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Thyroxine Stimulation of Amino Acid Incorporation into Protein*

LOUIS SOKOLOFF AND SEYMOUR KAUFMAN
WITH THE TECHNICAL ASSISTANCE OF GLADYS E. DEIBLER AND PHYLLIS L. CAMPBELL

From the Laboratory of Clinical Science and Laboratory of Cellular Pharmacology, National Institute of Mental Health, National Institutes of Health, United States Department of Health, Education and Welfare, Public Health Service, Bethesda, Maryland

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There is abundant clinical and physiological evidence to suggest an intimate role of the thyroid hormone in the regulation of protein metabolism. In man cretinism is usually associated with dwarfism (3), and thyroidectomy in immature animals results in retarded growth which can be corrected by L-thyroxine administration (4, 5). In mature animals thyroid dysfunction is generally accompanied by changes in nitrogen metabolism as prominent and characteristic as those observed in oxidative and energy metabolism (3).

A possible relationship between thyroxine and protein synthesis has been suggested by the studies of DuToit (6), who reported an increased rate of amino acid incorporation into protein in liver slices from rats pretreated *in vivo* with L-thyroxine. The present paper describes a similar stimulation of amino acid incorporation into protein in cell-free rat liver homogenates after the administration of L-thyroxine *in vivo* or its addition *in vitro*. A reduction in the rate of amino acid incorporation has been observed after thyroidectomy. Some characteristics of the thyroxine effect are described, and evidence is presented linking it to the mitochondrial fraction and possibly to oxidative phosphorylation.

EXPERIMENTAL PROCEDURE

Materials

Chemicals—The following compounds of the highest grade of purity available were purchased from commercial sources. AMP (Pabst Laboratories and Sigma Chemical Company); creatine phosphate (California Corporation for Biochemical Research and Sigma); α-ketoglutarate and succinate (California Corporation and Nutritional Biochemicals Corporation); DL-β-hydroxybutyrate (Nutritional Biochemicals); DL-leucine-1-C^{14} and DL-valine-1-C^{14} (Nuclear-Chicago Corporation); sodium L-thyroxine (California Corporation, Sigma, and Nutritional Biochemicals). For many of the experiments L-thyroxine was recrystallized once or twice from dilute ethanol-water solutions of the commercial preparations; the effects observed with recrystallized L-thyroxine were at least as great and usually greater than those observed with the original commercial preparations.

Tetraiodothyroacetic acid and sodium L-triiodothyronine were generously provided by Dr. A. E. Heming of the Smith, Kline and French Laboratories. D-Thyroxine was obtained similarly from Dr. L. Ginger of Travenol Laboratories, Inc. and Dr. T. F. Macrae of Glaxo Laboratories, Ltd., Middlesex, England. Crystalline creatine phosphokinase, prepared by the method of Kuby, Noda, and Lardy (7), was the gift of Dr. F. Friedberg.

Animals—Sprague-Dawley male rats were used in all these studies. Animals weighing between 90 and 150 g were preferred and were used in the experiments on the effects of thyroxine and its analogues *in vitro*. For studies involving pretreatment procedures *in vivo*, animals weighing between 70 and 100 g were selected, but they frequently attained weights up to 250 g by the end of the pretreatment schedule. The specific pretreatment procedures are described below. All animals were fed Purina laboratory chow *ad libitum* except for a 12- to 18-hour period immediately before they were killed, during which time they were deprived of food.

Methods

Preparation of Homogenates—Homogenates were prepared fresh for each experiment, and the homogenization procedures were performed in a room in which the temperature was maintained at 4°. The rats were decapitated, and their livers were quickly removed and transferred to a 0.25 M sucrose solution previously cooled to 0°. When once chilled to that temperature, tissue fractions were maintained between 0° and 2° through all subsequent operations. The livers were wiped dry on absorbent paper, weighed, and homogenized in portions of 1 to 2 g; each portion was first minced with scissors and then homogenized in 5 ml of 0.25 M sucrose per g of tissue by means of a motor-driven, loose-fitting, all-glass Potter-Elvehjem homogenizer. The motor speed was the minimum required to prevent binding of the homogenizer pestle, and homogenization was continued for only about 35 seconds regardless of the degree of completeness.

Fractionation and Reconstitution of Homogenates—Fractionation of the crude homogenate was accomplished by modifications of the method of Schneider and Hogeboom (8). Three different procedures were followed according to the nature of the experiment; these will be referred to as Procedures A, B, and C. All centrifugations below 15,000 × *g* were performed in a Servall refrigerated centrifuge; for higher centrifugal forces a Spinco model L preparative ultracentrifuge was used.

Procedure A: This procedure was used in most experiments. Intact cells, nuclei, and cell debris were removed from the crude homogenate by centrifugation for 10 minutes at 700 × *g*. The

* Preliminary reports of portions of this work have been presented (1, 2).

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TABLE I

Effect of L-thyroxine pretreatment in vivo on DL-leucine-1-C^{14} incorporation into protein[a]

The system contained the following components (in μmoles): sucrose, 150; AMP, 5; potassium phosphate buffer, pH 7.4, 20; $MgCl_2$, 5; potassium α-ketoglutarate, 50; DL-leucine-1-C^{14} (specific activity 5.33 or 5.47 μc/μmole), 0.8. In addition, 0.45 ml of homogenate prepared by Procedure A or 0.15 ml of each of the homogenate fractions prepared by Procedure B was added. The reaction mixture was brought to a final volume of 1.7 ml with water. Incubation time at 37° was 60 minutes. Radioactivity measured with end window Geiger-Müeller counter.

Assay	Control rats	L-Thyroxine pretreated rats	Effects of pretreatment	
			Δ	%[a]
c.p.m. per mg of protein	30.4±2.8	41.4±3.7	+11.0±3.8[b]	+42
mg of protein N per flask	2.23±0.11	2.12±0.30	−0.11±0.30	

[a] Rats were paired according to age and weight. One of each pair received almost daily intraperitoneal injections of 100 μg of sodium L-thyroxine in 1 ml of 0.01 N NaOH for 6 to 16 days (mean = 10 days); the other received equivalent amounts of the NaOH solution alone. On the day after the last dose, liver homogenates were prepared simultaneously from both animals, and DL-leucine-1-C^{14}-incorporation activity in both preparations was assayed in a single combined experiment. The values presented are the means ± standard errors of 8 such paired experiments; % represents the mean of the individual per cent effects.

[b] Statistically significant effect; $p < 0.02$ as determined by method of paired comparison.

supernatant fluid was decanted and centrifuged at 54,000 × *g* for 60 minutes. The 54,000 × *g* supernatant fluid was decanted, and the combined mitochondrial and microsomal fractions contained in the sediment were resuspended in 0.25 M sucrose to a final volume of 1.4 ml per g of original liver weight. The final homogenate was reconstituted by mixing the mitochondrial-microsomal suspension and the 54,000 × *g* supernatant fluid in proportions of 2:1, respectively; its composition was such that 0.45 ml, the aliquot added per flask, contained mitochondria and microsomes equivalent to the yield from 200 mg and supernatant fluid equivalent to the yield from 30 mg of liver.

Procedure B: This procedure was followed in experiments requiring more complete separation of mitochondrial, microsomal, and supernatant fractions. After removal of the intact cells, cell debris, and nuclei as in Procedure A, the mitochondria were separated by centrifugation of the homogenate at 15,000 × *g* for 15 to 20 minutes. The 15,000 × *g* supernatant fluid was then decanted and centrifuged for 60 minutes at 105,000 × *g* to remove the microsomes. The mitochondrial and microsomal fractions were each suspended separately in sufficient amounts of 0.25 M sucrose to yield final volumes of 0.7 ml per g of original liver weight. When this procedure was used, 0.15 ml of each of the fractions, the mitochondrial suspension, the microsomal suspension, and the 105,000 × *g* supernatant fluid were added separately to each flask; their combined contents were approximately equivalent to those of 0.45 ml of homogenate prepared by Procedure A.

Procedure C: This procedure was used in experiments designed to test the requirement of mitochondria for the thyroxine effect *in vitro*. The crude homogenate was centrifuged for 15 minutes at 10,000 to 13,000 × *g* to remove intact cells, nuclei, cell debris, and mitochondria. The sediment was discarded, and the supernatant fluid was centrifuged at 54,000 × *g* for 60 minutes to separate the microsomes. These were then resuspended in 0.25 M sucrose to a final volume of 0.7 ml per g of original liver weight, and the microsomal suspension and 54,000 × *g* supernatant fluid were mixed in equal proportions.

While the 54,000 × *g* centrifugation was in progress, an additional crude liver homogenate was prepared from another rat. Intact cells, cell debris, and nuclei were removed as in Procedure A and discarded. The remaining homogenate was centrifuged for 10 minutes at 8,000 × *g* to separate the mitochondria which were then resuspended in 0.25 M sucrose to a final volume of 0.7 ml per g of original liver weight. Preparation of fresh mitochondria from a second homogenate was necessary because no thyroxine effects *in vitro* on amino acid incorporation into protein could be obtained with mitochondria prepared from the first homogenate and allowed to remain isolated while the microsomes were being seperated.

The composition of the two mixtures obtained by Procedure C was such that 0.15 ml of the mitochondrial suspension and 0.30 ml of the microsomal-supernatant mixture contained amounts of their respective fractions approximately equivalent to those present in 0.45 ml of the combined mixture prepared by Procedure A.

Incubation—Incubations were carried out in air in 25-ml Erlenmeyer flasks shaken at a rate of 92 oscillations per minute in a Dubnoff water bath maintained at a temperature of 37°. The components of the standard system are described in Table I. All solutions were prepared in glass-distilled water and were brought to pH 6.6 to 7.6 before addition. When added *in vitro*, thyroxine solutions were prepared fresh for each experiment as described in Fig. 1 and were added after the addition of homogenate but

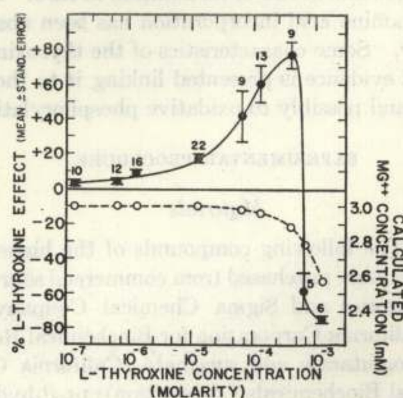

FIG. 1. Effects of various concentrations of L-thyroxine added *in vitro* on DL-leucine-1-C^{14} incorporation into protein. The assay conditions were the same as those described in Table I, except that in addition, experimental flasks received appropriate amounts of sodium L-thyroxine dissolved in 0.1 ml of 0.01 N NaOH for final thyroxine concentrations of 1.3×10^{-4} M or less and in 0.3 ml of 0.01 N or 0.1 ml of 0.04 N NaOH for higher concentrations. Control flasks received equivalent amounts of NaOH. Homogenates were prepared by Procedure A. Incubation time at 37° was 25 minutes. ●——●, experimentally determined percentage of L-thyroxine effects; numbers adjacent to points represent number of experiments from which mean and standard errors were calculated; thyroxine effects at all concentrations were statistically significant ($p < 0.02$). ○——○, theoretically calculated Mg^{++} concentrations on basis of known additions of Mg^{++} and L-thyroxine and assumption of the Mg^{++}-thyroxine compound reported by Lardy (11) containing 3 thyroxine moieties per atom of magnesium and having a solubility product of 1×10^{-17}.

before the addition of $MgCl_2$. Potassium α-ketoglutarate and DL-leucine-1-C^{14} were added last, and incubation was begun immediately after addition of the latter. Flasks and solutions were kept in ice between all additions. The reaction was terminated by precipitation of the protein with an equal volume of 12% trichloroacetic acid.

Purification and Counting of Protein Samples—The precipitated protein samples were purified according to the procedure described by Siekevitz (9), homogenized in acetone, and plated by suction on metal-ringed filter-paper (Whatman No. 1) planchets. Sample weight was determined from difference in planchet weights before and after plating. Radioactivity was measured with an end window gas flow counter, except where expressly stated otherwise. Sufficient total counts were collected to obtain a 3% coefficient of variation after correction for background, and counting rates were corrected for self-absorption to infinite thinness. Corrections were also made for zero time control values obtained in separate flasks in which the reaction was terminated with the trichloroacetic acid immediately after the addition of the radioactive amino acid; zero time values were almost always well below 1 c.p.m. per mg.

Miscellaneous Methods—Protein nitrogen contents of the homogenates were determined by the micro-Kjeldahl technique.

RESULTS

Effect of L-Thyroxine Pretreatment in Vivo—In order to determine the effects of hyperthyroidism on the rate of DL-leucine-1-C^{14} incorporation into protein, experiments were performed on matched pairs of control rats and rats made hyperthyroid by pretreatment with L-thyroxine *in vivo* as described in Table I. From these results it can be seen that L-thyroxine pretreatment leads to an increased rate of amino acid incorporation into protein which cannot be attributed to a difference in the protein nitrogen contents of the two homogenates.

Effects of Thyroidectomy—If the observed effect of thyroxine administration to the animals represented a physiological action of the thyroid hormone, then hypothyroidism might be expected to result in a reduced rate of amino acid incorporation into protein. To test this possibility, matched pairs of control and thyroidectomized rats were prepared and studied as described in Table II. As can be seen from the results summarized in Table II, thyroidectomy resulted in a significant reduction in DL-leucine-1-C^{14} incorporation activity which could not be explained by any difference in the protein nitrogen contents of the homogenates.

Effects of Various Concentrations of L-Thyroxine in Vitro—The addition of L-thyroxine directly to the flasks also resulted in accelerated rates of DL-leucine-1-C^{14} incorporation into protein.[1] In Fig. 1 are graphically summarized the effects of various concentrations of L-thyroxine in experiments with normal rat liver homogenates. It can be seen that the thyroxine effect increases with increasing thyroxine concentrations, ranging from +3.5% at 1.3×10^{-7} M to +77% at 3.9×10^{-4} M. It should be noted that these are average results; in more active preparations considerably greater effects have been observed at all concentrations within this range. At a concentration of 6.5×10^{-4} M, the thyroxine effect abruptly changes from stimulation to marked inhibition. The mechanism of this striking reversal is unknown, but if one assumes the formation of the magnesium-thyroxine compound described by Lardy (11), containing three thyroxine moieties per magnesium ion and having a solubility product of 1×10^{-17}, then it can be shown by the appropriate calculations that the reversal occurs at the thyroxine concentration at which the Mg^{++} concentration begins to decrease rapidly because of its precipitation by thyroxine (Fig. 1).

TABLE II

Effect of thyroidectomy on DL-leucine-1-C^{14} incorporation into protein[a]

The conditions were the same as those described in Table I. Homogenates were prepared according to Procedure A. Incubation time at 37° was 25 minutes.

Assay	Control rats	Thyroidectomized rats	Effects of thyroidectomy	
			Δ	%[a]
c.p.m. per mg of protein	29.4±3.4	20.1±3.4	−9.3±3.8[b]	−28
mg of protein N per flask	2.24±0.10	2.33±0.12	+0.09±0.10	

[a] Rats weighing less than 100 g were paired for age and weight; one of each pair was surgically thyroidectomized and the other subjected to sham operation. Paired rats were treated identically after operation until they were killed. Possible complications of the associated parathyroidectomy were avoided by either the oral administration of 0.6 mg of dihydrotachysterol every second day or the addition of 1 g of calcium lactate per 100 ml of drinking water during the first 2 postoperative weeks. Completeness of the thyroidectomy was evaluated by growth curves based on almost daily weight (10). In a few instances, the presumably thyroidectomized rats failed to show the characteristic retardation of growth (4, 5); they and their matched partners were then excluded from further study. Liver homogenates were prepared simultaneously from matched rats 28 to 41 days after operation (mean = 32 days), and their DL-leucine-1-C^{14}-incorporation activities were assayed in paired flasks. The values presented are the means ± standard errors of 8 such paired experiments; % represents the mean of the individual per cent effects.

[b] Statistically significant effect; $p < 0.05$ as determined by method of paired comparison.

In contrast to the usual metabolic responses to thyroid administration in hypothyroidism, the L-thyroxine effect *in vitro* on amino acid incorporation into protein was considerably lower in homogenates from thyroidectomized rats than in the normal rat preparations.[2] Possible reasons for this reduction in sensitivity after thyroidectomy are currently under investigation.

Amino Acid Specificity—As can be seen from the results of the experiments summarized in Table III, the L-thyroxine effect *in vitro* on amino acid incorporation into protein is not limited to leucine; it occurs to an equal degree with valine.

Substrate Specificity—In most of the experiments described in

[1] The additional C^{14} incorporated into protein as a result of the action of thyroxine appears to be almost wholly present in the form of carboxyl- or α-amino-bound leucine-1-C^{14}. Treatment of the purified, precipitated protein with ninhydrin, alkali, or thioglycollic acid as described by Siekevitz (9) results in small decreases in the specific activities of the protein from both control and experimental flasks, but these decreases are proportionate, and the percentage thyroxine effects remain essentially unchanged. On the other hand, almost all the radioactivity present in acid hydrolysates of both types of protein is released as $C^{14}O_2$ by ninhydrin, retained by the cation exchange resin, Dowex, 50W-X8 (hydrogen form), and travels in a single peak with the same R_F as the DL-leucine reference compound on Whatman No. 1 paper chromatograms developed in either of two solvent systems (*n*-butanol-acetic acid-water, 4:1:1, volume per volume, and 80% aqueous phenol).

[2] L. Sokoloff and S. Kaufman, unpublished observations.

TABLE III

Comparative effects of L-thyroxine in vitro on DL-leucine-1-C^{14} and DL-valine-1-C^{14} incorporation into protein

The assay conditions were the same as those in Fig. 1, except that the DL-leucine-1-C^{14} (specific activity = 5.47 μc/μmole) was replaced by equivalent molar quantities of DL-valine-1-C^{14} (specific activity = 3.05 μc/μmole) in the flasks indicated. Homogenates were prepared according to Procedure A. L-Thyroxine additions to experimental flasks were sufficient to yield the final concentrations indicated. The incubation time at 37° was 25 minutes.

L-Thyroxine concentration	Amino acid	Control	+ L-Thyroxine	L-Thyroxine effect	
		c.p.m./mg protein		Δ *c.p.m./mg*	%
Experiment 1					
1.3×10^{-5} M	DL-Leucine-1-C^{14}	37.7	47.0	+9.3	+25
	DL-Valine-1-C^{14}	19.4	23.2	+3.8	+20
Experiment 2					
6.5×10^{-5} M	DL-Leucine-1-C^{14}	33.6	43.2	+9.6	+29
	DL-Valine-1-C^{14}	11.7	15.7	+4.0	+34

TABLE IV

Substrate specificity of L-thyroxine effect in vitro on DL-leucine-1-C^{14} incorporation into protein

The assay conditions were the same as those in Fig. 1, except that the nature and quantity of substrate were as indicated. Homogenates were prepared according to Procedure A. The quantity of L-thyroxine added to the experimental flasks was sufficient to yield a final concentration of 6.5×10^{-5} M. Incubation time at 37° was 25 minutes.

Substrate	Control	+ L-Thyroxine	L-Thyroxine effect	
	c.p.m./mg protein		Δ *c.p.m./mg*	%
None	13.5	14.9	+1.4	+10
Succinate (50 μmoles)	24.5	24.9	+0.4	+2
α-Ketoglutarate (50 μmoles)	19.3	32.2	+12.9	+67
DL-β-Hydroxybutyrate (50 μmoles)	44.8	57.7	+12.9	+30
DL-β-Hydroxybutyrate (100 μmoles)	56.0	66.6	+10.6	+19

this report, α-ketoglutarate was used as the oxidizable substrate. Thyroxine effects *in vitro* were also observed, however, with other substrates, but the magnitude and consistency of the effect varied considerably with the nature of the substrate used (Table IV). When succinate was used, the results were erratic. With some homogenates the thyroxine effect with succinate equaled or even exceeded that obtained with α-ketoglutarate, but usually it was much lower, often being absent or no greater than occurred without any added substrate. Differences in the control rates of amino acid incorporation did not appear to be involved in this variability, for very similar rates were achieved with both substrates. In contrast to the inconsistent effects of thyroxine *in vitro* when succinate was used as substrate, the effects of thyroid pretreatment of the animal *in vivo* were just as great and consistent with succinate as with α-ketoglutarate.

Of the substrates tested, DL-β-hydroxybutyrate was associated with the highest rates of amino acid incorporation into protein, and the thyroxine effects *in vitro* obtained with it were almost always at least equivalent on an absolute basis to those obtained with α-ketoglutarate (Table IV). Frequently the effects with DL-β-hydroxybutyrate were greater, sometimes so much greater that despite the higher rate of amino acid incorporation, the percentage effect also exceeded that obtained with α-ketoglutarate. Doubling the total DL-β-hydroxybutyrate concentration did not materially alter the results.

Surprisingly high rates of amino acid incorporation and thyroxine effects *in vitro* were occasionally observed in the absence of added substrate. Since the chief source of endogenous substrate was probably the glycogen contained in the liver homogenate, it is likely that glycolytic processes were contributing to these results. In order to determine whether it was glycolysis itself or the oxidation of its products by the tricarboxylic acid cycle which was supporting the amino acid incorporation, the effects of fluoroacetate were investigated. In Table V it is seen that 1.2×10^{-3} M fluoroacetate reduces the amino acid incorporation rate only slightly but virtually eliminates the thyroxine effect in the absence of added substrate although, if anything, enhancing it in the presence of added α-ketoglutarate. The results obtained with this inhibitor suggest that although glycolysis can support amino acid incorporation, it cannot lead to a thyroxine effect *in vitro* unless its products enter into the tricarboxylic acid cycle. This interpretation is in agreement with the results of experiments described below indicating that both mitochondria and a substrate for oxidative phosphorylation are required for the thyroxine effect.

Specificity of Thyroactive Compound—In order to evaluate further the physiological significance of the thyroxine enhancement of amino acid incorporation into protein, the effects of several thyroxine analogues and derivatives with varying degrees of physiological activity were investigated. D-Thyroxine is known to have only a fraction of the calorigenic effect of the naturally occurring L-isomer (12, 13). The effects of the two optical isomers on amino acid incorporation after pretreatment of the animals *in vivo* are compared in Table VI. In agreement with its relative lack of physiological activity, D-thyroxine administration *in vivo* in doses in which L-thyroxine was quite active,

TABLE V

Effects of fluoroacetate on L-thyroxine effect in vitro on DL-leucine-1-C^{14} incorporation into protein in presence and absence of added substrate

The assay conditions were the same as those in Fig. 1, except for the absence of α-ketoglutarate or the addition of fluoroacetate in the flasks indicated. Final fluoroacetate concentration was 1.2×10^{-3} M. Homogenates were prepared according to Procedure A. L-Thyroxine additions to experimental flasks were sufficient to yield a final concentration of 6.5×10^{-5} M. Incubation time at 37° was 25 minutes.

Substrate	Inhibitor	Control	+ L-Thyroxine	L-Thyroxine effect	
		c.p.m./mg protein		Δ *c.p.m./mg*	%
None	None	13.5	15.4	+1.9	+14
None	Fluoroacetate	12.0	12.5	+0.5	+4
α-Ketoglutarate	None	26.7	34.2	+7.5	+28
α-Ketoglutarate	Fluoroacetate	23.4	33.7	+10.3	+44

TABLE VI

Effects of L-thyroxine, D-thyroxine, and L-triiodothyronine pretreatment in vivo on DL-leucine-1-C[14] incorporation into protein[a]

The conditions were the same as those described in Table I. Homogenates were prepared according to Procedure A. Incubation time at 37° was 25 minutes.

Pretreatment agent	Protein N per flask	Protein	Pretreatment effect	
	mg	*c.p.m./mg*	Δ *c.p.m./mg*	%[a]
7 Matched rat sets[b]				
Control	2.14 ± 0.12	33.4 ± 3.9		
L-Thyroxine	2.13 ± 0.10	40.7 ± 4.2	+7.3 ± 2.2[c]	+23
D-Thyroxine	2.21 ± 0.14	30.1 ± 5.5	−3.3 ± 4.0	−6
9 Matched rat pairs[d]				
Control	2.25 ± 0.14	30.0 ± 3.1		
3,5,3′-Triiodo-L-thyronine	2.08 ± 0.13	35.5 ± 3.6	+5.4 ± 2.3[c]	+22

[a] All values are means ± standard errors; % represents mean of individual per cent effects.

[b] Rats were matched into sets of three according to age and weight. One served as a control, and the other two were pretreated with L- or D-thyroxine. The pretreated rats received daily intraperitoneal injections of 100μg of sodium L- or D-thyroxine for 4 to 11 days (mean = 8 days). All rats of a set were simultaneously killed 1 to 4 days (mean = 2 days) after the last dose, and their amino acid-incorporating activity assayed in parallel flasks.

[c] Statistically significant effect; $p < 0.05$ as determined by method of paired comparison. None of the changes in protein N was statistically significant.

[d] Rats were paired according to age and weight; one served as a control, and the other was pretreated with L-triiodothyronine. Pretreated rats received 150 μg of sodium L-triiodothyronine intraperitoneally on the first day and 75 μg on the second day. Paired rats were simultaneously killed and studied on the third day.

had no significant effects on amino acid incorporation into protein (Table VI). On the other hand, when added *in vitro*, D-thyroxine was found to be about as effective as L-thyroxine (Table VII), a phenomenon which has also been described in reference to the uncoupling of oxidative phosphorylation by thyroxine (14). The striking discrepancy in the relative activities of L- and D-thyroxine when administered to the whole animal and when added directly to cell-free tissue preparations suggests that the lesser activity of the D-isomer *in vivo* may be more a matter of cell membrane permeability or its more rapid degradation and excretion (15, 16) than of stereospecificity on the part of the enzymes involved.

The effects of the physiologically active analogue, 3,5,3′-triiodo-L-thyronine, were almost opposite to those of D-thyroxine. Homogenates from rats pretreated with triiodothyronine incorporated amino acids into protein at substantially higher rates than preparations from matched control rats (Table VI). *In vitro*, however, the effect of triiodothyronine, though statistically significant ($p < 0.05$) was only a fraction of the L-thyroxine effect (Table VII). Tetraiodothyroacetic acid, another potent thyroactive compound, was found to be even more active *in vitro* than L-thyroxine (Table VII). Preliminary results indicate that it is also active *in vivo*.

Mitochondrial Requirement—In an attempt to localize the source of the increased amino acid-incorporating activity in hyperthyroid rat liver homogenates, mitochondria, microsomes, and supernatant fluid were prepared by Procedure B from the livers of both normal rats and rats pretreated with L-thyroxine as described above. Incubations were carried out with all possible combinations of mitochondria, microsomes, and supernatant fluid derived from these two sources. The results of a representative experiment are presented in Table VIII. It is seen that the

TABLE VII

Relative effects of L-thyroxine, D-thyroxine, L-triiodothyronine, and tetraiodothyroacetic acid in vitro on DL-leucine-1-C[14] incorporation into protein

The assay conditions were the same as those in Fig. 1. L-Thyroxine or analogues added to flasks in quantities needed to yield final concentrations indicated. Homogenates prepared by Procedure A. Incubation time at 37° was 25 minutes.

Compound	Relative effect[a]	
	1.3 × 10^{-5} M	1.3 × 10^{-4} M
	%	%
L-Thyroxine	100 (+18%)[b]	100 (+87%)[b]
D-Thyroxine	98	
Tetraiodothyroacetic acid	129	149
3,5,3′-Triiodo-L-thyronine	23	6

[a] Mean values of 5 to 16 experiments in which effects of L-thyroxine and each analogue were compared in parallel flasks. L-Thyroxine effect considered to be 100.

[b] Numbers in parentheses are the means of the actual percentage L-thyroxine effects obtained in this series of experiments.

TABLE VIII

Localization of increased amino acid-incorporating activity in homogenate fractions from L-thyroxine pretreated rats

The assay conditions were the same as described in Table I. Incubation time at 37° was 60 minutes. Radioactivity was measured with an end window Geiger-Müeller counter.

Flask No.	Source of homogenate fractions added to flasks[a]			Total protein[b]
	Mitochondria	Microsomes	Supernatant fluid	
				c.p.m./mg
1	Normal	Normal	Normal	17.4
2	Hyperthyroid	Hyperthyroid	Hyperthyroid	29.3
3	Normal	Normal	Hyperthyroid	15.3
4	Hyperthyroid	Hyperthyroid	Normal	32.2
5	Normal	Hyperthyroid	Normal	17.9
6	Hyperthyroid	Normal	Hyperthyroid	28.1
7	Hyperthyroid	Normal	Normal	31.2
8	Normal	Hyperthyroid	Hyperthyroid	16.7

[a] Mitochondria, microsomes, and supernatant fluid were prepared simultaneously by Procedure B (see text) from both normal rats and rats made hyperthyroid by the daily intraperitoneal injection of 100 μg of sodium L-thyroxine for 7 days preceding the day of experiment. The three homogenate fractions derived from both types of rats were added to the flasks in the combinations indicated.

[b] Represents amino acid incorporation into total homogenate protein and not protein of any specific homogenate fraction.

increased amino acid-incorporating activity follows the distribution of the mitochondria from the hyperthyroid rat, indicating that the thyroxine effect *in vivo* is associated with the mitochondrial fraction.

This association did not necessarily prove that mitochondria were intimately involved in the mechansim of the thyroxine effect; it could as readily have been explained by a concentration of the injected thyroxine in the mitochondrial fraction (17, 18).

TABLE IX

Requirement of mitochondria and oxidizable substrate for L-thyroxine effect in vitro on DL-leucine-1-C^{14} incorporation into protein

Homogenate fractions were prepared by Procedure C (see text). The complete system contained the same components as the standard system described in Table I and Fig. 1, including 0.15 ml of the mitochondrial suspension and 0.30 ml of the microsomal-supernatant mixture added separately. The contents of the flasks without mitochondria and α-ketoglutarate were identical, except that the mitochondrial suspension was replaced by 0.15 ml of 0.25 M sucrose solution and the α-ketoglutarate was replaced by 40 μmoles of creatine phosphate and 0.25 mg of crystalline creatine phosphokinase contained in an equivalent volume. Sufficient L-thyroxine was added to obtain a final concentration of 6.5×10^{-6} M. Incubation time at 37° was 25 minutes.

Experiment No.	System	Control	L-Thyroxine	L-Thyroxine effect	
		c.p.m./mg protein		*Δ c.p.m./mg*	*%*
1	Complete	26.9	33.0	+6.1	+23
	Minus mitochondria, minus α-ketoglutarate, plus creatine phosphate, plus creatine phosphokinase	15.9	16.2	+0.3	+2
2	Complete	28.4	39.8	+11.4	+40
	Minus mitochondria, minus α-ketoglutarate, plus creatine phosphate, plus creatine phosphokinase	40.6	42.8	+2.2	+5

TABLE X

Effects of L-thyroxine in vitro on DL-leucine-1-C^{14} incorporation into proteins of various fractions of total homogenate

Incubations in all flasks were carried out under the same conditions as in Fig. 1. Homogenate was prepared according to Procedure A (see text). Sufficient L-thyroxine was added to attain a final concentration of 1.3×10^{-4} M. Incubation times at 37° was 25 minutes. At the end of the incubations, the contents of one pair of control and experimental flasks were separated into mitochondrial and microsomal-supernatant fractions by differential centrifugation (see text). The protein of each fraction was then precipitated, purified, and counted as described in the text. Total homogenate protein was obtained from the contents of a parallel pair of flasks which were not fractionated but were otherwise similarly treated.

Sample	Control	+ L-Thyroxine	L-Thyroxine effect	
	c.p.m./mg protein		*Δ c.p.m./mg*	*%*
Total homogenate protein	19.7	26.0	+6.3	+32
Mitochondrial protein	14.7	14.9	+0.2	+1
Microsomal-supernatant protein	23.0	33.9	+10.9	+47

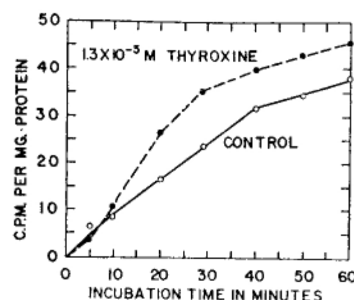

FIG. 2. Time course of L-thyroxine effect *in vitro* on DL-leucine-1-C^{14} incorporation into protein. The conditions were the same as those in Fig. 1. Homogenates were prepared according to Procedure A. Incubation was at 37°. Each point represents a separate flask. ○—○, controls; ●-----●, plus 1.3×10^{-5} M L-thyroxine.

In order to determine if mitochondria were actually essential for the effect, experiments were performed in which thyroxine effects *in vitro* were compared in the presence of mitochondria and a substrate for oxidative phosphorylation and in their absence and replacement by a creatine phosphate-ATP generating system. The results of two such experiments are presented in Table IX. In one experiment the control rate of amino acid incorporation was higher in the presence of the mitochondrial system; in the other the rate was higher with the creatine phosphate system; but in both experiments the thyroxine effect, clearly apparent in the presence of mitochondria and the oxidizable substrate, was virtually eliminated by their replacement with a system generating ATP from creatine phosphate. These results, when combined with those of the fluoroacetate experiments previously described (Table V), indicate that mitochondria and a substrate for oxidative phosphorylation are essential for the thyroxine effect on amino acid incorporation into protein.

Site of Proteins Containing Additional Amino Acids Incorporated—Bates *et al.* (19) and McLean *et al.* (20) have described systems which incorporate labeled amino acids into cytochrome *c* and other proteins of mitochondria, and Drabkin (21) has reported increased cytochrome *c* levels in liver and other tissues of hyperthyroid animals. In order to determine if the mitochondrial requirement reflected only an increased amino acid incorporation into cytochrome *c* or other mitochondrial proteins, experiments were carried out in which the mitochondria were separated from the microsomal-supernatant fractions at the end of a standard incubation by centrifugation of the flask contents at $8,000 \times g$ for 10 minutes.[3] The protein from the individual fractions was then precipitated, purified, and assayed for radioactivity in the usual manner as described above. Amino acid incorporation into total homogenate protein was determined in parallel flasks by the standard method. From the results of the experiment presented in Table X, it is seen that the entire thyroxine effect *in vitro*, apparent in the total homogenate protein, can be accounted for by the increased amino acid incorporation into the protein of the microsomal-supernatant fraction. It would appear then that although mitochondria are essential for the thyroxine effect, the actual effect is the enhancement of amino acid incorporation into the protein of the microsomal-supernatant fraction, probably mainly the microsomal protein.

[3] Before centrifugation the reaction was stopped by the addition of 10 ml of ice-cold 0.25 M sucrose solution containing 1 mg of nonisotopic DL-leucine per ml.

Time Course of Thyroxine Effect in Vitro—In order to determine whether the thyroxine effect *in vitro* represented a true stimulation of amino acid incorporation into protein or merely a preservation of the initial rate, complete time-course studies were carried out. The results of a representative experiment are graphically illustrated in Fig. 2. It is clear that the thyroxine effect is stimulatory rather than preservative, for it appears while the control rate of amino acid incorporation is still linear with respect to time. There is, however, a short latent period before the thyroxine effect appears. This lag period is followed by a 20- to 25-minute period of stimulation, after which continued thyroxine stimulation ceases, and the control and experimental curves become parallel. This pattern has been repeatedly observed in a large number of experiments, which have delineated the duration of the lag to a period between 5 and 7 minutes. The same time course is observed with all thyroxine concentrations tested between 1.3×10^{-5} M and 1.3×10^{-4} M. Although the degree of stimulation is greater, higher concentrations neither shorten the lag period nor prolong the period of stimulation, and the addition of more thyroxine after the initial stimulation is over does not appear to restore the effect. The effect of tetraiodothyroacetic acid, which is more potent than thyroxine in this system, has a similar time course including the lag period.

Elimination of Lag Period by Preincubation—When the entire system is preincubated for 4 minutes at 37° before the addition of the labeled amino acid, the lag period is eliminated, and the thyroxine stimulation is immediately apparent (Fig. 3).

To determine whether the elimination of the lag by preincubation was thyroxine-dependent or the result of some other effect unrelated to the presence of thyroxine, the thyroxine addition was delayed until after 10 minutes of incubation of the entire system. As seen from the results illustrated in Fig. 4, the lag period is still present under these circumstances, indicating that thyroxine must be present during the preincubation for the lag to be eliminated. The effect of preincubation on the lag is not merely a matter of duration of contact between thyroxine and the homogenate (22, 23); preliminary exposure of the homogenate to thyroxine at 0° for as long as 22 minutes does not affect the lag.

Dependence of Elimination of Lag by Preincubation on Oxidizable Substrate—When the substrate for oxidative phosphorylation is left out of the system during the 4-minute preincubation at 37° and added at the end of the preincubation together with the labeled amino acid, then the lag period is not eliminated (Fig. 5). In fact, under these conditions the lag period is frequently prolonged or the initial thyroxine effect may even be inhibitory, but ultimately the amino acid incorporation in the thyroxine flasks is accelerated and surpasses that of the control flasks. These results indicate that the thyroxine-dependent reactions proceeding during the preincubation, which are responsible for the elimination of the lag, are also dependent on the presence of an oxidizable substrate. They support the conclusions already reached from the results of the experiments with fluoroacetate (Table V) and creatine phosphate (Table IX) that both mitochondria and a substrate for oxidative phosphorylation are necessary for the thyroxine stimulation *in vitro* of amino acid incorporation into protein.

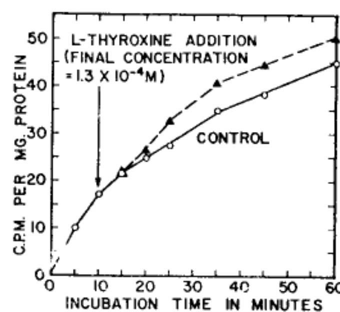

FIG. 4. Time course of L-thyroxine effect *in vitro* on DL-leucine-1-C^{14} incorporation into protein when L-thyroxine was added after 10 minutes of incubation of the total system. The conditions were identical to those in Fig. 2 except that the L-thyroxine was added at the time indicated by the arrow; control flasks received equivalent volumes of the 0.01 N NaOH solvent at the same time. Incubation was at 37°. ○——○, controls; ▲——▲, plus 1.3×10^{-4} M L-thyroxine.

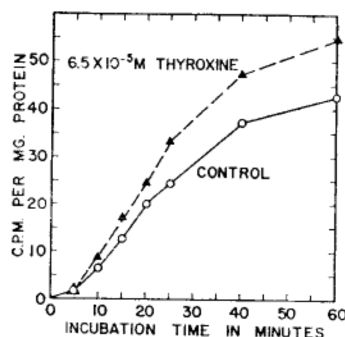

FIG. 5. Time course of L-thyroxine effect *in vitro* on DL-leucine-1-C^{14} incorporation into protein after a 4-minute preincubation of the total system at 37° in the presence of L-thyroxine but in the absence of the radioactive amino acid and added oxidizable substrate. The conditions were identical to those in Fig. 3 except that the α-ketoglutarate was added together with the DL-leucine-1-C^{14} at the end of the preincubation. Incubation temperature was 37°. ○——○, control; ▲——▲, plus 6.5×10^{-5} M L-thyroxine.

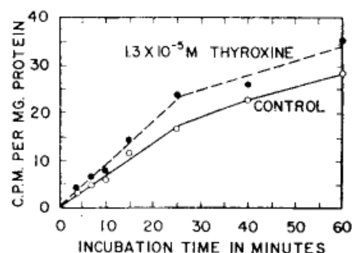

FIG. 3. Time course of L-thyroxine effect *in vitro* on DL-leucine-1-C^{14} incorporation into protein after a 4-minute preincubation at 37° of the complete system in the presence of thyroxine and absence of the radioactive amino acid. The conditions were identical to those in Fig. 2 except that the radioactive amino acid was added and the incubation begun after the 4-minute preincubation at 37°. Incubation was at 37°. ○——○, controls; ●——●, plus 1.3×10^{-5} M L-thyroxine.

DISCUSSION

The finding that thyroxine stimulates amino acid incorporation into protein in cell-free homogenates is further evidence of a role of the thyroid hormone in protein biosynthesis. It does not exclude the possibility of a similar role in other synthetic processes, and in fact there have been reports suggesting comparable effects on the synthesis of fatty acids (24), cholesterol (25), and glycogen (26). Increased amino acid incorporation into protein is not in itself proof of a stimulation of net or protein synthesis *de novo*,

but it is compatible with the physiological role of thyroxine in growth and development (3–5), processes in which protein synthesis is almost certainly involved. Also, Paik and Cohen (27) have recently reported that carbamyl phosphate synthetase activity is increased in the tadpole liver by thyroxine treatment, and they demonstrated by immunological and C^{14}-leucine-incorporation studies that the increased activity is the result of an accelerated synthesis *de novo* of this specific enzymatically active protein.

Previous demonstrations of increased tissue levels of specific proteins (21, 27) or enhanced activity of synthetic processes (24–26) were all obtained after pretreatment of the animal *in vivo*. Such studies fail to distinguish between a primary effect of the hormone and a secondary or adaptative change in response to the gross metabolic alterations known to occur in hyperthyroidism. In the present study, the ability of thyroxine to stimulate amino acid incorporation into protein was demonstrated not only after its administration *in vivo* but also *in vitro*, indicating more strongly a close relationship to the direct action of the hormone.

Uncoupling of oxidative phosphorylation has been suggested as the mechanism of action of thyroxine (18, 22). Leucine and valine incorporation into protein in the system used in these studies is an energy-dependent process, and its stimulation by thyroxine is inconsistent with the mechanisms generally implied by the concept of uncoupling of oxidative phosphorylation. Indeed, uncoupling agents, such as dinitrophenol,[4] in concentrations which measurably depress oxidative phosphorylation, also inhibit amino acid incorporation into protein (9).[2] The abrupt switch from stimulation to inhibition of amino acid incorporation observed with very high concentrations of thyroxine (Fig. 1) may reflect its uncoupling action; but this effect appears to be a qualitatively different phenomenon which may, perhaps, be operative in extreme thyrotoxic states but cannot explain many of the physiological effects of the thyroid hormone.

Recently, Bronk (29) has reported that thyroxine stimulates oxidative phosphorylation in submitochondrial particles, and Dallam and Howard (30) have observed similar effects under special conditions with intact mitochondria. It is possible that the thyroxine stimulation of amino acid incorporation is related to this phenomenon, but we have been unable to demonstrate thus far that the amino acid-incorporation rate in our system is limited by the amount of available ATP. In fact, lowering the AMP concentration, which probably lowers the steady-state concentration of ATP, enhances the rate, and replacement of the AMP by an equivalent quantity of ADP or ATP fails to increase and may even decrease the rate of amino acid incorporation.[2] These results, however, are difficult to interpret because of possible complicating influences such as Mg^{++} binding by the nucleotides, and further studies of the question are still in progress.

The possibility has been considered that the thyroxine effect is related to the phosphorylation of some other nucleotide required in the incorporation of amino acids into protein. GTP has been reported to be an essential cofactor in the rate-limiting step of the over-all process (31), and the greater and more consistent thyroxine effects obtained wtih α-ketoglutarate as compared with succinate could conceivably have been related to the coupled generation of GTP in the oxidation of the former substrate (32). The addition of GTP to the total system does indeed increase the rate of amino acid incorporation, but identical stimulations are observed with GDP, and neither nucleotide has any systematic influence on the thyroxine effect.[2]

Although the effect of thyroxine on amino acid incorporation into protein cannot be explained by its uncoupling action on oxidative phosphorylation, certain aspects of the results suggest that it is related to some interaction between thyroxine and oxidative phosphorylation. The requirement of both mitochondria and a substrate for oxidative phosphorylation for the over-all effect and the lag period which can be eliminated by preincubation only if both thyroxine and the oxidizable substrate are present, indicate a preliminary or intermediate reaction involving both thyroxine and oxidative phosphorylation preceding the effect on amino acid incorporation. These observations are equally compatible with an oxidative phosphorylation-dependent effect of thyroxine on mitochondrial membrane permeability (33, 34) or some other mitochondrial component, or, conversely, an effect of oxidative phosphorylation on thyroxine. An activated thyroxine intermediate, tetraiodothyroacetyl-CoA, has already been suggested by LeBreton *et al.* (35, 36) on the basis of a CoA-dependent thyroxine stimulation of glycolysis. No evidence has been obtained to indicate the involvement of CoA in the thyroxine effect reported here. It is hoped, however, that studies currently in progress on the nature of the interaction between thyroxine and oxidative phosphorylation may help to elucidate the mechanism of the thyroxine stimulation of amino acid incorporation into protein.

SUMMARY

1. L-Thyroxine pretreatment *in vivo* or addition *in vitro* increases the rate of amino acid incorporation into the protein of cell-free rat liver homogenates. Thyroidectomy results in a reduction of this rate.

2. The increased amino acid-incorporating activity in the L-thyroxine pretreated rats has been found to be associated with the mitochondrial fraction. The L-thyroxine effect *in vitro* is dependent on the presence of mitochondria and a substrate for oxidative phosphorylation; it is not observed when the oxidative phosphorylation system is replaced by a creatine phosphate-ATP generating system. Although mitochondria are essential for the L-thyroxine effect, the actual effect is to accelerate the amino acid incorporation into the protein of the microsomal-supernatant fractions.

3. Time-course studies have demonstrated that the L-thyroxine effect *in vitro* is a true stimulation of the rate of amino acid incorporation into protein and not merely a preservation of the initial rate. A short lag period in the appearance of the effect has been observed which can be eliminated by preincubation of the system with L-thyroxine, provided an oxidizable substrate is present; preincubation in the absence of added substrate fails to eliminate the lag.

4. D-Thyroxine, which is physiologically relatively inactive, also fails to stimulate amino acid incorporation into protein when injected into the animal in doses in which L-thyroxine is quite active. When added *in vitro* directly to the cell-free homogenates, D-thyroxine is as effective as L-thyroxine. On the other hand, the physiologically active analogue, 3,5,3′-triiodo-L-thyronine, is effective when administered *in vivo* but has very low activity

[4] We have recently observed that very low concentrations of dinitrophenol and salicylate also stimulate amino acid incorporation into protein in normal rat liver homogenates. However, in view of the recent finding by Christensen (28) that these agents inhibit protein binding of thyroxine, it is uncertain whether their stimulation of amino acid incorporation is a direct effect or secondary to the release of endogenous thyroxine bound on protein.

when added *in vitro*. Tetraiodothyroacetic acid is more effective *in vitro* than L-thyroxine.

5. The possible relationship of the L-thyroxine effect on amino acid incorporation into protein to the physiological role of the thyroid hormone in the processes of growth and development is discussed.

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