuged twice at 1000 × g for 15 min, and the supernatant liquid was then spun at 6500 × g for 10 min. The pellet was washed four times, centrifugation being at 6500 × g each time, and suspended in the same medium for studies on respiratory control. The mitochondria were suspended in 0.15 M KCl at...
25 to 40 mg of protein per ml, when further fractionation was necessary.

Fractionation of Mitochondria—The mitochondrial suspension in 0.15 m KCl was subjected to sonic oscillations in a Branson sonifier for 2 min and then centrifuged at 5,000 × g for 10 min to remove unbroken mitochondria. The supernatant fraction, referred to as mitochondrial protein, was centrifuged at 100,000 × g for 1 hour. It was then passed through a Sephadex G-25 (fine) column (2.5 × 35 cm) to reduce the salt concentration and to remove any free amino acids. The column was previously equilibrated with 5 mM potassium phosphate, pH 7.5. The filtered protein, representing the fraction called total soluble protein, was fractionated on a DEAE-cellulose column (1.5 × 15 cm). Based on preliminary fractionation experiments by gradient elution, five main fractions were eluted with 5 mM, 20 mM, 40 mM, 50 mM, and 250 mM potassium phosphate, pH 7.5. The pattern of elution is shown in Fig. 1. It should be noted that the absorbance scan came to the base line after each peak. The routine procedure involved equilibrating the column with 5 mM buffer, loading with 60 to 70 mg of Sephadex-filtered protein, and fractionating into the above five fractions.

Preparation of Samples for Radioactivity and Protein Determination—The fractions were precipitated with 10% trichloroacetic acid (final concentration), and the precipitates were washed with 50% ethanol (v/v) and then with ether. In some experiments, the precipitates were extracted with hot 5% trichloroacetic acid to remove RNA. The dried precipitates were dissolved in formic acid, and an aliquot of the formic acid solution was evaporated on planchet under infrared lamp and counted in a proportional gas flow counter at 30% efficiency.

Protein Determination—Protein was determined by either biuret (11) or Lowry’s method (12). Results from both methods were in good agreement. For this purpose, part of the formic acid solution of the protein was separately evaporated and dissolved in 0.5 N NaOH.

Unit—Specific radioactivity refers to counts min⁻¹ mg⁻¹ protein and specific enzyme activity μmoles substrate utilized × min⁻¹ × mg⁻¹ protein.

Purification of Mitochondria on Sucrose Density Gradient—The mitochondria, washed four times as described above, were further purified by linear sucrose density gradient centrifugation. The gradient was prepared by mixing 1 m and 2.2 m sucrose solutions. Centrifugation was carried out in an SW-25 rotor with the Beckman model L-2 centrifuge for 2 hours at 60,000 × g. About five diffuse bands with small amounts of protein and a major mitochondrial protein band were observed. These purified mitochondria were subsequently disrupted by exposure to sonic oscillation and fractionated as usual.

Enzyme Activities in DEAE-cellulose Fractions—Spectrophotometric determinations of glutamate-oxaloacetate transaminase (13), malate dehydrogenase (14), glutamate-pyruvate transaminase (15), lactate dehydrogenase (16), glycerol 1-phosphate dehydrogenase (17), and glutamate dehydrogenase (18) activities were carried out in a Gilford multiple sample absorbance recorder.

The cytochrome content of the mitochondria was determined in a dual wave length spectrophotometer with the wave lengths and extinction coefficients recommended by Chance and Haggihara (19).

Studies on Respiratory Activities of Mitochondria—Respiratory measurements were made polarographically with a Clark oxygen electrode in a medium containing 250 mM sucrose, 10 mM Tris-HCl, 0.2 mM EDTA, 10 mM KCl, 4 mM potassium phosphate, and 33 mM MgSO₄ at pH 7.4. Temperature of the reaction was 30°. Two to 3 mg of mitochondrial protein and 200 to 300 nmoles of ADP were used per assay. The substrate was 5 mM glutamate plus 5 mM malate.

Respiratory control ratio refers to the State 3-State 4 ratio or ratio of active to controlled rates of respiration. The oxygen consumption is expressed in micromoles of oxygen × mg⁻¹ × hr⁻¹.

Thyroxine Dose—In all of these experiments 0.52 μg of L-thyroxine per g of body weight was injected intraperitoneally. This dose of thyroxine is considered to be in the physiological and noncatabolic range (2, 6). The duration of therapy and exposure to ¹⁴C-leucine are given in the tables.

Leucine Analysis—The leucine analysis was kindly performed by Dr. M. Elzinga in a Phoenix amino acid analyzer. Samples of protein (0.5 mg) were hydrolyzed in evacuated sealed tubes in 1 ml of 6 N HCl for 22 hours at 110°.

RESULTS

Incorporation of ¹⁴C-leucine into Mitochondrial Fractions—The specific radioactivity (counts min⁻¹ per μg of protein) of all the mitochondrial fractions reached a plateau within 30 min after administration of ¹⁴C-leucine in a single dose (Table I). The specific radioactivities of the 20, 40, and 50 mM fractions (designated Group I proteins for convenience) were about 2- to 3-fold higher compared to the 5 and 250 mM fractions (Group II proteins) or the “mitochondrial protein.” Since the leucine content of these fractions was nearly the same, the greater incorporation in Group I proteins is related to differences in the uptake of the amino acids.

Two different experiments were carried out to exclude the contribution of contaminating microsomal proteins to the radioactivity in the different mitochondrial fractions. In one experiment the four times washed mitochondria were further purified on a sucrose density gradient and then fractioned. The labeling pattern was similar to that observed with the washed mitochondria. The average specific activities from two sets of experiments were as follows: mitochondrial protein, 147; soluble protein, 150; 5 mM fraction, 140; 20 mM fraction, 232; 40 mM fraction, 205; and 50 mM fraction, 308. One of the 250 mM fractions was lost. In the other experiment the labeling pattern of fractions derived from microsomes by sonic oscillation and fractionation in an identical manner was determined, and a cor-

Fig. 1. Chromatographic pattern of mitochondrial soluble protein on DEAE-cellulose column (1.5 × 15 cm). The experimental details are described in the text.
Incorporation of $^{14}$C-leucine in mitochondrial protein fractions in normal rats

Each group consisted of pooled livers from three rats injected intraperitoneally with 5 $\mu$C of $^{14}$C-leucine per 100 g of body weight. Leucine determinations were carried out after hydrolysis with HCl by Dr. M. Elainga. The 5 mM, 20 mM, 40 mM, 50 mM, and 250 mM refer to the protein fractions eluted by the respective concentrations. Other experimental details were as in the text.

<table>
<thead>
<tr>
<th>No. of groups</th>
<th>Interval between injection and death</th>
<th>$^{14}$C-Leucine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mitochondrial soluble protein fraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM</td>
</tr>
<tr>
<td>2</td>
<td>min</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>140±5</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>150</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>144</td>
</tr>
<tr>
<td>Total soluble protein in fractions, %</td>
<td>12</td>
<td>4.3</td>
</tr>
<tr>
<td>Leucine in protein, %</td>
<td>9.5</td>
<td>9.0</td>
</tr>
</tbody>
</table>

It is of interest to determine the distribution of some typical mitochondrial enzymes relative to the distribution of the radioactivity. The 5 mM eluate had malate dehydrogenase, lactate dehydrogenase, and glutamate-oxaloacetate transaminase activities. Glutamate dehydrogenase, glycerol 1-phosphate dehydrogenase, and glutamate-pyruvate transaminase activities were present in the 250 mM fraction. The increase in specific activities of these enzymes (micromoles of substrate consumed $\times$ min$^{-1}$ $\times$ mg$^{-1}$ of protein) compared to the soluble protein from mitochondria was in agreement with the protein recoveries in these fractions. None of these activities was present in the rapidly labeled Group I proteins (Table III). It should be pointed out that all the fractions were assayed for each enzyme.

There was no significant change in the cytochrome content of the thyroidectomized mitochondria as compared to the normal (Table IV), and the levels were essentially unaltered after a 60- or 180-min thyroxine treatment.
**Table IV**

Cytochrome contents of mitochondrial fraction

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome b</th>
<th>Cytochrome a, a3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol/mg protein</td>
<td>mol/mg protein</td>
</tr>
<tr>
<td>Normal</td>
<td>0.087±0.001</td>
<td>0.363±0.07</td>
</tr>
<tr>
<td>Hypothyroid (thyroidectomized)</td>
<td>0.083±0.004</td>
<td>0.260±0.06</td>
</tr>
<tr>
<td>Thyroidectomized + thyroxine, 60 min</td>
<td>0.095±0.001</td>
<td>0.299±0.05</td>
</tr>
<tr>
<td>Thyroidectomized + thyroxine, 180 min</td>
<td>0.098±0.001</td>
<td>0.309±0.05</td>
</tr>
</tbody>
</table>

**Table V**

Oxygen consumption of isolated liver mitochondria from normal, hypothyroid, and hypothyroid rats treated with L-thyroxine

The thyroidectomized animals (T) were treated with 0.52 μg of L-thyroxine per g of body weight (T). The figures in parentheses show the number of animals in each group. In some experiments with thyroidectomized animals the State 4 oxygen consumption was 0.52 to 0.65 μatom X mg⁻¹ X hour⁻¹, which can be attributed to the more severe depletion of thyroxine. The substrate was a mixture of glutamate and malate.

<table>
<thead>
<tr>
<th>State 3</th>
<th>State 4</th>
<th>ADP:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>μatoms O₂/mg/hour</td>
<td>μatoms O₂/mg/hour</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>3.7 (3)</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>4.0 (4)</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>4.2 (3)</td>
<td>1.2</td>
</tr>
<tr>
<td>Thyroidectomized (control)</td>
<td>2.4 (3)</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2.0 (3)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2.6 (3)</td>
<td>0.8</td>
</tr>
<tr>
<td>Thyroidectomized + thyroxine (60 min)</td>
<td>2.4 (3)</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>3.2 (3)</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>2.3 (3)</td>
<td>1.45</td>
</tr>
</tbody>
</table>

**Effect of Thyroxine on Respiratory Activities of Mitochondria**

There was no remarkable difference observed in ADP:O ratios of mitochondria from normal, thyroidectomized, or thyroxine-treated rats. The active (State 3) and controlled (State 4) respiratory rates were depressed to about the same extent (about 30%); thus the respiratory control ratio remains unaffected by thyroidectomy. But after administration of a single dose of thyroxine, the State 4 rate was increased. There was no change in the State 3 rate so that the respiratory control ratio was appreciably lowered (Table V).

**Discussion**

**Respiratory Control**—Maley and Lardy had shown that the State 4 respiration (controlled respiration) in thyroidectomized animals was abnormally low whereas State 3 respiration remained unaffected, giving rise to a very high respiratory control ratio (21). Similar findings have been recently reported by Hoch (5). He also showed that only State 4 respiration was increased by a single dose of thyroxine. During our experiments on respiratory control, it was observed that the State 3 and State 4 respiration of mitochondria from thyroidectomized rats were both depressed by about 30%; thus no effect on respiratory control ratio is apparent. Similar findings were made by Bronk (4).

However, the increase in State 4 respiration observed in our experiments after thyroxine treatment was in good agreement with the earlier reports (4, 5). In confirmation of the earlier work, no change in P:O ratio was observed either during thyroidectomy or after thyroxine treatment (4, 5). The levels of cytochrome b, a, and a3 were unchanged during thyroidectomy, but thyroxine treatment did not change the levels (4).

These results reveal an approximate correlation in time between the appearance of changes in respiratory control and stimulation of amino acid incorporation into certain mitochondrial protein fractions under the influence of thyroxine treatment. Experiments with much shorter periods of time would be technically difficult since incorporation does not reach a maximum for 30 min.

**Differential Labeling of Mitochondrial Proteins**—Fletcher and Sanadi (22) found that several rat liver mitochondrial components, including cytochrome c, had the same turnover rate and proposed the hypothesis that the mitochondrion turned over as an entity. These results have been reproduced by some (23, 24) and questioned by others (25-27). The recent careful experiments of Swick, Rexroth and Stange (28) showed that several mitochondrial fractions had the same half-life but the two inducible enzymes, glutamate-pyruvate transaminase and ornithine-ketoglutarate transaminase, declined in activity with much shorter half-lives. Tschudy, Waxman and Collins (29) had shown earlier that the synthesis of δ-aminolevulinate synthetase also was extremely rapid in mitochondria. The present finding that some mitochondrial protein fractions (Group I proteins) were labeled much faster than others (Table I) is consistent with these more recent results. This type of differential labeling of the soluble proteins might have been overlooked in some of the earlier experiments in which mitochondrial protein and total soluble protein were counted (23, 30). The observation that thyroxine increased the labeling in the same Group I proteins without simultaneously affecting the other proteins gives a clue to at least one reason for the discrepancies in the turnover results obtained in the different laboratories. The metabolic state of the animal could apparently affect the synthesis and breakdown of some mitochondrial components while others appear to be more resistant. The more labile components may represent a set of "inducible" proteins with important metabolic and regulatory functions in mitochondrial respiration. The role of these proteins in mitochondrial metabolism remains to be elucidated. None of the six enzymatic activities examined in these experiments was found in these rapidly labeled Group I
proteins. A further speculation might be that these inducible proteins may be in the mitochondrial matrix since the membrane-bound proteins of mitochondria (e.g., succinate dehydrogenase, cytochrome oxidase, cytochrome b) are more difficult to obtain in solution.

The more rapid labeling of Group I proteins in these experiments could be due either to more rapid protein synthesis or to faster labeling of their precursor pools. Additional experiments would be needed to distinguish rigorously between these alternatives.

Effect of Thyroxine on Leucine Uptake into Proteins—Administration of thyroxine to thyroidectomized rats produced a marked stimulation of 14C-leucine incorporation into the mitochondrial Group I proteins without affecting the other fractions. It may be more than fortuitous that they respond to thyroxine treatment as rapidly as does mitochondrial respiratory control. These active proteins appear in three distinct fractions and probably represent at least three different proteins, suggesting multiple regulatory responses to thyroxine administration.

Stimulation of amino acid uptake into microsomal protein was observed 26 hours after administration of thyroid hormone in the experiments of Tata et al. (1). This effect may not be related to our results, which have shown much faster response. While our experiments were in progress (10), Sokoloff et al. (9) observed stimulation of the labeling in vitro of microsomal protein by triiodothyronine administration to euthyroid animals. The effect was small but apparent within 2 hours. These results may have bearing on the results in vivo reported here. Experiments with much shorter periods of time would be technically difficult since incorporation does not reach a maximum for 30 min. On the other hand, the rapid (within minutes) stimulation by thyroxine of amino acid incorporation in vitro into acid-precipitable material of mitochondria reported by Buchanan and Tapley (8) probably involves proteins different from the Group I proteins, since only the insoluble "membranous" protein of mitochondria is labeled during incubations in vitro while the Group I proteins are derived from the soluble protein fraction. Further experiments are needed before the role of these rapidly labeled proteins in the regulation of mitochondrial activity can be determined.

REFERENCES
