Stimulation of Peptide Elongation by Thyroxine

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This study suggests that thyroxine stimulates peptide elongation in a cell-free rat liver polyribosome system. The thyroxine effect persists in the presence of sufficient aurointricarboxylic acid to prevent polyuridylic acid-stimulated peptide initiation. In addition, thyroxine stimulates elongation of pre-existing polyphenylalanine chains providing conclusive evidence that the effect does not depend on peptide initiation. Thyroxine does not stimulate release of nascent peptides from ribosomes into the supernatant phase of the reaction mixture. Therefore in this protein-synthesis system the thyroxine effect is expected to occur at one or more of the reactions of peptide chain elongation, which include aminoacyl-tRNA binding, peptide bond synthesis and translocation.

This paper examines the site at which thyroxine stimulates the reactions of protein synthesis *in vitro*. The thyroxine effect can be observed with aminoacyl-tRNA precursors, indicating that amino acid activation and aminoacyl-tRNA formation are not sites of thyroxine stimulation (Carter *et al.*, 1975; Sokoloff *et al.*, 1963). Synthesis of peptide from aminoacyl-tRNA precursors involves three groups of reactions: peptide chain initiation, elongation and termination (Lucas-Lenard & Lipmann, 1971). Studies reported in this paper suggest that thyroxine added *in vitro* stimulates peptide elongation. Thyroxine stimulation remains even though peptide initiation is prevented, and thyroxine does not stimulate release of nascent peptides from ribosomes.

Materials and Methods

Materials

The disodium salt of creatine phosphate, creatine phosphokinase, AMP, L-thyroxine and poly(U) (polyuridylic acid) were from Sigma Chemical Company (St. Louis, MO, U.S.A.). L-[U-¹⁴C]Valine (sp. radioactivity 228 $\mu\text{Ci}/\mu\text{mol}$) and L-[U-¹⁴C]phenylalanine (sp. radioactivity 360 $\mu\text{Ci}/\mu\text{mol}$) were from New England Nuclear (Boston, MA, U.S.A.). Aurintricarboxylic acid was from Eastman Chemical Company (Rochester, NY, U.S.A.) and the NH_4^+ salt of aurintricarboxylic acid was from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). All other chemicals were reagent grade. Male white rats (100–175 g) were from Charles Rivers Breeding Laboratories, Wilmington, MA, U.S.A. Animals were fed Purina laboratory chow except for the 18 h period immediately before death.

Methods

All procedures were carried out near 0°C. Membrane-free polyribosomes were prepared from rat liver by sucrose-gradient centrifugation by the method of Bloemendal *et al.* (1967) with the following modification. Rat liver was homogenized in 2.5 ml of 0.35 M-sucrose in medium B/g wet wt. (Medium B consists of 50 mM-Tris buffer, pH 7.4, 25 mM-KCl, and 10 mM-MgCl₂.) Approx. 4 ml of 13000g supernatant was layered over a discontinuous gradient consisting of 2.5 ml of 2.0 M-sucrose and 2.0 ml of 1.5 M-sucrose, both in medium B. Tubes prepared in this manner were spun at 105000g for 10 h in a Spinco model L ultracentrifuge with the no. 40 rotor. The membrane-free polyribosome fraction, recovered in a pellet at the bottom of the tubes, was resuspended in sufficient 0.25 M-sucrose to yield an RNA concentration of 0.5–2.0 mg/ml. The phospholipid phosphorus content of the polyribosomes prepared in this manner is approx. 5% that of unfractionated microsomes. A 105000g supernatant prepared as previously described (Carter *et al.*, 1971) was used as the source of soluble enzymes and cofactors. Equal parts of resuspended polyribosomes, 0.25 M-sucrose and 105000g supernatant were combined to yield the active protein-synthesizing preparation.

Incubation procedure. The composition of reaction mixtures is described in the legends to the Tables and Figures. Protein synthesis experiments used a creatine phosphate ATP-generating system as the energy source. Thyroxine was added in 10 mM-NaOH, with control reactions receiving equivalent volumes of NaOH. Thyroxine was added to reaction vessels after the addition of the tissue fraction, but before the addition of MgCl₂. Reaction vessels were kept on ice

between all additions. Zero-time samples were obtained and the incubation was begun by placing the vessels in a 37°C water bath. Incubation periods were terminated by removal of 0.1 ml of reaction mixture on to 23 mm Whatman filter-paper discs. Discs were immediately dried for 20 s in a stream of hot air and placed in an ice-cold solution of 10% (w/v) trichloroacetic acid.

Measurement of ^{14}C -labelled amino acid incorporation. The incorporation of radiolabelled amino acid into hot-trichloroacetic acid-insoluble residue was measured by use of a filter-paper-disc technique (Mans & Novelli, 1961). Details of the procedures used in calculating amino acid incorporation have been described previously (Carter *et al.*, 1971). By using this filter-paper-disc technique ^{14}C was counted at an efficiency of 66% in a Packard Tri-Carb liquid-scintillation counter.

Measurement of release of nascent peptides from ribosomes. Protein-synthesis reaction mixtures having a volume of 1 ml prepared as usual. Zero-time samples were obtained and reaction mixtures incubated at 37°C for 30 min. At this time 0.1 ml samples of reaction mixtures were taken for measurement of total amino acid incorporation. The reaction mixtures were then centrifuged at 105000g for 60 min to sediment the

ribosomes. Samples of the supernatant phase were taken for measurement of radiolabelled amino acid incorporation. The presence of hot-trichloroacetic acid-insoluble radioactivity in the supernatant was taken to represent nascent peptides released from the ribosomes.

Analytical procedures. Protein, RNA and lipid phosphorus were measured by established techniques (Lowry *et al.*, 1951; Schneider, 1957; Chen *et al.*, 1956).

Results and Discussion

Thyroxine effect on phenylalanine incorporation in the presence of aurintricarboxylic acid and poly(U)

The inhibitor of peptide chain initiation, aurintricarboxylic acid, has been used to test thyroxine effect on the peptide initiation process. This compound prevents attachment of synthetic and natural messenger RNA to ribosomes in both prokaryotic and eukaryotic systems (Stewart *et al.*, 1971; Lebleu *et al.*, 1970). To show that the 20 μM -aurintricarboxylic acid used in these experiments does indeed prevent peptide initiation, poly(U)-stimulated peptide initiation has been examined. Table 1 shows the effect of

Table 1. *Thyroxine effect on ^{14}C phenylalanine incorporation in the presence of poly(U) and aurintricarboxylic acid*

Reaction mixtures contained the following additions: sucrose, 156 $\mu\text{mol/ml}$; potassium phosphate buffer (pH 7.0), 12.0 $\mu\text{mol/ml}$; AMP, 2.9 $\mu\text{mol/ml}$; MgCl_2 , 12.0 $\mu\text{mol/ml}$; creatine phosphate (disodium salt), 21 $\mu\text{mol/ml}$. Each flask also received 50 μg of creatine phosphokinase, approx. 50 μg of polyribosomal RNA, 1.0 mg of supernatant protein and 0.2 μCi of L-[^{14}C]phenylalanine (final sp. radioactivity 160 $\mu\text{Ci}/\mu\text{mol}$). The poly(U) addition was 250 μg per flask. When indicated, sufficient aurintricarboxylic acid was added in 25 μl of 95% (v/v) ethanol to provide a final concentration of 20 μM and sufficient L-thyroxine was added in 25 μl of 10 mM-NaOH to provide a final concentration of 50 μM . Control flasks received identical volumes of NaOH or ethanol. In each experiment, duplicate reactions containing each described addition were run in parallel by using identical reagents and tissue fractions. The final volume of each reaction mixture was 0.5 ml. After addition of [^{14}C]phenylalanine, all reaction mixtures were incubated aerobically at 37°C for 30 min and samples obtained for measurement of hot-trichloroacetic acid-insoluble radioactivity.

Phenylalanine incorporation (pmol/mg of RNA)									
Experiment no.	No aurintricarboxylic acid added No poly(U) added			No aurintricarboxylic acid added + 500 μg of poly(U)/ml			+20 μM -aurintricarboxylic acid + 500 μg of poly(U)/ml		
	Control	+Thyroxine	Thyroxine effect (%)	Control	+Thyroxine	Thyroxine effect (%)	Control	+Thyroxine	Thyroxine effect (%)
1	63.5	105	65.4	98.4	165	67.7	64.7	76.3	17.9
2	186	257	38.2	506	781	54.4	184	247	34.2
3	263	295	12.2	484	964	99.2	201	283	40.8
4	377	477	26.5	1353	1780	31.6	389	454	16.7
5	97.1	113	16.4	349	591	69.3	88.4	106	19.9
6	100	118	18.0	323	517	60.1	111	132	18.9
7	92.5	126	36.2	682	792	16.1	81.5	104	27.6
Mean	168	213	30.4*	542	799	56.9**	160	200	25.1**
±s.e.			±7.5			±11			±3.8

* $P < 0.02$, paired t test.

** $P < 0.01$, paired t test.

thyroxine on peptide synthesis in control reactions, reactions containing poly(U) and reactions containing poly(U) plus 20 μ M-aurintricarboxylic acid. In each experiment, duplicate protein synthesis reactions containing each indicated addition have been run in parallel. Thyroxine (50 μ M) increases baseline [14 C]-phenylalanine incorporation $30 \pm 7.5\%$ (mean $\pm$ S.E.).

Mean [14 C]phenylalanine incorporation in control reactions is 168 pmol/mg of polyribosomal RNA and in thyroxine reactions 213 pmol/mg of polyribosomal RNA. In the presence of poly(U) control [14 C]-phenylalanine incorporation increases threefold with $57 \pm 11\%$ thyroxine stimulation. Mean [14 C]-phenylalanine incorporation in control reactions is

Table 2. Effect of thyroxine on synthesis of preformed polyphenylalanine chains

Reaction mixtures were prepared as described in Table 1, with the following exceptions. When indicated, sufficient NH_4^+ salt of aurintricarboxylic acid was added in 25 μ l of water to provide a final concentration of 50 μ M and sufficient L-thyroxine was added in 25 μ l of 10 mM-NaOH to provide a final concentration of 50 μ M. Control flasks received identical volumes of NaOH. In each experiment, duplicate reactions containing each described addition were run in parallel with identical reagents and tissue fractions. The final volume of each reaction mixture was 0.5 ml. In reactions involving poly(U) preincubation, reaction mixtures were preincubated with 250 μ g of poly(U) in a volume of 0.425 ml for 6 min at 37°C before addition of [14 C]phenylalanine, aurintricarboxylic acid and thyroxine. After addition of [14 C]phenylalanine, all reaction mixtures were incubated aerobically at 37°C for 24 min and samples obtained for measurement of hot-trichloroacetic acid-insoluble radioactivity.

(a)

[14 C]Phenylalanine incorporation (pmol/mg of RNA)

Experiment no.	No additions		+Poly(U)		+Poly(U) and aurintricarboxylic acid	+Aurintricarboxylic acid and thyroxine after poly(U) preincubation	
	Control (a)	+Thyroxine (b)	Control (c)	+Thyroxine (d)	Control (e)	Control (f)	+Thyroxine (g)
1	184	262	1027	1369	235	730	909
2	79	120	223	374	94	188	284
3	102	135	248	387	90	175	206
4	125	144	321	449	124	218	301
5	126	160	394	469	123	222	281
6	144	159	360	535	129	230	297
7	186	205	1035	1217	195	486	591
Mean	135	169	515	686	141	321	410
Thyroxine effect (%) $\pm$ S.E.	$27.1 \pm 6.7\%^*$		$40.3 \pm 7.6\%^*$			$29.8 \pm 4.6\%^*$	

b)

Poly(U) stimulated phenylalanine incorporation (pmol/mg of RNA)

Experiment no.	No aurintricarboxylic acid added		+Aurintricarboxylic acid	+Aurintricarboxylic acid and thyroxine after poly(U) preincubation	
	Control (c - a)	+Thyroxine (d - b)	Control (e - a)	Control (f - a)	+Thyroxine (g - b)
1	843	1107	51.0	546	647
2	144	254	15.3	109	164
3	146	252	-12.0	73	71
4	196	305	-1.0	93	157
5	268	309	-3.0	96	121
6	216	376	-15.0	86	138
7	849	1012	9	300	386
Mean	380	516	6.3	186	241
Thyroxine effect $\pm$ S.E.	$49.2 \pm 11\%^*$			$35.7 \pm 10\%^{**}$	

* $P < 0.01$, paired t test.

** $P < 0.02$, paired t test.

542 pmol/mg of polyribosomal RNA and in thyroxine reactions 799 pmol/mg of polyribosomal RNA. When 20 μ M-aurintricarboxylic acid is added to the poly(U)-stimulated reaction, all poly(U) stimulation of phenylalanine incorporation is lost, yet a $25 \pm 3.8\%$ thyroxine stimulation remains. Mean [14 C]-phenylalanine incorporation in control reactions is 160 pmol/mg of polyribosomal RNA and in thyroxine reactions 200 pmol/mg of polyribosomal RNA. These studies demonstrate that the thyroxine effect persists in the presence of sufficient aurintricarboxylic acid to prevent poly(U)-stimulated peptide initiation.

Since aurintricarboxylic acid may not completely inhibit peptide initiation caused by endogenous messenger RNA (Mathews, 1971), the effect of thyroxine on the elongation of preformed polyphenylalanine chains has been examined (Table 2). Polyribosomes were preincubated with poly(U) for 6 min, followed by the addition of 50 μ M-aurintricarboxylic acid, thyroxine and [14 C]phenylalanine. Table 2(a) shows the effect of 50 μ M-thyroxine on [14 C]phenylalanine incorporation in baseline protein synthesis reactions, in reactions containing poly(U) (500 μ g/ml), and in reactions preincubated with poly(U) before addition of aurintricarboxylic acid and thyroxine. In all reactions, incubation has been carried out for 24 min following addition of the radiolabelled amino acid and thyroxine. In baseline reactions, thyroxine increases [14 C]phenylalanine incorporation $27 \pm 6.7\%$, from a mean value of 135 to 169 pmol/mg of RNA; in poly(U) containing reactions, thyroxine increases incorporation $40 \pm 7.6\%$, from a mean value of 515 to 686 pmol/mg of

RNA; and in reactions preincubated with poly(U) before addition of aurintricarboxylic acid and thyroxine, thyroxine increases incorporation $30 \pm 4.6\%$, from a mean value of 321 to 410 pmol/mg of RNA. In addition, the fourfold stimulation of phenylalanine incorporation caused by poly(U) is abolished when aurintricarboxylic acid and poly(U) are added together at the beginning of the incubation. Table 2(b) shows the thyroxine effect on poly(U)-stimulated phenylalanine incorporation. The increase in phenylalanine incorporation obtained by subtracting baseline incorporation from that in reactions containing poly(U) is taken to represent poly(U)-stimulated polyphenylalanine synthesis. In the absence of aurintricarboxylic acid, thyroxine causes a $49 \pm 11\%$ increase in polyphenylalanine synthesis, from a mean value of 380 to 516 pmol/mg of RNA. In this instance thyroxine could be stimulating either initiation or elongation of polyphenylalanine chains. However, thyroxine causes an equivalent $36 \pm 10\%$ stimulation of polyphenylalanine synthesis, from a mean value of 186 to 241 pmol/mg of RNA, when added together with aurintricarboxylic acid to polyribosomes preincubated with poly(U). Since aurintricarboxylic acid prevents initiation of polyphenylalanine chains in this protein synthesis system, thyroxine must be stimulating elongation of preformed polyphenylalanine chains.

Thyroxine effect on peptide release from ribosomes

Labelled peptide in the soluble phase of the reaction mixture has been measured to determine whether

Table 3. *Effect of thyroxine on [14 C]valine incorporation into total-reaction-mixture protein and soluble protein*

Reaction mixtures were prepared as described in Table 1 with the following exceptions. Each flask received 0.40 μ Ci of L-[U- 14 C]valine (final sp. radioactivity 76 μ Ci/ μ mol), approx. 150 μ g of polyribosomal RNA and 2.0 mg of supernatant protein. The MgCl_2 concentration was 5.8 mM. When added, L-thyroxine was present at a final concentration of 50 μ M. In each experiment shown in the table, duplicate reactions containing no additions and thyroxine were run in parallel with identical reagents and tissue fractions. The final volume of each reaction mixture was 1.0 ml. Flasks were incubated aerobically at 37°C for 30 min with samples removed for determination of [14 C]valine incorporation into hot-trichloroacetic acid-insoluble residue in the total reaction mixture and 105 000g supernatant.

Experiment no.	Radioactivity in reaction mixture (c.p.m./ml)					
	Total reaction mixture			105 000g supernatant		
	Control (a)	+Thyroxine (b)	(b-a)	Control (a)	+Thyroxine (b)	(b-a)
1	376	699	323	81	68	-13
2	423	1104	681	95	49	-46
3	646	1797	1151	156	163	+7
4	740	1164	424	72	108	+36
5	562	806	244	105	100	-5
Mean	549	1114	565*	102	98	-4.2
$\pm$ S.E.			± 184			± 15

* $P < 0.05$, paired t test.

thyroxine stimulates the release of completed peptides from ribosomes. Table 3 shows [^{14}C]valine incorporation into protein in the total reaction mixture and 105000g reaction mixture supernatant. Hot-trichloroacetic acid-insoluble radioactivity in the supernatant is taken to represent nascent peptides released from ribosomes. As shown in Table 3, thyroxine increases [^{14}C]valine incorporation into the total-reaction-mixture protein from 549 to 1114 c.p.m./ml after 30 min incubation. Radio-labelled valine incorporation into the soluble phase of the reaction mixture is 19% of total incorporation and is not stimulated by thyroxine. Therefore the thyroxine effect cannot be due to enhanced release of completed peptides from ribosomes.

Evidence presented in this paper indicates that thyroxine stimulates peptide elongation in the rat liver polyribosome system studied. Thyroxine stimulates peptide synthesis in the presence of sufficient aurintricarboxylic acid to prevent poly(U)-stimulated peptide initiation. Since thyroxine stimulates elongation of preformed polyphenylalanine chains, the thyroxine effect does not depend on peptide initiation caused by endogenous messenger RNA, which may not be completely inhibited by aurintricarboxylic acid (Mathews, 1971). Since thyroxine does not enhance release of labelled peptides from ribosomes, it does not stimulate protein synthesis by facilitating peptide termination. Therefore thyroxine increases peptide synthesis without increasing peptide initiation or termination. In this rat liver polyribosome system, thyroxine is stimulating peptide elongation. The

reactions of peptide elongation include aminoacyl-tRNA binding, peptide bond synthesis and translocation.

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