Beta-Adrenergic Potentiation of the Increased In Vitro Accumulation of Cycloleucine by Rat Thymocytes Induced by Triiodothyronine

JAMES ETZKORN, PATRICIA HOPKINS, JANET GRAY, JOSEPH SEGAL, and
SIDNEY H. INGBAR, *Thorndike Laboratory of Harvard Medical School, and
Department of Medicine, Beth Israel Hospital, Boston, Massachusetts 02215*

ABSTRACT We have previously demonstrated that 3,5,3'-triiodothyronine (T_3), whether administered in vivo or added to suspending media in vitro, promptly stimulates the in vitro accumulation of the non-metabolized amino acids, alpha-aminoisobutyric acid, and cycloleucine (CLE) by thymocytes isolated from weanling rats. In these studies, we have examined the in vitro interaction between catecholamines and T_3 with respect to this effect. The previously reported enhancement of CLE accumulation in thymocytes by T_3 in vitro ($1 \mu\text{M}$) was confirmed. When added alone in concentrations ranging between 10 nM and 0.1 mM, the adrenergic agonists, epinephrine and norepinephrine, had no effect on CLE accumulation. At a concentration of $1 \mu\text{M}$, isoproterenol, terbutaline, and phenylephrine were also without effect. However, the effect of T_3 was clearly potentiated by the concomitant addition of epinephrine, norepinephrine, and possibly isoproterenol, whereas terbutaline and phenylephrine were without effect. Neither basal nor T_3 -enhanced CLE accumulation was affected by the addition alone of the adrenergic blocking agents, propranolol (0.1 mM), phentolamine ($10 \mu\text{M}$), or practolol (0.1 mM). Nevertheless, the β_1 - and β_2 -antagonist, propranolol, and the β_1 -antagonist, practolol, blocked the increment in CLE accumulation produced by epinephrine; the α -antagonist, phentolamine, was without effect.

The enhancement of CLE accumulation that occurred in the presence of T_3 , with or without epinephrine, was seen to be a result of an inhibition of CLE efflux, because T_3 alone inhibited CLE efflux, and this effect was increased when epinephrine was also present. On

the other hand, neither T_3 alone nor T_3 plus epinephrine appreciably altered the rate of inward transport of CLE. As judged from studies of the ability of thymocytes to exclude trypan blue, neither T_3 alone nor T_3 plus epinephrine either enhanced or impaired viability of cells during 3-h periods of incubation. Cell water content, measured with [^3H]urea, was unaffected by T_3 , either alone or in the presence of epinephrine. In confirmation of previous results, the stimulatory effect of T_3 on CLE accumulation was unaffected by concentrations of puromycin sufficient to inhibit protein synthesis by at least 95%, and the potentiating action of epinephrine on the response to T_3 was similarly unaffected.

From these findings, it is concluded that the effect of T_3 to increase CLE accumulation by thymocytes in vitro, though itself independent of adrenergic mediation, is potentiated by β_1 -adrenergic stimulation. This interaction appears distinctly different from other thyroid hormone-catecholamine interactions, in which thyroid hormones enhance physiological responses to catecholamines. Its mechanism remains unclear, but the properties of the T_3 effect, and possibly the interaction itself, suggest that T_3 enhances CLE accumulation by an action at the level of the cell membrane.

INTRODUCTION

A number of questions concerning the actions of the thyroid hormones have long been under investigation but remain, nevertheless, incompletely clarified. Among these are questions concerning the primary mechanism of thyroid action at the cellular and subcellular levels; others concern the nature of the interaction between thyroid hormones and catecholamines that is so apparent in many physiological and clinical settings (1-3). With respect to the primary locus of thyroid hormone action, numerous data suggest

This study was presented in part at The Eighth Annual Meeting of the European Thyroid Association, Lyon, France, October 1977.

Received for publication 21 July 1978 and in revised form 6 December 1978.

a direct action at the nuclear level to modify gene expression (4–7). Still other data raise the possibility that high affinity receptors for thyroid hormone exist within the mitochondrion and that thyroid hormones exert a direct effect on mitochondrial function (8–10). Data recently acquired by ourselves and others also support a direct, extranuclear action of thyroid hormones. Thus, *in vitro*, thyroid hormones enhance the accumulation of the nonmetabolized amino acids, alpha-aminoisobutyric acid, and 1-amino-cyclopentane-1-carboxylic acid (cycloleucine [CLE])¹ by both chick embryo cartilage (11, 12) and rat thymocytes (13, 14), and also increase the transport of the glucose analogue, 2-deoxy-D-glucose, by chick embryo myocardium in culture (15) and by freshly isolated rat thymocytes (16). These effects of thyroid hormone appear very promptly and are unaffected by inhibitors of protein or RNA synthesis, or both. Both the nature and promptness of these effects point to an action of thyroid hormone on the cell membrane or on membrane-mediated function.

In these studies, we have extended our earlier observations concerning the stimulatory effect of 3,5,3'-triiodothyronine (T₃) on the accumulation of CLE by rat thymocytes *in vitro* and have explored the interactions between T₃ and catecholamines within this system. The data reveal a previously unknown and possibly unique interaction in which certain catecholamines, though themselves without effect, act promptly to enhance the increase in CLE accumulation produced by T₃.

METHODS

Experiments were performed in thymocytes isolated from female CD rats purchased from Charles River Breeding Laboratories, Wilmington, Mass. Animals were 21 d old at the time of arrival and were fed Purina Laboratory Chow (Ralston Purina Co., Inc., St. Louis, Mo.) and given tap water to drink *ad libitum* until the time of sacrifice, which occurred between 2 and 8 d after arrival of animals in the laboratory.

On the day of each experiment, a single rat was decapitated and allowed to exsanguinate. The thymus gland was then quickly but gently removed, and thymocytes were isolated by the general technique previously employed in this laboratory (17). The gland was placed into a Petri dish standing on crushed ice and containing ice-cold buffer. The buffer employed contained the following: 120 mM NaCl; 5 mM KCl; 1 mM CaCl₂; 2.5 mM MgCl₂; 1.5 mM NaH₂PO₄; 25 mM Tris-HCl; 20 mM Hepes; and 22 mM glucose at pH 7.5.

Cells were freed from the thymus by gentle teasing with forceps, and when the liquid phase had become cloudy it was aspirated and filtered through nylon mesh. The filtrate was then centrifuged at 100 g for 20 min at 4°C, the supernate discarded, and the thymocyte pellet resuspended in 20 ml of cold buffer. Cells were then counted in a hemocytometer, and

buffer was added to yield a final thymocyte concentration of $2\text{--}4 \times 10^7$ cells/ml.

Accumulation of CLE. 1-ml aliquots of cell suspension were pipetted into Erlenmeyer flasks and were allowed a 15-min period of equilibration in a metabolic shaker at 37°C under room air. At this time, 50- μ l aliquots of buffer which contained T₃, adrenergic agonists, or adrenergic antagonists² were added in varying combinations to the experimental flasks while equal volumes of buffer were added to the controls. Incubations were then continued for an additional 30 min. At that time, 50 μ l of a solution of [¹⁴C]CLE was added to all vessels, together with 50 μ l of a [³H]mannitol solution to serve as an extracellular marker. Final concentration of CLE was 30 μ M, and that of mannitol was 4 nM. In all experiments, duplicate vessels for each experimental group and for the control group were prepared.

Suspensions were then incubated for 2 h, at which time triplicate 100- μ l aliquots were pipetted from each flask and transferred to microfuge tubes. Suspensions were then centrifuged for 15 s at 10,000 g in a Beckman microfuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.), the supernates were carefully aspirated, and 100 μ l of 10% perchloric acid was added to the cells. Each tube was then agitated vigorously in a Vortex mixer (Scientific Industries, Inc., Bohemia, N. Y.), and its contents were transferred to a scintillation vial. Each tube was washed with scintillation fluid, which was then transferred into its corresponding vial, and samples were counted in a liquid scintillation counter.

Uptake of CLE by thymocytes was calculated from the ¹⁴C counts contained in each vial, the known total counts added, and the known final concentration of CLE in the medium. Values for the uptake were corrected for the volume of the occluded extracellular phase, as judged from the ³H counting rate, and corrected values for CLE uptake were expressed as nanomoles per 10⁶ cells per 2 h.

Influx and efflux of CLE. Experiments were performed to ascertain the effect of various agents on the rate of influx of CLE into thymocytes. In these experiments, incubations were carried out exactly as described above, except that they were terminated 5 min after the addition of [¹⁴C]CLE.

Other experiments were performed to assess the effect of various agents on the rate of efflux of accumulated [¹⁴C]CLE from thymocytes. In these experiments, large volumes of thymocyte suspensions in media containing the agents to be tested were incubated with [¹⁴C]CLE for 1 h. Three 1-ml aliquots from each of the experimental groups were then pipetted into centrifuge tubes and spun for 5 min at 2,000 g. The pellets were then resuspended in buffer that contained the same agents with which the thymocytes had originally been incubated, but lacking [¹⁴C]CLE. Immediately, and at 5-min intervals thereafter, 100- μ l aliquots were removed, centrifuged, and prepared for counting as in the accumulation experiments described above.

Effect of puromycin. Four experiments were performed in which the effect of puromycin on the response of CLE accumulation by thymocytes to T₃, with or without

¹Abbreviations used in this paper: CLE, cycloleucine; T₃, 3,5,3'-triiodothyronine.

²The following reagents were obtained from commercial sources: [¹⁴C]CLE and [³H]mannitol were from New England Nuclear, Boston, Mass.; T₃, propranolol, epinephrine, norepinephrine, phenylephrine, and isoproterenol were from Sigma Chemical Co. St. Louis, Mo. Phentolamine was a gift from the Ciba-Geigy Corp., Pharmaceuticals Div., Summit, N. J., practolol was from Ayerst Laboratories, N. Y., and terbutaline was from Astra Pharmaceutical Products, Inc., Framingham, Mass. Optically active compounds among those listed above were all studied in the levorotatory form.

epinephrine, was assessed. Cells were preincubated for 15 min, after which puromycin was added to one-half of the vessels at a concentration (100 $\mu\text{g/ml}$) previously shown to inhibit amino acid incorporation into thymocyte protein under similar conditions of incubation by 95% (16). After 10 min, certain of the vessels were enriched with T_3 (1 μM) or T_3 plus epinephrine (1 μM). 30 min later, [^{14}C]CLE and [^3H]mannitol were added to all vessels, as described above. Aliquots of cell suspensions were removed for measurement of CLE accumulation 30 min and 2 h after addition of CLE. In each of these experiments, all experimental groups were studied in quadruplicate.

Cell integrity and viability. Additional experiments were performed to assess the effects of T_3 , with or without epinephrine, on cellular integrity and viability. Four experiments were performed in which triplicate vessels containing thymocytes were incubated for varying periods with or without T_3 (1 μM) or T_3 plus epinephrine (1 μM), together with [^3H]mannitol, as above, and [^{14}C]urea (4 $\mu\text{Ci/ml}$; 47.1 mCi/nmol sp act). At the end of incubations, cells were processed for the measurement of intracellular [^{14}C]urea content as for the measurement of [^{14}C]CLE content, described above.

In other experiments, control vessels and vessels containing either T_3 (1 μM) or T_3 plus epinephrine (1 μM) were incubated for 3 h with trypan blue (0.3%). Aggregation or disaggregation of the cells, as well as their ability to exclude trypan blue, was then assessed microscopically.

RESULTS

Effect of adrenergic agonists (Table I). Epinephrine and norepinephrine, in concentrations ranging between 10 nM and 100 μM , as well as isoproterenol, terbutaline, and phenylephrine at 1 μM , all failed to induce a significant change in CLE accumulation.

Effect of adrenergic agents on the action of T_3

(Table II). Studies were performed to ascertain whether the adrenergic agonists, though inactive alone, would alter the increase in CLE accumulation in thymocytes induced by T_3 . In these groups of experiments, with one exception, T_3 was employed at a concentration of 1.0 μM , and a similar concentration was employed in the case of the adrenergic agonists. The previously described effect of 1.0 μM T_3 to increase CLE accumulation was again observed, and both epinephrine and norepinephrine significantly increased CLE accumulation to values higher than those seen with T_3 alone. In the case of isoproterenol, values for CLE accumulation in the presence of both T_3 and isoproterenol were higher than those found with T_3 alone in each of the six experiments performed. However, the magnitude of this difference was quite variable, with the result that mean values were not significantly different by the paired *t* test that was employed in statistical analyses (18). Terbutaline and phenylephrine were entirely without effect.

Three experiments were performed in which a lower dose of T_3 (0.1 μM) was employed. As expected, no effect of T_3 alone on CLE accumulation was observed, but, despite the small number of experiments performed, CLE accumulation in the presence of epinephrine was significantly higher than in control vessels.

Effect of adrenergic antagonists (Table III). Experiments were also performed to determine the effect of adrenergic antagonists on both basal and T_3 -stimulated CLE accumulation. Propranolol (0.1 mM), phentolamine (10 μM), and practolol (0.1 mM) all failed to influence basal CLE accumulation. Concen-

TABLE I
The In Vitro Effect of Adrenergic Agonists on CLE Accumulation by Rat Thymocytes

Agonist	n*	CLE accumulation					
		Concentration of agonist					
		0	10 nM	0.1 μM	1 μM	10 μM	0.1 mM
nmol/10 ⁶ cells/2 h							
Epinephrine	5	12.1±1.6‡	11.5±1.5	11.4±1.6	12.0±1.4	11.1±1.4	11.6±1.4
	8	10.3±1.5			10.3±1.4		
Norepinephrine	3	9.5±1.2	9.9±2	8.2±1.4	10.2±1.9	10.1±1.8	9.9±1.7
	4	9.4±2.1		8.6±1.3	9.8±1.7	9.8±1.6	9.9±1.2
	6	9.1±0.7			9.6±1.1		
Isoproterenol	3	8.3±1.6			7.7±0.8		
Terbutaline	5	10.1±0.3			10.2±0.4		
Phenylephrine	5	7.7±0.7			6.9±0.8		

* Number of experiments performed in which all the variables shown were tested in duplicate at the same time in identical thymocyte suspensions.

† Mean $\pm$ SE. For each agonist, none of the values shown is significantly different from control or from any of the others.

TABLE II
*The Effect of Adrenergic Agonists on T₃-Stimulated Accumulation of CLE by Rat Thymocytes**

Agonists	n	CLE accumulation			Statistical analysis†		
		(a) Control	(b) T ₃	(c) T ₃ + agonist	(b) - (a)	(c) - (a)	(c) - (b)
		nmol/10 ⁶ cells/2 h			mean difference ± SEMD		
Epinephrine, 1 μM§	3	8.4 ± 1.3	8.1 ± 0.9	10.2 ± 1.4	-0.4 ± 0.6 NS	1.7 ± 0.3 P < 0.05	2.0 ± 0.4 NS
Epinephrine, 1 μM	20	9.6 ± 0.6	11.8 ± 0.9	13.8 ± 1.2	2.1 ± 0.7 P < 0.01	4.2 ± 0.8 P < 0.01	2.1 ± 0.6 P < 0.01
Norepinephrine, 1 μM	5	8.5 ± 1.1	11.3 ± 1.4	14.1 ± 1.3	2.8 ± 0.9 P < 0.05	5.6 ± 1.2 P < 0.05	2.8 ± 0.9 P < 0.05
Isoproterenol, 1 μM	6	9.8 ± 0.6	13.1 ± 1.1	14.5 ± 2.2	3.3 ± 0.7 P < 0.01	4.7 ± 1.8 P < 0.05	1.4 ± 1.3 NS
Terbutaline, 1 μM	6	10.1 ± 0.7	13.7 ± 0.8	13.5 ± 0.7	3.6 ± 0.3 P < 0.02	3.4 ± 0.1 P < 0.01	-0.1 ± 0.4 NS
Phenylephrine, 1 μM	5	7.5 ± 1.4	9.9 ± 1.2	9.4 ± 1.2	2.4 ± 0.4 P < 0.01	1.8 ± 0.3 P < 0.01	-0.4 ± 0.1 NS

* Values for CLE accumulation shown are mean ± SE for the number of separate experiments, indicated by n, in which all variables shown were tested in duplicate at the same time in identical thymocyte suspensions.

† Statistical analyses by paired *t* test.

§ In these experiments, T₃ was added at a concentration of 0.1 μM. In the remaining experiments, the concentration of T₃ employed was 1 μM.

trations of these agents lower than the above were also without effect (data not shown). Results shown for phentolamine are those obtained with a concentration of 10 μM, because a 0.1-mM concentration of this agent inhibited basal CLE accumulation.

In experiments seeking a direct interaction between T₃ and the adrenergic blocking agents, significant stimulation of CLE accumulation by T₃ alone was observed in those experiments with propranolol and phentolamine. In each of four experiments concerning the effects of practolol, T₃ increased CLE accumulation above basal values, but owing to variation in the magnitude of the response to T₃, the effect of T₃ in these experiments was not statistically significant.

As had been the case with basal CLE accumulation (Table II), none of the adrenergic antagonists appreciably affected the increased CLE accumulation observed in the presence of T₃.

Effect of adrenergic antagonists in the presence of T₃ plus epinephrine (Table IV). Experiments were then conducted to ascertain whether the adrenergic antagonists would influence the synergistic interaction between T₃ and epinephrine already demonstrated. In these experiments, CLE accumulation was measured in control vessels, as well as in vessels containing T₃ (1 μM), T₃ plus epinephrine (1 μM), and T₃ plus both epinephrine and adrenergic antagonist. The concentrations of antagonists employed were the highest

concentrations previously shown to be without effect on either basal or T₃-increased CLE accumulation.

In these experiments, the well-documented effect of T₃ to increase CLE accumulation was again observed (statistical significances not shown), and in each of the three groups of experiments dealing with the three antagonists studied, CLE accumulation in the presence of T₃ plus epinephrine was significantly greater than that seen with T₃ alone. Phentolamine (10 μM) had no effect on the accumulation of CLE seen in the presence of T₃ plus epinephrine, with values remaining significantly higher than those seen in the presence of T₃ alone. In contrast, propranolol (0.1 mM) and practolol (0.1 mM) both blocked the potentiating effect of epinephrine on the action of T₃, significantly reducing CLE accumulation to values almost identical to those seen with T₃ alone.

Effect of T₃ with and without epinephrine on CLE influx and efflux. In four experiments in which thymocytes were allowed to accumulate [¹⁴C]CLE in control vessels, others containing T₃ (1 μM), and still others containing T₃ plus epinephrine (1 μM), the rate of efflux of [¹⁴C]CLE was assessed after transfer of thymocytes to analogous media devoid of [¹⁴C]CLE. In each of the four experiments, the rate of efflux of [¹⁴C]CLE was greatest in control samples, less in samples containing T₃, and least in samples containing T₃ plus epinephrine. Covariance analysis (18) of pooled

TABLE III
*The Effect of Adrenergic Antagonists on T₃-Stimulated CLE Accumulation by Rat Thymocytes In Vitro**

Antagonists	n	CLE accumulation				Statistical analysis‡		
		(a) Control	(b) T ₃	(c) Antagonist	(d) Antagonist + T ₃	(b) – (a)	(c) – (a)	(d) – (b)
		nmol/10 ⁶ cells/2 h				Mean difference ± SEMD		
Propranolol, 0.1 mM	8	7.6±0.3		7.2±0.2			–0.5±0.2 NS	
	8	8.2±0.5	10.0±0.6		9.5±0.7	1.8±0.5 P < 0.01		–0.4±0.5 NS
Phentolamine, 10 μM	3	11.4±3.1		10.6±2.9			–0.7±0.4 NS	
	6	10.6±1.4	12.3±1.3		12.1±1.3	1.7±0.4 P < 0.02		–0.2±0.2 NS
Practolol, 0.1 mM	6	9.6±0.6		9.3±0.9			–0.3±0.6 NS	
	4							
	4	9.6±0.7	12.8±1.7		12.2±1.9	3.2±1.2 NS		–0.6±0.6 NS

* Values for CLE accumulation are mean ± SE for the number of experiments, indicated by *n*, in which all variables shown were tested in duplicate at the same time in identical thymocyte suspensions. T₃ was employed at a concentration of 1 μM.

† Statistical analyses by paired *t* test.

data from the four experiments revealed significant differences in the efflux rates among all three experimental groups (Fig. 1).

No significant effect of T₃, with or without epinephrine, on the influx of [¹⁴C]CLE into thymocytes was observed. In six experiments, the accumulation of CLE after 5 min averaged 6.0 ± 2.4 (nanomoles per 10⁶ cells;

mean ± SD) in control specimens, 6.4 ± 2.9 in specimens incubated with T₃ (1 μM), and 5.2 ± 2.4 in specimens incubated with T₃ plus epinephrine (1 μM).

Effect of puromycin. In four experiments, preincubation with puromycin (100 μg/ml) inhibited the 30-min accumulation of CLE in T₃- and epinephrine-free control thymocytes by 11.4 ± 0.3%, and the

TABLE IV
*Effect of Adrenergic Antagonists on the Stimulation of CLE Accumulation in Rat Thymocytes Produced by T₃ and Epinephrine**

Antagonist	n	CLE accumulation				Statistical analysis‡		
		(a) Control	(b) T ₃	(c) T ₃ + Epi§	(d) T ₃ + Epi + Antag	(c) – (b)	(d) – (b)	(d) – (c)
nmol/10 ⁶ /2 h						Mean difference ± SEMD		
Propranolol, 0.1 mM	6	10.6±0.7	13.0±1.4	18.2±2.5	12.6±1.6	5.2±1.6 <i>P</i> < 0.05	–0.4±0.6 NS	–5.6±1.6 <i>P</i> < 0.02
Phentolamine, 10 μM	4	10.7±2.2	12.4±2.0	13.9±1.9	13.9±2.1	1.5±0.2 <i>P</i> < 0.01	1.5±0.2 <i>P</i> < 0.01	0.0±0.2 NS
Practolol, 0.1 mM	5	10.0±0.7	12.7±1.4	15.2±1.4	12.2±1.5	2.5±0.8 <i>P</i> < 0.05	–0.6±0.4 NS	–3.1±1.1 <i>P</i> < 0.05

* Values for CLE accumulation are mean ± SE for the number of separate experiments, indicated by *n*, in which all the variables shown were tested in duplicate at the same time in identical thymocyte suspensions. T₃ and epinephrine were employed at concentrations of 1 μM, antagonists were tested at the concentration indicated.

† Statistical analyses by paired *t* test.

§ Epi, epinephrine; Antag, antagonist.

FIGURE 1 The effect of T_3 , with and without epinephrine, on the efflux of $[^{14}\text{C}]$ CLE from rat thymocytes in vitro. Thymocytes were incubated with $[^{14}\text{C}]$ CLE at 37°C for 1 h in control medium, medium containing T_3 ($1\ \mu\text{M}$), or medium containing T_3 plus epinephrine ($1\ \mu\text{M}$). Cells were then transferred to analogous media that lacked $[^{14}\text{C}]$ CLE. Aliquots of suspensions were removed immediately and at 5-min intervals thereafter for measurement of cellular $[^{14}\text{C}]$ CLE content. Results shown are the means of those obtained in four experiments in which duplicate samples from each group were studied. Covariance analysis (18) revealed significant differences ($P < 0.01$) among efflux slopes of all three groups.

2-h accumulation of CLE by $48.9 \pm 1.6\%$. Nevertheless, puromycin did not alter the proportionate stimulation of CLE accumulation produced by T_3 ($1\ \mu\text{M}$) or T_3 plus epinephrine ($1\ \mu\text{M}$) at either time period. In the absence of puromycin, T_3 increased the 30-min accumulation of CLE significantly, by $27.7 \pm 4.5\%$; the percentage of increase ($25.3 \pm 3.2\%$) was almost identical in the cells treated with puromycin. A significantly ($P < 0.01$) greater proportionate enhancement of 30-min CLE accumulation was seen in vessels containing T_3 plus epinephrine ($38.0 \pm 6.8\%$), and this increase ($38.1 \pm 6.5\%$) was also uninfluenced by puromycin.

Similar results were obtained with respect to the 2-h accumulation of CLE. T_3 increased CLE accumulation by $31.0 \pm 2.8\%$ in the absence of puromycin and by $29.2 \pm 5.4\%$ in the presence of puromycin. A significantly ($P < 0.05$) greater increment in CLE accumulation was produced by T_3 in the presence of epinephrine ($40.8 \pm 3.8\%$), and this too was unaltered by puromycin ($39.3 \pm 5.8\%$).

Cell integrity and viability. Effects of T_3 ($1\ \mu\text{M}$) and of T_3 plus epinephrine ($1\ \mu\text{M}$) on CLE accumulation could not be explained by changes in the intracellular

diffusion space of CLE, i.e., the intracellular water content of thymocyte suspensions, as judged from intracellular:extracellular ratios of $[^{14}\text{C}]$ urea. In four experiments in which cells were incubated with or without T_3 , epinephrine, or T_3 plus epinephrine for 2 h, ratios expressed as a percentage of those found in untreated controls were 99.3 ± 3.1 in cells exposed to T_3 alone, 100.6 ± 3.0 in cells exposed to epinephrine alone, and 95.7 ± 1.0 in cells exposed to epinephrine plus T_3 .

After 3 h of incubation, thymocytes remained unaggregated in control vessels, as well as in those containing T_3 or T_3 plus epinephrine, and trypan blue was excluded by 93–96% of cells in all three experimental groups.

DISCUSSION

In these studies we have confirmed our earlier observations indicating that T_3 in vitro is capable of enhancing the accumulation of the nonmetabolized amino acids, alpha-aminoisobutyric acid and CLE, by thymocytes harvested from weanling rats, and that this effect is mainly a result of an inhibition of amino acid efflux, rather than an enhancement of inward transport (13, 14). We have, in addition, demonstrated that a variety of adrenergic agonists, thought to be active at either the alpha- or beta-adrenergic receptor, do not in themselves, over a wide range of concentrations, significantly affect CLE accumulation. Despite this, certain of the adrenergic agonists, notably epinephrine and norepinephrine, proved capable of increasing the response to T_3 , an effect seen to result from an increase in the T_3 -mediated inhibition of CLE efflux. Moreover, at the concentrations tested, several adrenergic antagonists were themselves without effect on basal or T_3 -increased CLE accumulation, but propranolol and practolol among them proved capable of abolishing the synergistic effect of epinephrine.

Among the variety of reported interactions between the thyroid hormones and adrenergic agonists (1–3), the presently described response appears, at least superficially, to be unique. Previously described interactions, certain of which are discussed more fully below, can be categorized as reflecting an enhanced response to catecholamines in the presence of excess or additional thyroid hormone and a depressed response in states of thyroid hormone lack. It is generally stated, therefore, that thyroid hormones increase the sensitivity of certain physiological systems that are responsive to catecholamines (2, 3). In these studies of the in vitro accumulation of CLE by thymocytes, in contrast, T_3 was stimulatory when added alone, and its effect was increased by the addition of epinephrine (or norepinephrine). However, concentrations of

epinephrine 100 times higher than those which increased the effect of T_3 failed to affect CLE accumulation when added alone. In this system, therefore, catecholamines potentiated the response to thyroid hormones, rather than vice versa.

As judged from conventional criteria (19), the potentiating effect of the catecholamines on this action of T_3 appeared to be mediated at the β_1 -receptor. A consistent enhancement of the T_3 effect was produced by both epinephrine and norepinephrine, which interact with both α - and β -receptors. However, phenylephrine, which is solely an α -agonist, did not increase the effect of T_3 ; and phentolamine, an α -antagonist, did not inhibit the potentiating action of epinephrine. Moreover, the effect of epinephrine was abolished by propranolol, a β_1 - and β_2 -antagonist. Finally, mediation at the β -receptor was indicated both by the ability of practolol, a β_1 -blocking agent, to abolish the effect of epinephrine and by the failure of terbutaline, a β_2 -agonist, to potentiate the effect of T_3 .

The promptness of the interaction between T_3 and catecholamines that we have described is not without precedent in previous reports. In rat epididymal adipose tissue, enhancement of epinephrine-induced lipolysis by T_3 in vitro is evident in 15–30 min (20–22). Furthermore, in the morphinized dog receiving a continuous infusion of isoproterenol, small doses of T_3 have been shown to increase the maximum rate of left ventricular tension development within 16 min, though similar doses of T_3 in the absence of isoproterenol infusion were without effect.³ Both this interaction in the heart and that in adipose tissue (23), like the converse interaction that we now describe, apparently involve the β -receptor, but little further is known of their mechanism.

A greater number of studies have been undertaken in efforts to explain the manner in which thyroid hormones act to enhance certain responses to adrenergic agents, but the ultimate mechanism or mechanisms remain uncertain, and data are contradictory in some respects. A review of the voluminous literature related to this topic is clearly beyond the scope of this report and, in any event, would not necessarily apply to the converse effect that we have seen in the thymocyte. Because hypothyroidism decreases and hyperthyroidism increases the increase in cyclic AMP induced by epinephrine in adipose tissue in vitro (24), and because dibutyryl cyclic AMP increases the accumulation of CLE by thymocytes in vitro (13), it might be thought that a greater cellular accumulation of cyclic AMP in the presence of T_3 and epinephrine than of either alone might explain these findings. This

is unlikely, however, because dibutyryl cyclic AMP increases the accumulation of α -aminoisobutyric acid by increasing influx (13), whereas the potentiating action of epinephrine on CLE accumulation was demonstrably a result of an increase in the inhibition of CLE efflux that T_3 produces.

A central question concerning these observations relates to their physiological relevance. This question arises because to demonstrate an increased accumulation of CLE by rat thymocytes in vitro, either high concentrations of T_3 must be added directly ($1 \mu\text{M}$) or a large dose of T_3 ($20 \mu\text{g}/100 \text{ g body wt}$) must be given to rats before thymocyte isolation. Recently, however, we have been able to define conditions in which the T_3 -responsiveness of the thymocyte with respect to CLE accumulation is greatly increased. In thymocytes isolated from adrenalectomized rats, enhancement of CLE accumulation by T_3 in vitro is produced by concentrations as low as 0.1 nM , and this effect of adrenalectomy is abolished by pretreatment of the donor animals with physiological replacement doses of glucocorticoid.⁴ Moreover, in the adrenalectomized rat, the quantity of T_3 by single intravenous injection that is required to increase accumulation of CLE by subsequently isolated thymocytes is as low as the daily physiological replacement dose, $0.5 \mu\text{g}/100 \text{ g body wt}$. Such sensitization of thymocytes from adrenalectomized animals to the effect of T_3 is consonant with the well-documented in vivo antagonism between physiological quantities of thyroid hormones and glucocorticoids, as well as their contrary independent effects, with respect to the growth and function of the lymphoid tissue (25–27). Such consonance suggests, in turn, that the effect of T_3 on amino acid accumulation that we are studying in vitro has its counterpart under physiologic circumstances in vivo. Additional evidence to this point is provided by the findings of Daniel et al. (28), who report that after intravenous administration of leucine, valine, and lysine, accumulation of the free forms of these amino acids by brain and skeletal muscle is far lower in hypothyroid than in normal rats.

High concentrations of thyroid hormones are often, though not invariably (29–31), required in vitro to produce effects that appear to have a physiological counterpart in vivo. For example, physiologic concentrations of thyroid hormone clearly modulate the lipolytic response of the rat to epinephrine because hypothyroidism greatly damps this response (24), but concentrations of T_3 required to enhance epinephrine-

³ G. S. Kurland. Personal communication.

⁴ Etzkorn, J. R., P. Hopkins, D. Gluckin, J. Gray, I. D. Goldfine, and S. H. Ingbar. 1979. Enhancement of glucocorticoid deficiency of the increase in cycloleucine accumulation induced in rat thymocytes by triiodothyronine. Manuscript submitted for publication.

stimulated lipolysis by rat adipose tissue *in vitro* are the same as or higher than the high concentrations that we have employed, 0.1–1.0 μM (20–22). Similarly, hypothyroidism greatly damps a variety of cardiovascular responses to catecholamines (1–3), but in the fetal rat heart in organ culture, for example, enhancement of the contractile rate requires T_3 concentrations of 0.1–10 μM (32). Previous authors have discussed the problems posed by, and possible explanations for, this discrepancy between *in vivo* and *in vitro* responses (14, 20).

A further question concerning these observations is their possible implications with respect to the mechanism of action of the thyroid hormones. Neither the stimulatory effect of T_3 on CLE accumulation nor the potentiation of this effect by epinephrine can be ascribed to nonspecific cytotoxicity of these agents. Thus, as judged from the ability of cells to exclude trypan blue, the viability of thymocytes was neither enhanced nor diminished by T_3 , either alone or in combination with epinephrine. Neither can a more subtle cytotoxic effect of T_3 and epinephrine leading to an increase in cell water and in CLE diffusion space explain the stimulation of CLE accumulation because, as judged from studies with [^{14}C]urea, total cell water in thymocyte suspensions was unaffected by T_3 and epinephrine, either alone or in combination. Hence, more specific fundamental effects seem likely.

The stimulatory effects of T_3 on amino acid accumulation by chick embryonic bone and by thymocytes (12, 14), as well as the effect of T_3 to increase 2-deoxy-D-glucose uptake in both chick embryo myocardium (15) and rat thymocytes (16), like the effect of T_3 to enhance epinephrine-stimulated lipolysis (17), are all independent of new protein synthesis. The same is true of the potentiating action of epinephrine on the stimulation of CLE accumulation in thymocytes by T_3 that we have herein described. This, as well as the promptness of these effects, the fact that they relate to substrate influx or efflux, and the fact of their interaction with catecholamines, whose actions are thought to be mediated at the cell membrane, suggest that these actions of T_3 *in vitro* also occur at the cell surface. The likelihood that this is the case is increased, it would appear, by the recent demonstration of high affinity, limited capacity binding sites for T_3 in plasma membranes derived from rat liver (33).

If it be granted that these *in vitro* effects of T_3 have relevance to the physiologic actions of T_3 *in vivo*, then we would suggest that thyroid hormones act at the nuclear level to alter gene expression, probably act directly on the mitochondrion, and act at the cell membrane as well. In this light, thyroid hormones would be seen to act at multiple loci to induce a coordinated response in which increased availability of

substrate proceeds hand in hand with increased energy metabolism and accelerated synthesis of specific proteins.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Bernard J. Ransil for advice concerning statistical analysis of the data. Data analysis was performed in part on the PROPHET System, a national computer resource sponsored by the Chemical/Biological Information Handling Program, National Institutes of Health, Bethesda, Md.

This study was supported in part by research grant AM-18416 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md.

REFERENCES

1. Landsberg, L. 1977. Catecholamines and the sympatho-adrenal system. *In* The Year in Endocrinology, 1975–1976. S. H. Ingbar, editor. Plenum Publishing Corp., New York. 177–231.
2. Spaulding, S. W., and R. H. Noth. 1975. Thyroid-catecholamine interactions. *Med. Clin. N. Am.* **59**: 1123–1131.
3. Brodie, B. B., J. I. Davies, S. Hynie, G. Krishna, and B. Weiss. 1966. Interrelationships of catecholamines with other endocrine systems. *Pharmacol. Rev.* **18**: 273–289.
4. Tata, J. R., and C. C. Widnell. 1966. Ribonucleic acid synthesis during the early action of thyroid hormones. *Biochem. J.* **98**: 604–620.
5. Oppenheimer, J. 1975. Initiation of thyroid hormone action. *N. Engl. J. Med.* **292**: 1063–1068.
6. Kurtz, D. T., A. Sippel, and P. Feigelson. 1976. Effect of thyroid hormones on the level of the hepatic mRNA for $\alpha_2\text{u}$ globulin. *Biochemistry.* **15**: 1031–1036.
7. Seo, H., G. Vassart, H. Brocas, and S. Refetoff. 1977. Triiodothyronine stimulates specifically growth hormone mRNA in rat pituitary tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 2054–2058.
8. Hoch, F. L. 1974. Metabolic effects of thyroid hormone. *Handb. Physiol.* **3**(Sect. 7): 231–243.
9. Babor, B. M., S. Creagan, S. H. Ingbar, and R. S. Kipnes. 1973. Stimulation of mitochondrial adenosine diphosphate uptake by thyroid hormone. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 98–102.
10. Sterling, K., and P. O. Milch. 1975. Thyroid hormone binding by a component of mitochondrial membrane. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 3255–3259.
11. Adamson, L. F., and S. H. Ingbar. 1967. Selective alteration by triiodothyronine of amino acid transport in embryonic bone. *Endocrinology.* **81**: 1362–1371.
12. Adamson, L. F., and S. H. Ingbar. 1967. Some properties of the stimulatory effect of thyroid hormones on amino acid transport by embryonic chick bone. *Endocrinology.* **81**: 1372–1378.
13. Goldfine, I. D., C. G. Simons, and S. H. Ingbar. 1975. Stimulation of the uptake of α -aminoisobutyric acid in rat thymocytes by L-triiodothyronine: a comparison with insulin and dibutyryl cyclic AMP. *Endocrinology.* **96**: 802–805.
14. Goldfine, I. D., C. G. Simons, G. T. Smith, and S. H. Ingbar. 1975. Cycloleucine transport in isolated rat thymocytes: *in vitro* effects of triiodothyronine and thyroxine. *Endocrinology.* **96**: 1030–1037.

15. Segal, J., H. Schwartz, and A. Gordon. 1977. The effect of triiodothyronine on 2-deoxy-D-(1-³H) glucose uptake in cultured chick embryo heart cells. *Endocrinology*. **101**: 143–149.
16. Segal, J., and S. H. Ingbar. 1979. Stimulation by triiodothyronine of the in vitro uptake of sugars by rat thymocytes. *J. Clin. Invest.* **63**: 507–515.
17. Goldfine, I. D., C. J. Smith, C. G. Simons, S. H. Ingbar, and E. Jorgensen. 1976. Activities of thyroid hormone and related compounds in an *in vitro* thymocyte assay. *J. Biol. Chem.* **251**: 4233–4238.
18. Snedecor, G. W. 1956. Statistical Methods Applied to Experiments in Agriculture and Biology. The Iowa State University Press, Ames, Iowa. 5th edition.
19. Innes, I., and M. Nickerson. 1975. Epinephrine and the sympathomimetic amines. In *The Pharmacological Basis of Therapeutics*. L. Goodman and A. Gilman, editors. Macmillan Publishing Co., Inc., New York. 477–513.
20. Vaughn, M. 1967. An in vitro effect of triiodothyronine on rat adipose tissue. *J. Clin. Invest.* **46**: 1482–1491.
21. Challoner, D. R., and D. O. Allen. 1970. An *in vitro* effect of triiodothyronine on lipolysis, cyclic AMP-C¹⁴ accumulation and oxygen consumption in isolated fat cells. *Metab. Clin. Exp.* **19**: 480–487.
22. Ichikawa, A., H. Matsumoto, N. Sakato, and K. Tomita. 1971. Effect of thyroid hormones on epinephrine-induced lipolysis in adipose tissues of rats. *J. Biochem. (Tokyo)*. **69**: 1055–1064.
23. Robison, G. A., P. E. Langley, and T. W. Burns. 1972. Adrenergic receptors in human adipocytes—divergent effects on adenosine 3',5'-monophosphate and lipolysis. *Biochem. Pharmacol.* **21**: 589–592.
24. Malbon, C. C., F. J. Moreno, R. H. Cabelli, and J. N. Fain. 1978. Fat cell adenylate cyclase and α -adrenergic receptors in altered thyroid states. *J. Biol. Chem.* **253**: 671–678.
25. Marder, S. S. 1949. Effect of thyroxine on lymphoid tissue mass of adult male mice. *Proc. Soc. Exp. Biol. Med.* **72**: 42–45.
26. Munck, A., and D. A. Young. 1975. Corticosteroids and lymphoid tissue. *Handb. Physiol.* **6**(Sect. 7): 231–243.
27. Fabris, N. 1977. Hormones and aging. In *Immunology and Aging*. T. Makinodan and S. Yunis, editors. Plenum Publishing Corp., New York. 73–90.
28. Daniel, P. M., E. R. Love, and O. E. Pratt. 1975. Hypothyroidism and amino acid entry into brain and muscle. Letter. *Lancet*. **II**: 872.
29. Samuels, H. H., J. S. Tsai, and R. Cintron. 1973. Thyroid hormone action: a cell culture system responsive to physiological concentrations of thyroid hormones. *Science (Wash. D. C.)*. **181**: 1253–1256.
30. Tsai, J. S., and A. Chen. 1976. Thyroid hormones: effect of physiological concentrations on cultured cardiac cells. *Science (Wash. D. C.)*. **194**: 202–204.
31. Popovic, W. J., J. E. Brown, J. W. Adamson. 1977. The influence of thyroid hormones on in vitro erythropoiesis. *J. Clin. Invest.* **60**: 907–913.
32. Wildenthal, K. 1972. Studies of isolated fetal mouse hearts in organ culture. *J. Clin. Invest.* **51**: 2702–2709.
33. Pliam, N. B., and I. D. Goldfine. 1977. High affinity thyroid hormone binding sites on purified rat liver plasma membranes. *Biochem. Biophys. Res. Commun.* **79**: 166–172.