MECHANISMS OF STIMULATION OF PROTEIN SYNTHESIS BY THYROID HORMONES IN VIVO

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Numerous studies have established that thyroid hormones stimulate protein synthesis,1–8 but the mechanism of the stimulation remains obscure and controversial. The first observations of the stimulation in cell-free systems also provided evidence of mitochondrial participation in the mechanism of the effect.1 2 Mixing experiments with subcellular fractions from euthyroid and hyperthyroid rat liver implicated a change in the mitochondrial fraction as the cause of most of the increase in microsomal protein synthesis observed in hyperthyroid homogenates.

Thyroxine added directly in vitro to the identical cell-free system which exhibited the effects of in vivo hormone administration also stimulated amino acid incorporation into microsomal protein.1 2 The stimulation in vitro was localized to the transfer of transfer-RNA-bound amino acid to microsomal9 or ribosomal10 protein but was not dependent on any effect on RNA synthesis.11 Indeed, it was unaffected by inhibitors of RNA synthesis and was exerted equally well on synthetic polyribonucleotide-directed polypeptide synthesis.11 Recent studies with a reticulocyte lysate system have demonstrated that thyroxine can stimulate the synthesis of the α and β chains of hemoglobin and that the stimulation is a translational effect exerted primarily at a late stage in the assembly or elongation of the nascent polypeptide chain.12

In agreement with the evidence of mitochondrial involvement in the mechanism of the effect following its in vivo administration,1 2 the stimulation of microsomal protein synthesis by thyroxine in vitro exhibited an absolute requirement for mitochondria in the reaction mixture.2 9 11 Kinetic studies and studies of the partial reactions of the effect have demonstrated that it is not thyroxine itself but a consequence of a prior thyroxine-mitochondrial interaction which is responsible for the increased ribosomal protein synthetic activity.7 12 The studies carried out in this laboratory, both in vivo and in vitro, have provided several independent lines of evidence all consistently pointing to a stimulation of microsomal protein synthesis by thyroid hormones which is cytoplasmic in origin, localized at the level of translation and not of transcription, and secondary to a reaction between the hormone and the mitochondrial components of the cell.

More recently, other workers observed that the depressed rate of protein synthesis in cell-free preparations from thyroid-deficient rats could be raised toward normal by prior replacement therapy in vivo and that the effect was manifest in reaction mixtures devoid of mitochondria.3 4 The stimulation of protein synthesis did not become apparent, however, until at least 30 hours following a dose of hormone in vivo.4 In contrast, nuclear RNA turnover,13 RNA polymerase activity,14 and microsomal RNA content4 were found to be increased after latent
periods of only 3–4, 10, and 20 hours, respectively. These effects on RNA metabolism and protein synthesis were observed only after in vivo administration of the hormone; attempts to detect similar effects in vitro have been unsuccessful.\(^4\) On the basis of these and related observations, it was concluded that the stimulation of protein synthesis by thyroid hormones is secondary to the stimulation of nuclear synthesis of mainly ribosomal, and perhaps also messenger, RNA\(^1^5\) and that mitochondria are not involved in the mechanism of the effect.\(^4\)

There are then two apparently opposing concepts of the mechanism of action of thyroid hormones on protein synthesis and experimental evidence in favor of each. One proposes a cytoplasmic mechanism in which there is a mitochondria-dependent thyroid hormone stimulation of translational activity in the ribosomes independent of new RNA synthesis;\(^3\) \(^7\) \(^9\)–\(^1^2\) the other proposes a nuclear effect resulting in increased ribosomal synthesis.\(^4\) \(^1^4\) \(^1^5\) The results of the present studies may serve to resolve the conflict. They indicate that following in vivo administration there are two sequential mechanisms by which thyroid hormones increase protein synthesis: an early effect with essentially no latent period which is mitochondria-dependent and occurs before the reported changes in nuclear RNA metabolism; and a second, delayed effect which occurs several hours later in concert with an increase in the RNA content of the microsomes and is no longer dependent on the presence of mitochondria. The second effect may represent a secondary, nuclear-mediated, cellular response or adaptation to the effects of a primary cytoplasmic biochemical action of the thyroid hormone.

Materials and Methods.—Animals: Normal Osborne-Mendel male rats weighing between 100 and 160 gm were paired for age and weight. One of each pair was injected intraperitoneally with a single dose of 60 \(\mu\)g of sodium 3,5,3'-triiodo-L-thyronine (T\(_3\)) per 100 gm of body weight dissolved in 0.01 \(N\) NaOH (75 \(\mu\)g/ml); the other received an equal volume of 0.01 \(N\) NaOH alone. Paired animals were killed at either 2, 17, or 27 hr after injection. Paired cell-free preparations were made and assayed for protein-synthesizing activity simultaneously in parallel flasks. The animals were maintained on Purina laboratory chow and tap water until the last 16 hr before killing when they were allowed only 5% glucose in the drinking water ad libitum.

Chemicals and enzymes: Chemicals and enzymes were the same as those described previously.\(^2\) \(^9\) \(^1^1\) \(^1^2\)

Preparation of homogenates and cell fractions: Liver homogenates were prepared and fractionated fresh for each experiment by a combination of procedures B and C of Sokoloff and Kaufman.\(^2\) Microsomes and cell sap were separated from the liver homogenates of matched pairs of control and T\(_3\)-treated rats as in procedure B,\(^2\) and the mitochondria were prepared by procedure C\(^2\) from the homogenates of a second pair of animals, closely matched to the first and treated identically. Mitochondria and microsomes were isolated from separate homogenates by these two procedures to minimize contamination of one fraction by the other.

Assay of protein synthesis: Incubation conditions were the same as those previously described.\(^2\) The contents of the reaction mixtures are described in the legends to the tables and figure. The reactions were terminated by chilling and dilution with 5 ml of ice-cold 0.25 \(M\) sucrose solution containing 5 mg of unlabeled carrier amino acid per milliliter, and any mitochondria present were removed by centrifugation for 15 min at 12,800 \(\times\) \(g\) in the Servall refrigerated centrifuge. The supernatant fractions containing the microsomes and cell sap were then decanted into equal volumes of 12% trichloroacetic acid, and the precipitated protein was purified and assayed for specific activity as previously described.\(^1\) \(^2\) Microsomal protein specific activity was calculated by dividing
the total amino acid incorporation into the microsomal and soluble protein per flask by the amount of microsomal protein added per flask. The assumption that the soluble protein is negligibly labeled compared to the microsomal protein was verified in control experiments in which they were purified and assayed for specific activity separately.

**Miscellaneous assay procedures:** Protein contents of the cell fractions were assayed by the method of Lowry et al. or calculated from the protein nitrogen content determined by the micro-Kjeldahl technique. RNA was measured by the method of Fleck et al. 17, 18

**Results.**—Early effects of triiodothyronine on protein synthesis: Tata et al. have reported a latent period of 30 or more hours between the time of triiodothyronine administration *in vivo* and the appearance of the stimulation of protein synthesis in cell-free liver preparations *in vitro*. In contrast, in this laboratory much earlier stimulations are routinely observed. For example, a statistically significant increase in the rate of amino acid incorporation into microsomal protein is already well established within two hours following a single dose of T3 (Table 1). This is the earliest time studied, but it is already earlier than the reported stimulation of nuclear RNA turnover. 14

**Mitochondrial requirement for early effect of triiodothyronine on microsomal protein synthesis:** Because of the mitochondrial requirement in the stimulation of microsomal protein synthesis by thyroid hormones *in vitro*, 9, 11 mitochondria were also included in the system used to assay the effects of T3 administration *in vivo*. The assay system used by Tata et al. did not contain mitochondria.

**Table 1.** Role of mitochondria in the early stimulation of amino acid incorporation into microsomal protein by a single dose of triiodothyronine *in vivo*.

<table>
<thead>
<tr>
<th>Series</th>
<th>Time after T3 dose (hr)</th>
<th>No. of paired expts.</th>
<th>System</th>
<th>Microsomal Protein Specific Activity* (cpm/mg protein)</th>
<th>T3 effect (Acpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>11</td>
<td>C†</td>
<td>123 ± 7 145 ± 7</td>
<td>+22 ± 8†</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>8</td>
<td>C</td>
<td>126 ± 16 166 ± 17</td>
<td>+40 ± 16†</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>9</td>
<td>C</td>
<td>132 ± 5 155 ± 8</td>
<td>+23 ± 9†</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>12</td>
<td>M†</td>
<td>128 ± 7 123 ± 9</td>
<td>5 ± 9</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>11</td>
<td>C</td>
<td>130 ± 9 164 ± 11</td>
<td>+34 ± 11†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>153 ± 10 165 ± 13</td>
<td>+12 ± 11</td>
<td></td>
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</tbody>
</table>

The contents of the complete system were as follows: sucrose, 158 μmoles; potassium phosphate buffer, pH 7.4, 20 μmoles; MgCl2, 5 μmoles; guanosine 5′-phosphate, 0.25 μ mole; adenosine 5′-phosphate, 5 μmoles; sodium DL-β-hydroxybutyrate, 50 μmoles; DL-leucine-1-C14 (specific activity = 5.4 me/μmole), 0.8 μ mole; 0.15 ml of mitochondrial suspension, 0.15 ml of microsomal suspension, and 0.15 ml of cell sap prepared from the livers of either control or matched T3-treated rats (see Materials and Methods); and water sufficient to bring the reaction mixture to a final volume of 1.7 ml. The reaction mixtures lacking mitochondria and DL-β-hydroxybutyrate were assayed simultaneously in parallel flasks. The contents of these flasks were identical to those of the complete system, except that the mitochondrial suspension was replaced by 0.15 ml of 0.25 M sucrose solution, and the DL-β-hydroxybutyrate was replaced by 35 μmoles of creatine phosphate and 0.25 mg of creatine kinase contained in an equivalent volume. The incubation mixtures were incubated at 37°C for 25 min in a shaking waterbath. The reaction was terminated, and the microsomal protein was separated, purified, and assayed for specific activity as described in Materials and Methods. Radioactivity was measured with a thin-window, low-background, Geiger-Mueller gas-flow counter (Tracerlab Omni-Guard model BLB-575A). Sufficient counts were collected to achieve a coefficient variation of less than 2%.

* All values are means ± standard errors.
† Denotes statistically significant difference (p value less than 0.05 as determined by method of paired comparison).
‡ C = complete; M = minus mitochondria and β-hydroxybutyrate; plus creatine phosphate and creatine kinase.
Fig. 1.—Time course of the changes in liver microsomal protein synthesis, assayed in the presence and absence of mitochondria in the assay system, and in the cytoplasmic RNA contents following a single dose of triiodothyronine to euthyroid rats. The percentage effects are the means of the individual percentage effects obtained in the experiments described in Table 1 (series B) and Table 2. In each experiment, microsomal protein synthesis was assayed simultaneously in cell-free liver preparations from paired control and T₃-treated rats in the presence and absence of mitochondria in the assay system. The assay conditions are described in Table 1. The dotted line (mitochondria-dependent stimulation of microsomal protein synthesis) is calculated by subtracting the percentage effect in the absence of mitochondria from the percentage effect in their presence; it is assumed that the latter is the total effect representing the sum of the mitochondria- and nonmitochondria-dependent effects. The encircled points represent statistically significant changes (p < 0.05).

Therefore, experiments were carried out to determine whether the presence of mitochondria was responsible for the earlier effects observed in this laboratory. Microsomal protein synthesis was assayed in the complete system containing mitochondria and simultaneously in parallel flasks containing the identical system, except for replacement of the mitochondrial components by a creatine phosphate-adenosine 5'-triphosphate generating system. Control rates of amino acid incorporation into microsomal protein were essentially the same in both systems (Table 1, series B). In the system containing mitochondria, a progressively increasing stimulation by T₃ was already present and statistically significant within two hours after its administration (Table 1, series B; Fig. 1). In contrast, in the mitochondria-free system, statistically significant effects did not appear until 27 hours after the hormone administration. The latent period of the latter effect was, therefore, very similar to the one described by Tata et al.¹ The results of these experiments indicate, however, that thyroid hormones stimulate protein synthesis with almost no latent period, but that mitochondria are an essential requirement of the assay system in order to detect the earliest effect.

Effects of triiodothyronine administration on RNA contents of tissue fractions: The RNA contents of the cellular components of the protein synthesis assay system followed a time course almost identical to that of the delayed or nonmitochondria-dependent stimulation of protein synthesis. No statistically significant changes in RNA were observed until 17 hours after T₃ administration when the microsomal RNA:protein ratio was slightly increased (Table 2; Fig. 1). This ratio increased further at 27 hours. Total RNA content of the cellular fractions did not increase statistically significantly until 27 hours when the nonmitochondria-dependent stimulation of protein synthesis also first became significant.
protein synthesis occurred before any change in RNA content of the tissue fractions (Table 2; Fig. 1).

Discussion.—The present studies demonstrate that a single small dose of T₃ stimulates liver microsomal protein synthesis without any appreciable latent period. The over-all response extends over a number of hours and consists of at least two components which are separated in time, distinctive in properties, and probably caused by different mechanisms (Fig. 1). The initial effect occurs almost immediately and is characterized by a complete dependency on the presence of mitochondria in the assay system. The second component is a delayed effect which lags behind the onset of the first by several hours, does not require the presence of mitochondria, and appears almost in concert with an increase in the content of cytoplasmic RNA.

The initial effect commences and reaches a peak before any detectable change in cytoplasmic RNA contents and earlier than the reported increase in nuclear RNA turnover or RNA polymerase activity.14 It is not, therefore, secondary to increased RNA synthesis. Its time of onset and mitochondrial dependency are consistent with recent findings by Hoch15, 20 of comparably early thyroxine effects on mitochondrial respiratory control and energy-conserving functions. The early stimulation of protein synthesis can also be reproduced by thyroid hormones added in vitro to cell-free systems similar to the one used in the present studies.1, 2 The in vitro effect shows the same requirement for mitochondria in the assay system and independence of an effect on RNA synthesis.3, 11 It differs from the in vivo effect in regard to sensitivity; the maximum concentration of T₃

* Denotes statistically significant effect (p value less than 0.05 determined by method of paired comparison).

Table 2. Effects of a single dose of triiodothyronine on the RNA contents of the components of the protein synthesis assay system.

<table>
<thead>
<tr>
<th>Time</th>
<th>Total RNA Contents of Cell Sap and Microsomal Contents</th>
<th>Protein Synthesis of Cell Sap</th>
<th>Protein Synthesis of Microsomal Contents</th>
<th>Prophase Content per Flask</th>
<th>Postphase Content per Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Control (mg/mg flask)</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>17</td>
<td>T₃ (mg/mg flask)</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>27</td>
<td>T₃ (mg/mg flask)</td>
<td>0.017</td>
<td>0.017</td>
<td>0.017</td>
<td>0.017</td>
</tr>
</tbody>
</table>

The methods of assay for protein and RNA are described in Materials and Methods.
which could have occurred in the liver with the dose used in the present studies would, if added \textit{in vitro}, have been insufficient to achieve the degree of mitochondria-dependent stimulation which was observed.\textsuperscript{2} The results of the present studies suggest, therefore, that the \textit{in vitro} effect may be a relevant model of the initial action of thyroid hormones \textit{in vivo}. Studies with this model system have indicated that the thyroxine stimulation of protein synthesis is a translational effect at the level of elongation or completion of the nascent polypeptide chain which is not a direct action of thyroxine but secondary to a preceding mitochondria-thyroxine interaction.\textsuperscript{7, 9, 12, 13} It is this preliminary reaction which may account for the mitochondrial requirement in the thyroid hormone stimulation of protein synthesis.

It has been claimed that all early mitochondrial effects of thyroid hormones are manifestations of "toxic" actions and, therefore, physiologically irrelevant.\textsuperscript{21} The basis of this allegation is the purported failure to detect early changes in selected mitochondrial functions with small doses of thyroid hormones which were, nevertheless, sufficient to stimulate growth in stunted, athyreotic rats;\textsuperscript{4} only larger doses which retarded growth or caused loss of body weight produced such effects.\textsuperscript{4, 21} The distinction between physiological and "toxic" actions seems arbitrary because thyroid hormones also have a physiological function in fully grown animals; in these, negative nitrogen balance is a more characteristic manifestation of their action than increased growth. Moreover, there is no compelling evidence that gross toxicity in the animal as a whole in hyperthyroidism represents a qualitative rather than quantitative difference in the fundamental biochemical mechanism of action of the hormone. For example, hypoglycemia is a physiological effect of insulin which is beneficial in diabetes when quantitatively appropriate but causes coma and death when excessive; the mechanism of the hypoglycemia is, however, the same. Furthermore, the association of mitochondrial effects with "toxic" actions was conceived on the basis of essentially negative evidence. Failure to detect mitochondrial effects with small, "growth-promoting" doses might also be due to irrelevancy of the particular mitochondrial functions examined, inadequate sensitivity of the assay method, or inappropriate assay conditions. The last possibility is particularly pertinent to the studies purporting to prove lack of early mitochondrial effects with physiological doses of thyroid hormones.\textsuperscript{4} Effects on oxidative phosphorylation were sought in the presence of bovine serum albumin and relatively high Mg\textsuperscript{++} concentrations, both of which reverse the uncoupling action of thyroid hormones.\textsuperscript{22-24} Mitochondrial swelling was assayed in the presence of phosphate which is itself a potent swelling agent like thyroid hormones.\textsuperscript{25, 26} Even if present, the assay conditions would have minimized the possibility of finding such effects. It was also concluded that mitochondrial respiratory control was not affected, but the actual data showed a rise in respiratory control index in hypothyroid liver mitochondria and a lowered value in liver mitochondria from rats treated with chronic but still growth-promoting doses of thyroxine;\textsuperscript{4} the data were insufficient, however, to assess the statistical significance of the changes. In fact, Hoeh\textsuperscript{19} has found very early effects on mitochondrial respiratory control with minute, sub-calorogenic doses of thyroxine. In the present studies a single relatively small
dose of hormone for a euthyroid animal was used; although convenient, it was not the minimal dose required to produce the mitochondria-dependent stimulation of protein synthesis. There is, therefore, little reason to doubt its physiological relevance.

The second or delayed component of the T₃ stimulation of protein synthesis observed in the present studies has the same properties as the effect studied by Tata and his associates and which they attributed to increased nuclear RNA synthesis and ribosome formation. It differs from theirs essentially only in magnitude. Both the mitochondria-independent increase in protein synthesis and the associated changes in cytoplasmic RNA were smaller than those reported by them, partly, perhaps, because normal rather than athyreotic animals were used in the present studies, but probably mainly because the animals were fasted except for glucose in the drinking water. In contrast to the mitochondria-dependent stimulation of protein synthesis which is unaffected by fasting or feeding, Tata et al. have found all their observed effects on protein and RNA synthesis to be obliterated by mild fasting.

It appears then that the increased rate of protein synthesis which follows the administration of thyroid hormones in vivo is achieved by two distinct mechanisms operating sequentially. There is first a cytoplasmic mitochondria-dependent stimulation of polypeptide assembly at the level of translation, followed several hours later by a second effect at the level of transcription resulting in the formation of additional protein synthesizing units within the cell. The initial effect is probably closer to the primary chemical action of the hormone, since it occurs without any significant latent period and can be reproduced in cell-free systems in vitro. The thyroid hormone molecule may not even participate directly in the mechanism of the delayed response which lags behind the initial cytoplasmic effect and may therefore be initiated by intracellular changes arising from it. Attempts to stimulate nuclear RNA synthesis by thyroid hormones in vitro have been unsuccessful, suggesting that it is not the hormone itself which produces this effect. Fasting blocks the stimulation of RNA synthesis or the delayed effect on protein synthesis; it is difficult to conceive how the mild nutritional deficiency caused by a short fast could block the primary chemical action of the hormone. The properties of the delayed effects are more consistent with a secondary cellular response or adaptation to the consequences of the initial effect.

The complete effect of thyroid hormones on protein synthesis in vivo combines the elements of an initial and positive-feedback response. There is first a cytoplasmic stimulation of the existing protein-synthesizing machinery; the cell responds by increasing its content of protein-synthesizing machinery. Since it probably involves the genome, the secondary response may have a greater potential for specificity. For example, the obviously altered pattern of gene expression which occurs in thyroxine-induced amphibian metamorphosis is more likely mediated by the secondary nuclear-dependent response. On the other hand, in the normal, mature, adult mammal the primary cytoplasmic effect may be the more important one.
Summary.—A single dose of triiodothyronine to a euthyroid animal stimulates liver microsomal protein synthesis within less than two hours. This early effect is manifested only if mitochondria are present in the assay system, and it precedes any change in the cytoplasmic RNA content. The initial effect is followed several hours later by a second delayed increase which is no longer mitochondria-dependent and is associated with an increase in cytoplasmic, mainly microsomal, RNA. It is suggested that the stimulation of protein synthesis by thyroid hormones in vivo consists of two components: (1) an initial, cytoplasmic, mitochondria-dependent stimulation of the existing protein synthesizing apparatus, followed by (2) a secondary, nuclear-mediated, cellular response or adaptation which leads to an increase in the amount of protein-synthesizing machinery.

11 Sokoloff, L., C. M. Francis, and P. L. Campbell, these PROCEEDINGS, 52, 728 (1964).
20 Hoch, F. L., these PROCEEDINGS, 58, 506 (1967).