Studies on Triiodothyronine-Induced Synthesis of Liver Mitochondrial α-Glycerophosphate Dehydrogenase in the Thyroidectomized Rat

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SUMMARY

Administration of a single small dose of triiodothyronine (T_3) greatly increased liver mitochondrial L-α-glycerophosphate dehydrogenase activity of thyroidectomized rats.

The induction of liver mitochondrial L-α-glycerophosphate dehydrogenase by T_3 could be blocked by simultaneous administration of puromycin; in addition, the incorporation of L-leucine-^{14}C into total liver proteins and into proteins of all subcellular fractions was greatly inhibited. Furthermore, puromycin blocked further induction when it was administered after the administration of T_3. L-Ethionine prevented the induction of enzyme formation, and the inhibition could be partially reversed by L-methionine. "Pulse labeling" was used to study the incorporation of L-leucine-^{14}C into liver proteins, and the data indicate that an increased rate of protein synthesis precedes the maximal increase in liver mitochondrial L-α-glycerophosphate dehydrogenase produced by T_3-administration. These observations suggest that T_3-induction of L-α-glycerophosphate dehydrogenase in the thyroidectomized rat results from acceleration of enzyme synthesis. Similar observations were reported earlier with the euthyroid rat.

Administration of actinomycin D along with the T_3 abolished the increase of L-α-glycerophosphate dehydrogenase in liver mitochondria. The induction was also partially inhibited by 5-fluorouracil. These results indicate that the induction process depends on the formation of an adequate amount of renewable messenger-RNA molecules.

INTRODUCTION

A number of tissue enzyme concentrations are known to be altered by changing the thyroid status (1); hepatic cytochrome-linked L-α-glycerophosphate dehydrogenase, localized in the mitochondria (LM-GPDH), is the most responsive enzyme so far studied. The enhancement of the oxidation of L-α-glycerophosphate by liver mitochondria obtained from rats fed desiccated thyroid gland and the demonstration that thyroidectomized rats have a very low LM-GPDH activity which is rapidly restored by administration of thyroxine were first reported by Y.-P. Lee *et al.* (2) and Lardy *et al.* (3). The observations have since been confirmed in this laboratory (4) and by others (5, 6). Recently Y.-P. Lee and Lardy (7) have extended the study of this phenomenon and have noted that all organs known to respond to thyroid hormone administration by increased oxygen consumption also show an increase in their mitochondrial L-α-glycerophosphate dehydrogenase activities. Furthermore, there is good correlation between ability of thyroid hormone analogs to induce LM-GPDH activity and their hormonal activity (8). These observations, along with the finding that LM-GPDH activity is adaptively increased after administration of thyroid hormone to either euthyroid (9) or thyroid-

ectomized rats (10) and decreased after hypophysectomy (11), thyroidectomy (3, 12), or feeding a diet containing thiouracil (6, 8), strongly suggest that LM-GPDH activity is controlled by the hormone. The significance of this observation is, as suggested by Lardy *et al.* (3), that the increased L-α-glycerophosphate dehydrogenase activity may accelerate the oxidation of cytoplasmic NADH through the glycerophosphate cycle (13, 14). This enhanced oxidation of NADH may represent an advantage to the cell since mitochondrial oxidations yield high-energy phosphate bonds more efficiently than do nonmitochondrial oxidations. In addition, glycerophosphate is the precursor of phospholipids and triglycerides and possibly this induction may also influence lipid metabolism. Direct evidence to show the effect of glycerophosphate on fatty acid synthesis has been reported (15). The early report of Y.-P. Lee *et al.* (2) indicates that the increase in LM-GPDH by thyroid hormone in the euthyroid animal may result from acceleration of the synthesis of LM-GPDH as judged by the inhibitory effect of ethionine. Similarily, Sellinger and K.-L. Lee (9) reached the same conclusion based on their observations that the increase of LM-GPDH in euthyroid animals caused by a single injection of triiodothyronine (T_3) could be prevented by puromycin or actinomycin D. Recent observations in our laboratory indicate possible differences in biological half-life of the induced LM-GPDH in euthyroid and thyroidectomized rats. Therefore, the effect of puromycin, ethionine, actinomycin D, or 5-fluorouracil on T_3-induced synthesis of LM-GPDH has been studied in the thyroidectomized animal. These inhibitors prevent the induction of LM-GPDH by T_3-administration in thyroidectomized rats as effectively as in intact animals and indicate a basic similarity between euthyroid and thyroidectomized rats in response to T_3-administration.

MATERIALS AND METHODS

Animals and materials. All thyroidectomized animals utilized in these studies weighed 180–230 g and were obtained from the Charles River Laboratories. The rats, which were not used until 4 weeks after receipt, were fed ad libitum on Purina laboratory chow diet and were allowed free access to drinking water containing 1% calcium gluconate. The completeness of thyroidectomy was indicated by a slower gain in body weight and smaller liver weight than in intact animals. Occasionally the ability to retain $Na^{131}I$ was also tested.

T_3 and DL-α-glycerophosphate were purchased from Sigma Chemical Company. Puromycin hydrochloride was obtained from Nutritional Biochemicals Corporation, and L-ethionine from Mann Research Laboratories. Actinomycin D was kindly supplied by Merck, Sharp and Dohme. 5-Fluorouracil was purchased from Hoffmann-La Roche Company. NADH-linked glycerophosphate dehydrogenase from rabbit muscle and L-leucine-^{14}C (uniformly labeled, specific activity 74 mC/mmole) were obtained from Calbiochem Company.

Experimental conditions. T_3, prepared in an alkaline-saline solution (16), was injected at a dose level of 25 μg per 100 g body weight whereas control rats received an equal volume of alkaline-saline. In studying the effect of ethionine (25 mg per 100 g of body weight) and actinomycin D (100 μg per 100 g of body weight) on the increase of LM-GPDH activity after T_3-administration, a single dose of either inhibitor was given to the rats at zero time; that is, at the same time that T_3 was administered. Rats were sacrificed 18 hr (actinomycin D) or 22 hr (L-ethionine) after injection. The choice of the dosages of actinomycin D and ethionine was based on the observation of Schwartz *et al.* (17) that administration of actinomycin D at a dosage of 100 μg per 100 g body weight blocked 90% of the nuclear synthesis of RNA in rat liver 30 min after its injection, and also the observation of Villa-Trevino *et al.* (18) that the administration of DL-ethionine at a dosage of 50 mg per 100 g of body weight to rats resulted in 75% inhibition of L-leucine-^{14}C incorporation into liver protein (microsomal system) and caused the depletion of the hepatic ATP concentration to 35% of the control value. As

previously reported (9) puromycin, at a dosage of 3 mg per 100 g of body weight, was given to the rats at hourly intervals for 5 hours starting at zero time. 5-Fluorouracil was injected 4 times, at a dosage of 50 mg per 100 g body weight, at intervals of 4.5 hr starting at zero time. The choice of the frequency and the dosage of 5-fluorouracil was based on the observations of Nemeth (19), who observed that 5-fluorouracil at a dosage of 25–50 mg per 100 g body weight given 4 times did not interfere with the incorporation of valine-^{14}C into liver proteins but was able to prevent the developmental and adaptive formation of tryptophan pyrrolase. The rats were sacrificed 18 hr after the administration of 5-fluorouracil. L-Leucine-^{14}C was given (3 μC per 100 g body weight) either at zero-time plus 2 hr, or for pulse labeling (8 μC per 100 g body weight), 10 min before the rats were sacrificed. All substances were administered to rats intraperitoneally.

Methods. Rats were killed by decapitation. Livers were rapidly excised and chilled on crushed ice, weighed, and homogenized in 0.25 M sucrose in a homogenizer tube fitted with a Teflon pestle. The subcellular fractionation of liver homogenates was carried out according to the procedure of de Duve *et al.* (20) with Spinco Model L centrifuge. The mitochondrial pellet was washed twice and resuspended in 0.25 M sucrose so that 1 ml contained mitochondria from 0.3 to 0.5 g of liver (original wet weight).

The activity of LM-GPDH was determined by incubation of 0.5 ml to 1 ml of mitochondrial suspension with 0.5 ml of 0.3 M sodium phosphate buffer, pH 7.6; 0.7 ml of 0.23 M DL-α-glycerophosphate; 0.5 ml of 1.3 M sucrose, and water added to make the total volume of 3 ml. After 45 min of incubation (Dubnoff metabolic shaking incubator, 30°), 0.25 ml of 70% perchloric acid was added, the mixture was cooled, and water was added to make the total volume 5 ml. The precipitate was removed by centrifugation, 4-ml aliquots were neutralized with KOH (pH 7.1–7.3), and the insoluble potassium perchlorate was also removed by centrifugation. Aliquots of the supernatant solution, 0.1–0.2 ml, were used to assay for the dihydroxyacetone phosphate formed during the incubation using NADH-linked L-α-glycerophosphate dehydrogenase in the presence of 0.4 mM Tris buffer, pH 7.2, and following the disappearance of NADH with a Beckman DK spectrophotometer (21). The protein was determined by the method of Lowry *et al.* (22). In measuring the incorporation of L-leucine-^{14}C the radioactive protein was precipitated with cold trichloroacetic acid and purified according to Siekevitz (23). The purified protein samples were dissolved in 0.05 N NaOH at a concentration of approximately 5 mg/ml. One-half milliliter of this solution was added to scintillation fluid (8), and the radioactivity determined with a Packard Tri-Carb liquid scintillation counter.

RESULTS

Puromycin is an effective inhibitor of protein synthesis (24) and has been extensively used in studying enzyme induction in both bacterial (25) and mammalian systems (26). Figure 1 and Table 1 demonstrate the inhibitory effect of puromycin administration on the increase of LM-GPDH activity induced by T_3 administration. If puromycin was administered at zero time and hourly for the succeeding 5 hr there was essentially no enzyme induction and also the incorporation of L-leucine-^{14}C into proteins of the liver was greatly inhibited (Table 1). When the administration of puromycin was delayed for either 12 or 18 hr after T_3-administration, the induction process was stopped (Fig. 1). These observations are consistent with the hypothesis that protein synthesis is required during the induction process. In control experiments, puromycin had no effect on endogenous enzyme level over a period of 6 hr.

Ethionine, an analog of methionine, has been shown to inhibit protein synthesis and this inhibition can be reversed by administration of methionine (27). L-Ethionine proved to be an effective inhibitor of T_3-induction of LM-GPDH in thyroidectomized animals, and its inhibitory effect could be partially reversed by the simulta-

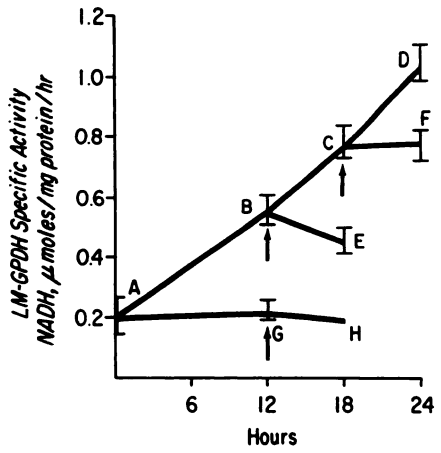

FIG. 1. *The effect of puromycin on the induction of LM-GPDH by T_3*

Rats were treated as follows: Group A was killed at zero time, groups B, C and D received T_3 at zero time and were killed after 12 hr, 18 hr, and 24 hr, respectively. Group E received T_3 at zero time and puromycin at zero plus 12 hr, and at hourly intervals for the succeeding 5 hr, and was killed at 18 hr. Group F received T_3 at zero time and puromycin at zero plus 18 hr, and at hourly intervals for the succeeding 5 hr, and was killed at 24 hr. Group G received saline at zero time and was killed after 12 hr. Group H received saline at zero time and puromycin at zero plus 12 hr and at hourly intervals for the succeeding 5 hr, and was killed at 18 hr. Vertical arrows indicate beginning of puromycin administration. Vertical bars indicate range of values taken from at least 3 rats (except *H* = one rat).

neous administration of an equimolar amount of L-methionine (Table 2). Ethionine had no effect on the endogenous level of LM-GPDH.

Actinomycin D has been shown to inhibit DNA-dependent RNA synthesis (28) and has been applied in the study of the nature of the induction process (29–31). This antibiotic did not decrease the endogenous LM-GPDH level for at least 18 hr. However, an identical dosage of actinomycin D, when administered along with T_3 to the animal, prevented the induction of LM-GPDH (Table 2). These results are consistent with the view that the induction process depends on the formation of an adequate amount of renewable messenger-RNA molecules. In this context, Sellinger and K.-L. Lee (9) have observed that approximately 8 hr is required for the formation of the messenger-RNA, presumably specific for the synthesis of LM-GPDH in the euthyroid rat. Presumably 8 hr is also the approximate time required to form such specific messenger-RNA in thyroidectomized rats.

5-Fluorouracil has been shown to inhibit mammalian as well as bacterial enzyme induction (19, 32). Its activity in preventing the increase of LM-GPDH activity produced by T_3 was similar to that of actinomycin D, although 5-fluorouracil is not

TABLE 1

Effect of puromycin on LM-GPDH induction and amino acid incorporation into proteins in thyroidectomized rat liver

T_3 was administered at zero time, and puromycin was given at 0, 1, 2, 3, 4, and 5 hr. L-Leucine-^{14}C (3μC/100g) was administered 4 hr after start of experiment. All rats were sacrificed at zero time plus 6 hr. For experimental details see Methods.

	Substance administered			Radioactivity of protein (cpm/mg protein)		
Expt. No.	T_3	Puromycin	LM-GPDH (NADH μmoles/mg protein/hr)	Total liver	Mitochondrial fraction	Microsomal and supernatant fraction
I	−	−	0.19	390	244	371
	+	−	0.34	453	287	484
	+	+	0.18	12	8	11
II	−	−	0.21	338	190	310
	+	−	0.36	483	267	418
	+	+	0.22	16	13	12

TABLE 2

The effect of L-ethionine, actinomycin D, and 5-fluorouracil on the onset of the induction of LM-GPDH by T_3

T_3 was prepared in alkaline saline (16) and administered at a dosage of 25 μg per 100 g body weight. A single dose of L-ethionine or actinomycin D at a dose level of 25 mg and 100 μg, respectively, was administered to the rats at the same time as T_3 was given. 5-Fluorouracil was injected 4 times, at a dosage of 50 mg per 100 g body weight at 4 and half-hour intervals starting at zero time. All substances were prepared in saline and administered intraperitoneally. Rats were sacrificed 18 hr after injection of either actinomycin D or 5-fluorouracil or 22 hr after injection of L-ethionine.

Expt. No.	Treatment: Inhibitors	Treatment: T_3	Number of animals	LM-GPDH activity (NADH μmoles/mg mitochondrial protein/hr)
I	None	−	3	0.31 ± 0.016[a]
	None	+	4	1.04 ± 0.074
	L-Ethionine	−	3	0.30 ± 0.021
	L-Ethionine	+	4	0.38 ± 0.052
	L-Ethionine and L-methionine	+	3	0.83 ± 0.085
II	None	−	4	0.20 ± 0.022
	None	+	4	0.75 ± 0.043
	Actinomycin D	+	3	0.25 ± 0.038
	Actinomycin D	−	3	0.19 ± 0.014
III	None	−	3	0.28 ± 0.013
	None	+	3	0.77 ± 0.019
	5-Fluorouracil	+	4	0.55 ± 0.025
	5-Fluorouracil	−	3	0.24 ± 0.012

[a] Standard error of mean.

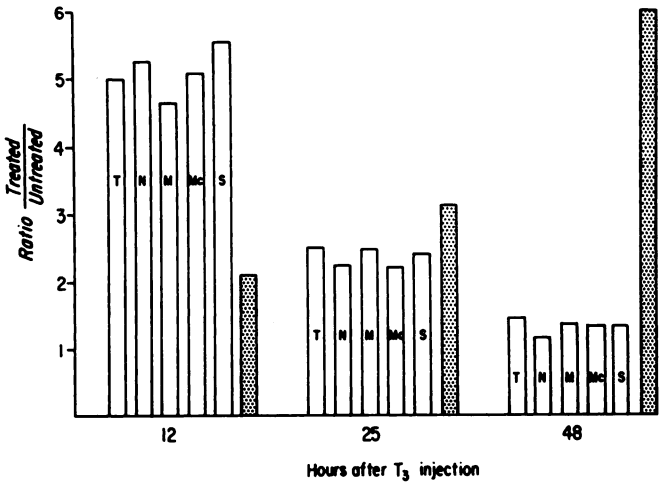

FIG. 2. *The effect of T_3 on the incorporation of L-leucine-^{14}C into liver proteins*

Rats were distributed into two groups. At zero time, one group received T_3 (25 μg/100 g) and the other group received an equal volume of alkaline saline. Ten minutes before each test interval two or three rats from each group were given an intraperitoneal injection of L-leucine-^{14}C (8 μC/100 g). After 10 min the rats were sacrificed and the specific radioactivity of purified proteins from liver and its subcellular fractions was determined. For other details see "Methods." The bar graph depicts the ratio of activities between the T_3-treated and saline-treated rats. Open bars represent the ratio of specific activity of protein; *T*, total liver; *N*, nuclear fraction; *M*, mitochondrial fraction; *Mc*, microsomal fraction; *S*, supernatant fraction. The shaded bars represent corresponding ratios of enzyme activities.

as effective as actinomycin D in blocking T_3-induction of LM-GPDH (Table 2).

Thyroid hormone has been reported, both *in vivo* (33) and *in vitro* (34), to increase the incorporation of radioactive amino acid into protein. In addition, Michels *et al.* (33) demonstrated that thyroxine stimulates L-leucine-^{14}C incorporation into proteins of the liver, kidney, and heart but has no effect on incorporation into the proteins of spleen, testis, and brain; this observation correlates well with the effects of thyroxine on oxygen consumption by these same tissues. Utilizing the pulse labeling technique, it was hoped to evaluate, after administration of T_3, the condition of the cellular organelles which synthesize protein. In agreement with the earlier findings, T_3 increased L-leucine-^{14}C incorporation into total liver proteins. However, this increase of L-leucine-^{14}C incorporation into liver protein rapidly decreased as a function of time (12–48 hr) after T_3 was administered. The increase in incorporation was approximately the same for all subcellular fractions and very similar to the value for total protein at each time studied (Fig. 2). Twelve hours after T_3-administration, the amino acid incorporation was increased 5-fold over control values; although, under the same conditions, the level of LM-GPDH of T_3-treated animals was enriched only twice as much as control animals. A 3-fold increase of LM-GPDH activity was noted by 25 hr after T_3-administration, whereas the L-leucine-^{14}C incorporation was increased only about 2.5-fold. The LM-GPDH reached the highest level, about a 6-fold increase in this case, 2 days after T_3-administration; the amino acid incorporation was only slightly increased at this time. These data demonstrate that an increased turnover of liver protein precedes the maximal increase in LM-GPDH produced by T_3-administration.

DISCUSSION

Although the resistance of LM-GPDH to solubilization and purification has prevented more definitive studies with the isolated and purified enzyme, the studies presented here strongly suggest that the increase of LM-GPDH enzyme activity may result from stimulation of the formation of more enzyme molecules from amino acids, as judged by the inhibitory effects of puromycin and ethionine. It has been repeatedly emphasized (35) that the level of an enzyme is determined by its rate of synthesis and its rate of breakdown. The increase of LM-GPDH activity after administration of T_3 probably does not result from the stabilization of LM-GPDH, for the following reasons: (1) When protein synthesis was blocked in the control animals (puromycin without T_3, Fig. 1, point *H*), and ethionine without T_3 (Table 2, experiment I) there was no degradation of LM-GPDH. (2) 5-Fluorouracil, an inhibitor of RNA synthesis which has no effect on the incorporation of radioactive amino acids into liver protein (19), partially blocked the response of LM-GPDH to T_3 (Table 2, experiment III). (3) Actinomycin D, which has been shown to block the increase of enzyme level caused by synthesis but not by stabilization (29, 36), completely inhibits LM-GPDH response to T_3 (Table 2, experiment II). Also, these observations suggest that degradation of the enzyme may not play the key role in regulating its intracellular concentration, which is in contrast to data reported for tryptophan pyrrolase (36). In fact, the administration of glycerophosphate or glycerol to the animals with or without T_3 has no effect on either LM-GPDH level or on T_3-induction (8). The inhibitory effect of actinomycin D and 5-fluorouracil on the T_3-induced synthesis of LM-GPDH suggests that this synthesis requires the simultaneous synthesis of RNA. These results are consistent with our earlier studies (4, 9) which indicated that T_3-induction of LM-GPDH in euthyroid animals is also sensitive to puromycin, ethionine, and actinomycin D inhibition. Recently, Tarentino *et al.* (37) reported that 5-fluorouracil has no effect on T_3-induced LM-GPDH synthesis. The discrepancy between their data and ours may arise from experimental factors such as dosage and frequency of administration of the antimetabolite to the animals.

To eliminate the possibility that the in-

crease in LM-GPDH activity by T_3 may result from alteration in catalytic activity of enzyme molecules, experiments were carried out in which varying volumes of the mitochondrial suspension from either normal or T_3-treated animals were cross-mixed or were added to various volumes of suitable postmitochondrial supernatant solutions. The results revealed the additivity of LM-GPDH activity from normal and T_3-treated livers (8). Furthermore, the Michaelis constants for α-glycerophosphate of the induced and normal enzyme were found to be essentially the same (8).

Preliminary results were obtained using euthyroid animals, which further supports the hypothesis that the increase in LM-GPDH activity after administration of T_3 results from an increase in population of enzyme molecules. Attempts were made recently to check the immunochemical similarity between induced and noninduced enzymes. A 10-fold purified enzyme preparation from a twice-washed mitochondrial suspension, was injected subcutaneously to immunize a rabbit; subsequently the antiserum was isolated. Although the antiserum showed two bands by immunoelectrophoresis and four bands by Ouchterlony's technique of double diffusion, the amount of antiserum required to precipitate the activity of the purified enzyme from T_3-induced and noninduced mitochondrial preparations was approximately proportional to the units of enzyme present in the two preparations (8).

The experiments in which the incorporation of L-leucine-^{14}C was studied (Table 2) indicate that the increased rate of protein synthesis precedes the maximal increase in LM-GPDH activity produced by T_3-administration. These results are consistent with the idea that the increased rate of protein synthesis is responsible for the increased LM-GPDH activity. The rapid decrease of the incorporation of L-leucine-^{14}C into liver proteins, under the described conditions, may result from a number of factors in addition to the change in rate of protein synthesis, such as a possible change of leucine-pool size in the liver, the blood supply to the liver, and the rate of leucine absorption from the abdominal cavity. However, Sternheimer (38) has demonstrated only a small and perhaps insignificant change in the total free amino acid nitrogen in the liver following the administration of thyroid hormone to euthyroid animals. These changes, even if significant, could not account for the data reported here. An increased heart rate was observed after administration of a small single dose of T_3 to thyroidectomized rats (39), which in turn might be expected to change the hepatic blood flow resulting from an increase in cardiac output. However, Sambhi (40) indicates that the increased systemic blood flow that accompanies hyperthyroidism is not reflected in hepatic blood flow, as estimated in hepatic vein catheterization experiments. The absorption of leucine from the abdominal cavity is unlikely to be different during the period of maximal incorporation (12 hr) from what it is during the period of lowest incorporation (48 hr), since the dosage of T_3 was about 50 times the amount of T_3 produced under physiological conditions.

The results reported here support the thesis that T_3-induction of GPDH results from acceleration of enzyme synthesis. However, it would be of interest to know whether or not T_3 increases the enzyme population in all the liver mitochondria. In view of recent observations which indicate that protein synthesis also occurs in mitochondria (41) and that administration of T_3 increases mitochondrial protein synthesis (42), the question arises whether or not T_3 increases the enzyme level in only those mitochondria which have been formed after T_3 administration. Investigation of this point is in progress.

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