

## Myocardial adenyl cyclase: activation by thyroid hormones and evidence for two adenyl cyclase systems

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### Research Article

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# Myocardial Adenyl Cyclase: Activation by Thyroid Hormones and Evidence for Two Adenyl Cyclase Systems

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**ABSTRACT** The mechanism responsible for the hyperdynamic circulatory state in hyperthyroidism has not been defined. Although certain cardiac manifestations resemble those caused by excessive adrenergic stimulation, recent evidence suggests that thyroid hormone exerts an effect on the heart that is independent of the adrenergic system. Since the inotropic and chronotropic effects of norepinephrine appear to be mediated by activation of adenyl cyclase, the possibility that thyroxine and triiodothyronine are also capable of activating adenyl cyclase was examined in the particulate fraction of cat heart homogenates.

L-thyroxine and L-triiodothyronine increased the conversion of adenosine triphosphate-<sup>32</sup>P (ATP-<sup>32</sup>P) to cyclic 3',5'-adenosine monophosphate-<sup>32</sup>P (3',5'-AMP-<sup>32</sup>P) by 60 and 45% respectively ( $P < 0.01$ ). A variety of compounds structurally related to the thyroid hormones, but devoid of thyromimetic activity did not activate adenyl cyclase: these included 3,5-diiodo-L-thyronine, L-thyronine, 3,5-diiodotyrosine, monoiodotyrosine, and tyrosine. D-thyroxine activated adenyl cyclase and half maximal activity was identical to that of the L-isomer. Although the beta adrenergic blocking agent propranolol abolished norepinephrine-induced activation of adenyl cyclase, it failed to alter activation caused by thyroxine. When maximal concentrations of L-thyroxine ( $5 \times 10^{-8}$  moles/liter) and norepinephrine ( $5 \times 10^{-8}$  moles/liter) were incubated together, an additive effect on cyclic 3',5'-AMP production resulted.

This investigation demonstrates: (a) thyroid hormone is capable of activating myocardial adenyl cyclase in vitro and (b) this effect is not mediated by the beta adrenergic receptor. Moreover, the additive effects of norepinephrine and thyroxine suggest that at least two separate adenyl cyclase systems are present in the heart, one responsive to norepinephrine, the other to thyroid hormone.

These findings are compatible with the hypothesis that the cardiac manifestations of the hyperthyroid state may, in part, be caused by the direct activation of myocardial adenyl cyclase by thyroid hormone.

## INTRODUCTION

Among the more striking changes caused by hyperthyroidism are those leading to the production of the hyperdynamic circulatory state, and include such alterations in cardiac performance as tachycardia, augmentation of the rate of myocardial tension development, and enhancement of myocardial contractility (1, 2). Although some evidence had suggested that these changes were caused by an increased sensitivity of the heart to sympathetic stimulation (3), more recent studies have not confirmed this hypothesis (1, 4, 5). Moreover, the finding that the intrinsic contractile state of papillary muscles from hyperthyroid cats was markedly augmented, even when myocardial norepinephrine stores were depleted by prior administration of reserpine, suggested that thyroid hormone might exert a direct effect on the heart (1).

The biochemical alterations responsible for mediating the positive inotropic and chronotropic effects of the catecholamines on the heart are thought to be dependent on activation of adenyl cyclase and the resultant increase in intracellular cyclic 3',5'-AMP<sup>1</sup> (6). In a recent communication we reported that L-thyroxine and L-triiodothyronine also augmented myocardial adenyl cyclase activity, and suggested that this effect might represent the mechanism by which thyroid hormone influences cardiac performance (7).

The present investigation describes the effects of the thyroid hormones and structurally related compounds on

<sup>1</sup> Abbreviations used are: AMP = adenosine monophosphate; ATP = adenosine triphosphate; EDTA = ethylenediaminetetraacetate.

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myocardial adenyl cyclase activity. Evidence is presented suggesting that separate receptor sites and separate adenyl cyclase systems exist in the heart, one responsive to thyroid hormone and the other to catecholamines.

## METHODS

Left ventricular muscle was obtained from normal cats, and a single cat was used for each experiment. After the animals were anesthetized with pentobarbital, 25–35 mg/kg, the heart was quickly excised. The left ventricle was dissected free of endocardium and epicardium, and approximately 220–250 mg of left ventricular muscle was homogenized in 4.5 ml of cold 0.25 M sucrose with a motor-driven homogenizer at 1°C. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant fluid decanted; the particles were washed with cold 0.25 M sucrose, resuspended, and recentrifuged at 10,000 rpm for 10 min. The washed particles were resuspended and homogenized in the cold 0.25 M sucrose. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (8) and adenyl cyclase was assayed by a recently developed method (9). The particulate fraction, containing 0.06–0.09 mg protein in a total volume of 0.06 ml, was incubated at 37°C for 3 min (except where noted in the text) with the following: ATP, 1.6 mmoles/liter; ATP- $\alpha$ - $^{32}$ P,  $2.5 \times 10^6$  cpm; theophylline, 8 mmoles/liter; MgCl<sub>2</sub>, 2 mmoles/liter; Tris-Cl, 21 mmoles/liter, (pH 7.7), human serum albumin, 0.8 mg/ml; and hormone at concentrations stated in the text. The incubations were started by adding the particulate fraction, which had been kept at 1°C, to the other components which were at 23°C. Hormone was added to the particles just before the incubations were initiated. DL-propranolol, when present, was added immediately before the addition of hormone. The incubations were stopped by adding 0.1 ml of a solution containing 4  $\mu$ moles of ATP, 1.25  $\mu$ moles of cyclic 3',5'-AMP, and 0.15  $\mu$ c of cyclic 3',5'-AMP- $^3$ H, and boiled for 3 min. The cyclic 3',5'-AMP- $^3$ H served to determine the recovery of cyclic 3',5'-AMP during the procedure; recoveries were 30–35%. After boiling, 0.4 ml of water was added, the precipitate removed by centrifugation, and the supernate applied to a 0.5  $\times$  2.0 cm Dowex 50 column. The column was washed with water, and the eluate, between 3.0

TABLE I  
Activation of Cardiac Adenyl Cyclase by  
L-Thyroxine and L-Triiodothyronine\*

	Cyclic 3',5'-AMP accumulated
	<i>p</i> moles/3 min per mg protein
Control	93 $\pm$ 4
L-Thyroxine ( $5 \times 10^{-6}$ moles/liter)	148 $\pm$ 4†
Control	99 $\pm$ 6
L-Triiodothyronine ( $5 \times 10^{-6}$ moles/liter)	145 $\pm$ 5‡

\* The values represent the mean  $\pm$ SE of 32 control and 32 hormone samples in 10 cats for the L-thyroxine experiments and 12 control and 12 hormone samples in five cats for the L-triiodothyronine experiments.

†  $P < 0.001$ .

‡  $P < 0.01$ .

TABLE II  
Specificity of L-Thyroxine-Mediated Conversion of ATP- $^{32}$ P  
to Cyclic 3', 5'-AMP- $^{32}$ P\*

	Cyclic 3',5'-AMP accumulated
	<i>p</i> moles/3 min per mg protein
Enzyme present‡	
Thyroxine absent	95 $\pm$ 1
Thyroxine absent, diluent present	96 $\pm$ 5
Thyroxine present	138 $\pm$ 1
Boiled enzyme	
Thyroxine absent	< 1
Thyroxine present	< 1
Enzyme absent	
Thyroxine absent	< 1
Thyroxine present	< 1

\* ATP, ATP- $^{32}$ P, theophylline, MgCl<sub>2</sub>, Tris-Cl, and human serum albumin were present in all incubations at concentrations noted under Methods.

‡ Values represent the mean  $\pm$ SE of three samples.

and 6.0 ml, was collected and precipitated with 0.17 M ZnSO<sub>4</sub> and 0.15 M Ba(OH)<sub>2</sub>. The mixture was centrifuged, and the cyclic 3',5'-AMP- $^{32}$ P and cyclic 3',5'-AMP- $^3$ H in the supernatant fraction were then counted in a liquid scintillation spectrometer.

The presence of cyclic 3',5'-AMP in the final barium-zinc supernatant fluid was confirmed by thin-layer chromatography in a solvent system containing a mixture of *n*-butanol, acetone, acetic acid, 5% ammonia, and water (7:5:3:3:2).

Results are expressed as the mean  $\pm$ SE. Statistical tests of significance were performed utilizing the Student's *t* test.

## RESULTS

*Effects of L-thyroxine and L-triiodothyronine on myocardial adenyl cyclase.* L-thyroxine and L-triiodothyronine at  $5 \times 10^{-6}$  moles/liter increased the conversion of ATP- $^{32}$ P to cyclic 3',5'-AMP- $^{32}$ P in the particulate fraction of cat heart homogenates by 60% and 45% respectively (Table I). Accumulation of cyclic 3',5'-AMP was detected as early as 1 min after the start of the incubation; formation of cyclic 3',5'-AMP was linear with time (Fig. 1) and protein concentration (Fig. 2). Although the increased accumulation of cyclic 3',5'-AMP could have been due to inhibition of phosphodiesterase activity rather than to activation of adenyl cyclase, previous studies demonstrated that under the experimental conditions employed phosphodiesterase activity was unaffected by either L-thyroxine or L-triiodothyronine (7). Incubations with a boiled enzyme preparation, with thyroid hormone diluent, or with thyroxine in the absence of enzyme failed to increase the conversion of ATP- $^{32}$ P to cyclic 3',5'-AMP- $^{32}$ P (Table II).

*Effects on adenyl cyclase of compounds structurally related to L-thyroxine and L-triiodothyronine.* The ca-

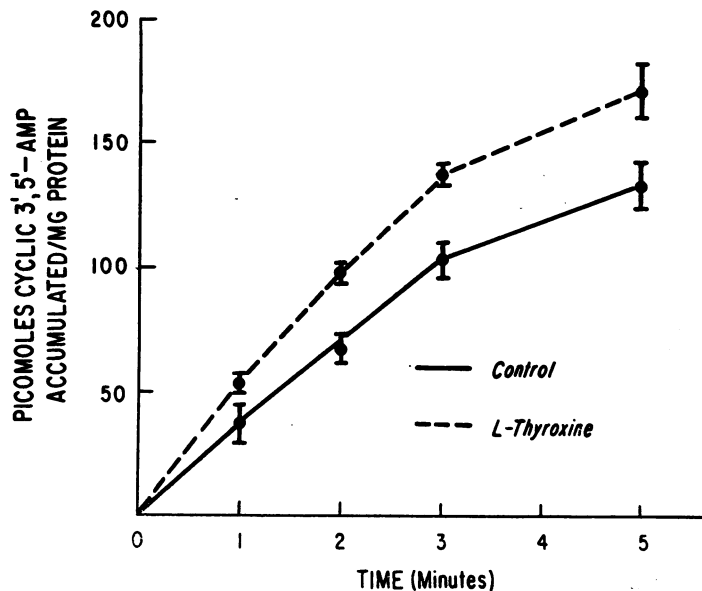


FIGURE 1 Activation of myocardial adenylyl cyclase by L-thyroxine as a function of time. L-thyroxine was present at  $5 \times 10^{-6}$  moles/liter. Each value represents the mean  $\pm$ SE of three samples.

capacity to activate myocardial adenylyl cyclase by a variety of compounds structurally related to the thyroid hormones was determined. These compounds included D-thyroxine, 3,3',5'-triiodo-DL-thyronine, 3,5-diiodo-L-thyronine,

L-thyronine, 3,5-diiodotyrosine, monoiodotyrosine, and tyrosine (Table III). Only the first two agents activated adenylyl cyclase. D-thyroxine was found to be equally as potent as L-thyroxine in mediating the conversion of

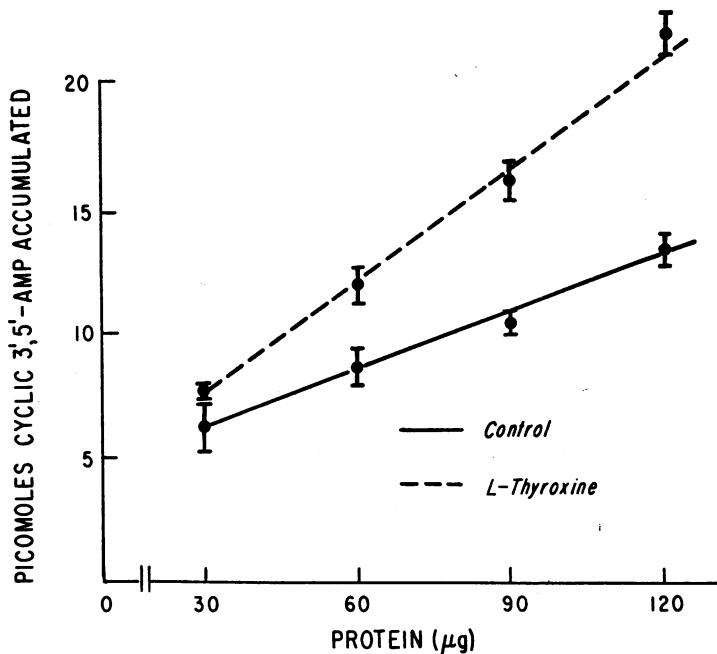


FIGURE 2 Activation of myocardial adenylyl cyclase by L-thyroxine as a function of protein concentration. L-thyroxine was present at  $5 \times 10^{-6}$  moles/liter. Each value represents the mean  $\pm$ SE of four samples.

ATP-<sup>32</sup>P to cyclic 3',5'-AMP-<sup>32</sup>P; the estimated  $K_m$  for D-thyroxine was  $4 \times 10^{-7}$  moles/liter, a value essentially identical to that previously reported for L-thyroxine and L-triiodothyronine in this system (7). Although 3,3',5'-triiodo-DL-thyronine also increased the accumulation of cyclic 3',5'-AMP ( $P < 0.02$ ), its potency was about 60% of that of D- or L-thyroxine ( $P < 0.05$ ) under the experimental conditions. Phosphodiesterase activity was unaffected by either D-thyroxine or 3,3',5'-triiodo-DL-thyronine. Thus, the increased accumulation of cyclic 3',5'-AMP produced by these agents would appear to be due to activation of adenylyl cyclase, rather than to inhibition of phosphodiesterase activity.

**Effect of pentabromophenol.** Pentabromophenol, a halophenol derivative that in vitro has been shown to produce effects similar to thyroxine on glutamic dehydrogenase and oxidative phosphorylation (10, 11), did not significantly increase the accumulation of cyclic 3',5'-AMP (Table IV).

**Effect of EDTA on L-thyroxine activation of cardiac adenylyl cyclase.** Thyroxine chelates divalent cations (12). To determine whether the activation of cardiac adenylyl cyclase was secondary to chelation of an inhibitor of adenylyl cyclase such as calcium, ventricular muscle was homogenized in the presence of EDTA,  $1 \times 10^{-3}$  moles/liter, washed, and then incubated with L-thyroxine (Table V). Pretreatment with EDTA did not alter control values of cyclic 3',5'-AMP production nor did it diminish the thyroxine-mediated increase in cyclic 3',5'-AMP production.

**Effects of DL-propranolol on activation of myocardial adenylyl cyclase by L-thyroxine and L-triiodothyronine.** The catecholamine-induced activation of myocardial adenylyl cyclase is effectively inhibited by propranolol, a

TABLE IV  
Effect of Pentabromophenol

	Cyclic 3',5'-AMP accumulated	P value
	<i>pmoles/3 min per mg protein</i>	
Control*	97 ± 4	—
Pentabromophenol ( $5 \times 10^{-6}$ moles/liter)	108 ± 6	NS
L-thyroxine ( $5 \times 10^{-6}$ moles/liter)	140 ± 6	<0.01

\* Each value represents the mean ±SE of eight samples in three cats.

beta adrenergic blocking drug (13). Activation of adenylyl cyclase by the maximal concentration of L-thyroxine ( $5 \times 10^{-6}$  moles/liter), however, was not diminished by a concentration of DL-propranolol ( $1 \times 10^{-5}$  moles/liter) that totally abolished the effects of L-norepinephrine ( $1 \times 10^{-5}$  moles/liter) on adenylyl cyclase activity (Fig. 3). The effects of triiodothyronine on adenylyl cyclase activation were also unaffected by DL-propranolol.

**Effects of combined maximal doses of L-thyroxine and L-norepinephrine on activation of myocardial adenylyl cyclase.** The combination of maximal stimulatory doses of L-thyroxine ( $5 \times 10^{-6}$  moles/liter) and L-norepinephrine ( $5 \times 10^{-6}$  moles/liter) produced an additive effect on cyclic 3',5'-AMP production when compared with the effects of the hormones incubated individually (Fig. 4). Thus, L-thyroxine and L-norepinephrine produced increases of  $51 \pm 5.5$  and  $129.4 \pm 4.8$  picomoles of cyclic 3',5'-AMP/3 min per mg of protein respectively. Both hormones in combination produced an increase of  $183.7 \pm 9.1$  picomoles, a value almost identical with the sum

TABLE III  
Effect of Compounds Structurally Related to Thyroid Hormone\*

	Cyclic 3',5'-AMP accumulated	P value
	<i>pmoles/3 min per mg protein</i>	
Control‡	92 ± 2	—
L-Thyroxine	122 ± 2	<0.001
D-Thyroxine	123 ± 1	<0.001
3,3',5'-Triiodo-DL-thyronine	111 ± 3	<0.02
3,5-Diiodo-L-thyronine	94 ± 4	NS
L-Thyronine	95 ± 3	NS
3,5-Diiodotyrosine	87 ± 13	NS
Monoiodotyrosine	92 ± 8	NS
Tyrosine	80 ± 10	NS

\* L-thyroxine and related compounds present at  $5 \times 10^{-6}$  moles/liter.

‡ Each value represents the mean ±SE of three to six samples.

TABLE V  
Effects of EDTA on the Activation of Cardiac Adenylyl Cyclase by L-Thyroxine\*

	Cyclic 3',5'-AMP accumulated
	<i>pmoles/3 min per mg protein</i>
EDTA absent‡	
Control	102 ± 5
L-Thyroxine ( $5 \times 10^{-6}$ moles/liter)	133 ± 7
EDTA present	
Control	101 ± 6
L-Thyroxine ( $5 \times 10^{-6}$ moles/liter)	160 ± 5

\* Ventricular muscle was homogenized in 0.25 M sucrose in the presence of  $1 \times 10^{-3}$  M EDTA. The particles were washed with 0.25 M sucrose in the absence of EDTA as noted under Methods.

‡ The values represent the mean ±SE of four samples.

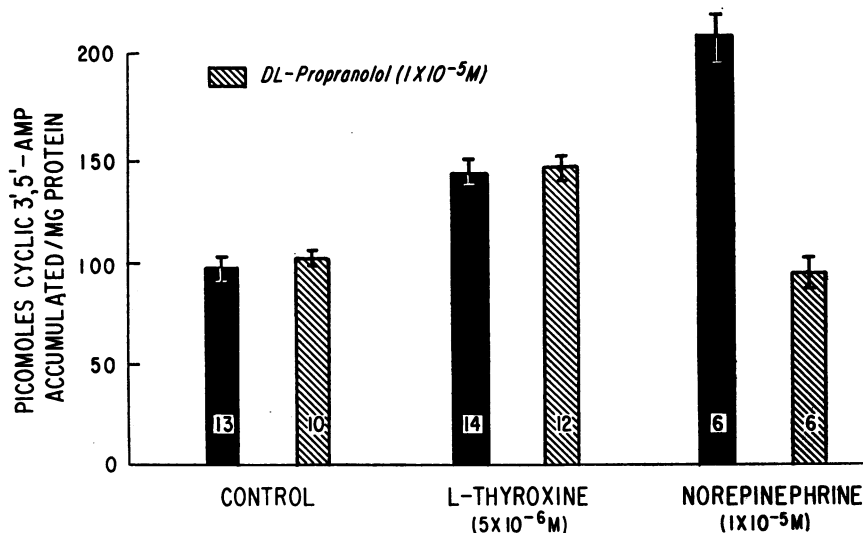


FIGURE 3 Effect of DL-propranolol on L-thyroxine and L-norepinephrine activation of adenylyl cyclase. Each bar represents the mean  $\pm$ SE; the number of determinations is shown at the base of each bar.

of the increments produced by the hormones individually. The increase produced by the combination of thyroxine and norepinephrine was significantly higher than that produced by the individual hormones ( $P < 0.01$ ).

Combined maximal doses of L-thyroxine and L-triiodothyronine did not result in any increase in cyclic 3',5'-AMP production above that seen with the individual hormones.

## DISCUSSION

Although it has been suggested that the cardiac manifestations of the hyperthyroid state are caused by an enhanced sensitivity of the heart to catecholamine stimulation, this hypothesis has not been confirmed in several carefully controlled studies in which it was shown that norepinephrine produces the same increases in heart rate (4) and myocardial contractility (1, 5) in hyperthyroid, as in euthyroid animals. Similar results were obtained when the effects of cardiac sympathetic nerve stimulation on heart rate and blood pressure were compared in euthyroid and hyperthyroid cats (4), and when the changes in heart rate produced by an infusion of isoproterenol in normal subjects were compared with the changes occurring in these same individuals after hyperthyroidism was induced pharmacologically (14). In addition to these studies showing that the *physiologic* effects of adrenergic stimulation on the heart are not augmented, it has recently been shown that the ability of norepinephrine to activate adenylyl cyclase, the enzyme believed to be responsible for mediating the cardiac

effects of catecholamines, is not enhanced in hyperthyroid animals.<sup>2</sup>

Further evidence suggesting that thyroid hormone exerts an effect on the heart independent of the adrenergic system was provided by the demonstrations that the addition of triiodothyronine to isolated myocardial cells of a 24 hr chick embryo culture produced an immediate increase in the rate of pulsation (15), and that depletion of norepinephrine by the administration of reserpine did not effect the augmentation of the intrinsic contractile state of isolated cat papillary muscle produced by hyperthyroidism (1).

The present investigation was undertaken in an attempt to define and characterize the mechanism through which thyroid hormone might exert a direct effect on the heart. Since the positive inotropic and chronotropic actions of catecholamines and glucagon are believed to be mediated by activation of adenylyl cyclase (6, 13), the observation that L-thyroxine and L-triiodothyronine are also capable of activating this enzyme suggests that the direct effect of thyroid hormone on myocardial performance may be mediated by activation of adenylyl cyclase.

There are, nevertheless, several findings that appear to be at variance with such a hypothesis. First, the acute administration of L-thyroxine or triiodothyronine does not alter the contractile state of the isolated cat papillary muscle.<sup>3</sup> Second, although D-thyroxine is a considerably less potent thyromimetic agent than L-thyroxine *in vivo* (16), it is equally as potent as L-thyroxine in activating adenylyl cyclase. Third, 3,3',5'-DL-triiodothyronine, or

<sup>2</sup>Levey, G. S., C. L. Skelton, and S. E. Epstein. Submitted for publication.

<sup>3</sup>Skelton, C. L. Personal Communication.

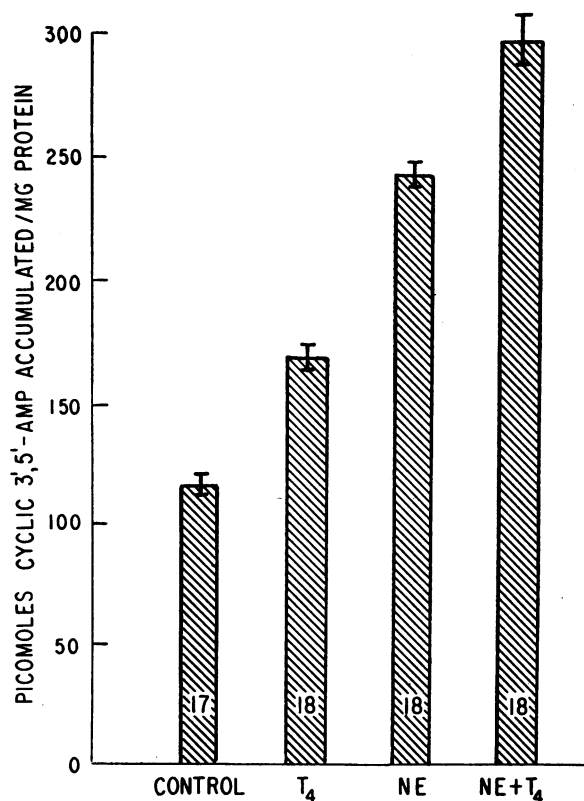


FIGURE 4 Effect of combined maximal doses of L-thyroxine and L-norepinephrine. L-thyroxine was present at  $5 \times 10^{-8}$  moles/liter and L-norepinephrine at  $5 \times 10^{-8}$  moles/liter. NE = norepinephrine; T<sub>4</sub> = L-thyroxine.

"reverse T<sub>3</sub>," a compound thought to have little if any thyromimetic activity *in vivo* (17), also activates myocardial adenylyl cyclase, although to a lesser extent than L- or D-thyroxine. On the basis of these observations, the physiologic significance of the thyroid-induced activation of myocardial adenylyl cyclase might be questioned. However, it is probably invalid to compare the effects of such compounds in broken cell preparations with the effects observed in whole tissue preparations. For example, there is a substantial delay between the administration of L-thyroxine or triiodothyronine *in vivo* and the appearance of the biochemical and physiologic changes characteristic of these hormones (18). This suggests that diffusion or metabolic factors may be responsible for prolonging the time it takes for sufficient concentrations of the active form of thyroid hormone to appear at the site responsible for initiating the end-organ response. It is therefore not surprising that papillary muscle contractility is unchanged by acute exposure to L-thyroxine or triiodothyronine. On the other hand, broken cell systems would provide ready access to the site of action of the hormone, and thus the demonstration of acute effects would not be unexpected.

Similar considerations apply when the relative potencies of D-thyroxine and L-thyroxine in *in vitro* preparations are compared with their relative potencies *in vivo*. For example, it has been shown that when administered parenterally, D-thyroxine is metabolized more rapidly and distributed differently than the L-isomer (19, 20). In addition, the discrepancy between the potencies of these agents as measured *in vivo* and *in vitro* is not limited to the adenylyl cyclase system (21-27). With the exception of D-thyroxine and 3,3',5'-triiodothyronine, none of the other compounds tested in this study that were structurally related to thyroid hormone were capable of activating myocardial adenylyl cyclase.

Several *in vitro* studies have shown that thyroid hormone can chelate certain divalent cations, including Ca<sup>2+</sup>, an inhibitor of adenylyl cyclase (12, 28). Conceivably, the effect of thyroid on adenylyl cyclase activation could therefore be due to the binding of such an inhibitor. If this were the case reduction of the ionic concentration of calcium with EDTA would be expected to diminish the thyroid-mediated activation of adenylyl cyclase. However, no diminution in activation was observed when ventricular muscle was homogenized in the presence of  $1 \times 10^{-8}$  M EDTA before incubation with thyroid hormone. This finding suggests that thyroid hormone does not activate myocardial adenylyl cyclase by chelating Ca<sup>2+</sup> or other divalent cations that bind to EDTA.

The activation of myocardial adenylyl cyclase by catecholamines has been shown to be blocked by drugs characterized pharmacologically as beta adrenergic blocking agents (6, 13). This finding provided further evidence that catecholamine activation of adenylyl cyclase is mediated by the beta receptor. However, propranolol at a concentration sufficient to block completely the effects of  $1 \times 10^{-8}$  M norepinephrine on adenylyl cyclase did not abolish the activation by L-thyroxine and L-triiodothyronine, indicating that there are separate adenylyl cyclase receptors in the heart for thyroid hormone and catecholamines.

In a previous study we found that additive effects on myocardial adenylyl cyclase activity were not produced when maximal concentrations of glucagon and norepinephrine were incubated together (13). Since additive effects would have been expected if two separate adenylyl cyclase systems were being stimulated, it was hypothesized that glucagon and norepinephrine acted on the same adenylyl cyclase system. In contrast, the present investigation demonstrates that maximal stimulatory doses of L-thyroxine and L-norepinephrine do produce additive effects on cyclic 3',5'-AMP production. The results of these investigations are therefore consistent with the hypothesis that at least two separate adenylyl cyclase systems are present in the heart, one responsive to thy-

roid hormone and the other to norepinephrine and glucagon.

#### ACKNOWLEDGMENT

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