EFFECT OF RNA SYNTHESIS INHIBITORS ON STIMULATION OF SULFATION BY L-3,5,3'-TRIIODOTHYRONINE

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Received January 27,1977

SUMMARY: Stimulation of sulfation by T_3[1] in chick embryo cartilage was blocked when actinomycin D or camptothecin was added to the incubation medium together with the T_3. When either inhibitor was added 2 hr after the T_3, the stimulation was not affected. The results suggest that there is an early step in the action of T_3 that involves synthesis of a stable species of RNA that is larger than 5S. α-Amanitin had no effect on the stimulation of sulfation by T_3, although it inhibited synthesis of poly(A)-rich RNA almost completely. This suggests that the action of T_3 is not associated with increased synthesis of RNA by RNA polymerase II. We conclude that the stimulation of sulfation by T_3 involves synthesis of a species of RNA that is larger than 5S but is not a messenger.

Incorporation of sulfate into glycosaminoglycans of chick embryo cartilage is stimulated by normal human serum (1), and this stimulation is markedly enhanced by addition *in vitro* of T_3 or L-thyroxine at concentrations in the physiological range (2). T_3 alone will also stimulate sulfation, although to a smaller extent than in the presence of serum (2). The stimulation by both serum and T_3 represents increased synthesis of at least the major proteoglycan fraction of the tissue (3). The action of T_3 in this system is rapid, a significant effect being observed within two hours. Stimulation of sulfation by T_3 is not part of a general increase in anabolic processes, since there is no significant increase in the incorporation of leucine into total cartilage protein (4). The rapid action of the hormone, the specificity of its effect and the fact that the concentration of hormone required is within the physiological range, make this system suitable for investigating thyroid hormone action at the cellular level.

Recently, it has been established by a number of investigators (5-7), using a variety of cells and tissues, that there are specific thyroid hormone binding

[1] Abbreviation: T_3, L-3,5,3'-triiodothyronine.

ISSN 0006-291X

receptors in the cell nucleus. Based on these observations, it has been suggested that thyroid hormone may act by regulating the synthesis of RNA (5,7). In this communication we present studies of the action of three inhibitors of RNA synthesis on sulfation in chick embryo sterna and its stimulation by T_3. Our results support the view that synthesis of RNA is involved in the action of T_3 in this tissue, but they also raise significant questions about the type of RNA involved.

MATERIALS AND METHODS

Camptothecin was a generous gift from Dr. Harry B. Wood, Jr., Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Sources of other chemicals were: α-amanitin, C.H. Boehringer; oligo(dT) cellulose, Collaborative Research Inc.; $H_3{}^{32}PO_4$(carrier-free), ^{3}H-uridine (6-7 Ci/mmol) and $H_2{}^{35}SO_4$ (carrier-free), New England Nuclear.

Incorporation of $^{35}SO_4{}^{2-}$ into chondroitin sulfate was measured as described in detail previously (3,8,9). Incorporation of ^{3}H-uridine into total RNA was measured by a modification of the method of Eisenbarth et al. (10). Sterna were incubated in 1 ml of incubation medium (3) to which was added 0.1 mM ^{3}H-uridine (100 mCi/mmol). The cartilages were homogenized in cold trichloroacetic acid; the acid precipitate was washed twice with 5% trichloroacetic acid and extracted with 5% trichloroacetic acid for 15 min at 90°C. The soluble material was collected by centrifugation, and its A_{260} and radioactivity were determined.

For extraction of total cytoplasmic RNA, cartilages were homogenized in 25 mM Tris-HCl (pH 7.5) - 25 mM NaCl - 5 mM $MgCl_2$, containing 1 mg/ml Na heparin and 2% Triton-X100. To the postmitochondrial supernatant, obtained by centrifugation at 27,000 x g for 5 min, were added Na desoxycholate and Na dodecyl sulfate to a final concentration of 1%, and the mixture was extracted three times with an equal volume of phenol-chloroform (1:1) with shaking for 20 min. RNA was precipitated by addition of NaCl to 0.2 M followed by ethanol to 67% (v/v). The RNA was fractionated by zone sedimentation on linear gradients of sucrose (15-30% w/v) in 0.02 M Tris-HCl (pH 7.5) - 0.15 M NaCl - 0.001 M EDTA. Portions (0.1 ml) were layered on 12.5 ml gradients and centrifuged at 35,000 rev/min, 25°C, in a SW40 rotor in a Spinco L2-65B Ultracentrifuge for approximately 10 hr. Integrated $\omega^2 t$ values, measured with an $\omega^2 t$ Integrator Accessory, were 4.5-4.6 x 10^{11} $rads^2$-sec. The gradients were removed by upward displacement and separated into fractions of approx. 0.5 ml.

Polysomes were prepared from homogenized cartilages and their RNA extracted exactly as described by Palmiter (11). Oligo(dT) cellulose chromatography was performed essentially as described by Aviv and Leder (12).

RESULTS AND DISCUSSION

Actinomycin D at a concentration of 5 μg/ml completely abolishes the stimulatory effect of T_3 on proteoglycan synthesis, but inhibits sulfation in control incubations by only 40% (8). This suggests that synthesis of some form of RNA might be involved in stimulation of proteoglycan synthesis by T_3. Since

TABLE 1. Effect of Actinomycin D and Camptothecin on Stimulation of Proteoglycan Synthesis by T_3.

Inhibitor (μg/ml)	Sulfate incorporation into glycosaminoglycans (pmol/μg chondroitin sulfate)	
	T_3 Omitted	T_3 Added
None	13.5 ± 0.22	18.8 ± 0.40
Actinomycin D (10)	9.8 ± 0.31	9.4 ± 0.31
Camptothecin (3)	9.9 ± 0.49	10.2 ± 0.22

Actinomycin D (10 μg/ml) or camptothecin (3 μg/ml) was added together with T_3 (4 nM) at the beginning of the incubation period. After 2 hr the cartilages were labeled with $^{35}SO_4^{2-}$ (10 mCi/mmol) for 60 min. Incorporation into chondroitin sulfate is expressed as mean ± S.E.M. for 5 sterna.

TABLE 2. Effect of Actinomycin D and Camptothecin on RNA Synthesis.

Exp. No.	Inhibitor	Counts/min/A_{260}	% Inhibition
1	None	24,960 ± 700	
	Actinomycin D (10 μg/ml)	2,079 ± 135	92
2	None	23,906 ± 364	
	Camptothecin (3 μg/ml)	9,869 ± 569	60

Actinomycin D (10 μg/ml) or camptothecin (3 μg/ml) was added at the beginning of the incubation period. After 30 min the cartilages were labeled with ^{3}H-uridine (100 mCi/mmol) for 6 hr. Incorporation into RNA is expressed as mean ± S.E.M. for 5 sterna.

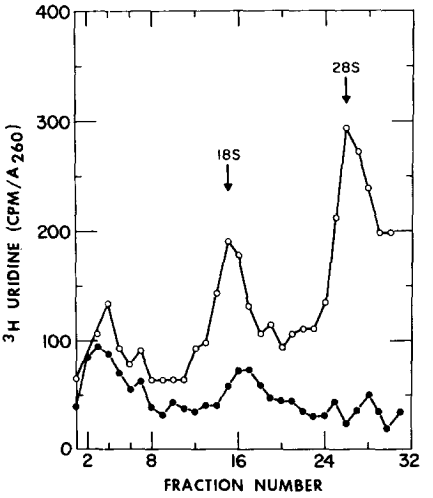

Figure 1. Effect of camptothecin on synthesis of stable RNA. Camptothecin (3 µg/ml) was added at the beginning of the incubation period. After 30 min the cartilages were pulse-labeled for 30 min with ^{3}H-uridine (5 Ci/mmol) and chased for 4 hr in the presence of unlabeled 10 mM uridine. Cytoplasmic RNA was extracted and fractionated by sedimentation on linear 15-30% sucrose gradients, as described in 'Materials and Methods'. Results are presented as counts/min/A_{260} of the RNA applied to the gradient.

actinomycin D inhibits synthesis of all types of RNA, we decided to investigate more selective inhibitors. One such inhibitor is camptothecin, which almost completely inhibits the synthesis of high molecular weight RNA, but has little or no effect on synthesis of 4S and 5S RNA (13). As shown in Table 1, the stimulatory effect of T_3 was completely abolished by camptothecin (3 µg/ml), as well as by actinomycin D (10 µg/ml); and sulfation in control incubations was decreased by the two inhibitors to the same extent. Table 2 shows the effect of the inhibitors on incorporation of ^{3}H-uridine into RNA, measured over a 6 hr period. Actinomycin D (10 µg/ml) almost completely inhibited incorporation of ^{3}H-uridine into RNA, whereas camptothecin (3 µg/ml) inhibited by only 60%. Increasing the concentration of camptothecin to 100 µg/ml did not significantly alter the inhibition of RNA synthesis. These results are very similar to those of Abelson and Penman (13), who found substantial inhibition of RNA synthesis in HeLa cells with 1 µg/ml of camptothecin, but little extra effect at higher

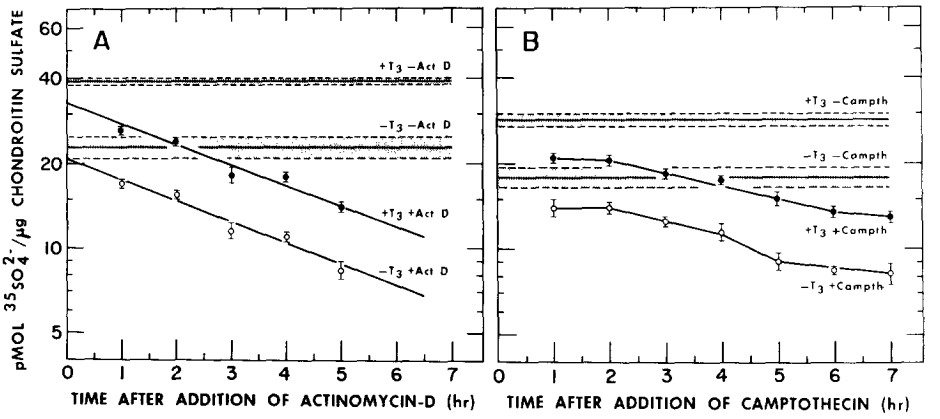

Figure 2. Decline of proteoglycan synthesis in the presence of actinomycin D or camptothecin. T_3 (4 nM) was added at the beginning of the incubation period. Actinomycin D (10 μg/ml) or camptothecin (3 μg/ml) was added after 2 hr of incubation. At hourly intervals, groups of cartilages were labeled with $^{35}SO_4^{2-}$ (10 mCi/mmol) for 60 min. Incorporation into chondroitin sulfate is expressed as mean ± S.E.M. for 5 sterna.

concentrations. To establish which classes of RNA were affected, stable RNA was labeled by incubating sterna with ^{3}H-uridine for 30 min and chasing for 4 hr with excess unlabeled uridine; cytoplasmic RNA was then fractionated on sucrose gradients. Camptothecin completely inhibited incorporation into 28S RNA, strongly inhibited incorporation into 18S RNA but had little effect on the labeling of low molecular weight RNA (Fig. 1). Taken together with the data in Table 1, this suggests that the RNA whose synthesis is required for the action of T_3 on sulfation is larger than 5S.

To determine whether there is a requirement for continuous RNA synthesis associated with the action of T_3, sterna were incubated for 2 hr in the presence or absence of T_3; actinomycin D (10 μg/ml) or camptothecin (3 μg/ml) was then added and at intervals groups of sterna were pulse-labeled with $^{35}SO_4^{2-}$. Figure 2 shows that once the stimulation of sulfation was established it persisted in the presence of the inhibitors. Also, with either inhibitor the rate of chondroitin sulfate synthesis declined in parallel in sterna that either

TABLE 3. Effect of α-Amanitin on Poly(A)-Rich RNA Synthesis.

Inhibitor	Preincubation Time	^{32}P CPM | A_{260} of RNA Applied to Oligo(dT) Cellulose					
		oligo(dT) bound	% inhibition	oligo(dT) non-bound	% inhibition	Total	% inhibition
None	1 hr	6,224		32,800		39,024	
α-amanitin 20 µg/ml	1 hr	1,696	73	23,800	27	25,496	35
None	3 hr	7,624		56,250		63,874	
α-amanitin 20 µg/ml	3 hr	942	88	14,500	73	15,442	76
None	5 hr	8,466		60,900		69,366	
α-amanitin 20 µg/ml	5 hr	552	93	9,668	84	10,220	85

α-Amanitin was added at the beginning of the incubation period. After 1, 3 or 5 hr the cartilages were pulse-labeled for 60 min with $H_2{}^{32}PO_4$ (60 µCi/ml, carrier-free). Polysomal RNA was extracted and fractionated on oligo(dT) cellulose columns, as described in 'Materials and Methods'. Results are presented as counts/min/A_{260} of the RNA applied to the columns.

had or had not been exposed to T_3, suggesting that the RNA whose synthesis is required for the action of T_3 is a relatively long-lived species.

One obvious possibility is that the species of RNA whose synthesis is involved in the action of T_3 is a particular mRNA involved in the synthesis of proteoglycan. To investigate this, we examined the effect of α-amanitin. In cell-free systems, α-amanitin is a potent inhibitor of the activity of RNA polymerase II, which is believed to be responsible for the synthesis of all types of mRNA (14). *In vivo*, and in undisrupted cells, synthesis of both mRNA and rRNA is inhibited (15). Table 3 shows the effect of α-amanitin on synthesis of polysomal RNA in chick embryo sterna. After 1 hr of exposure to α-amanitin, incorporation into oligo(dT)-bound, poly(A)-rich RNA was inhibited by 73%. After 3 hr of exposure to α-amanitin, incorporation into the bound, poly(A)-rich RNA

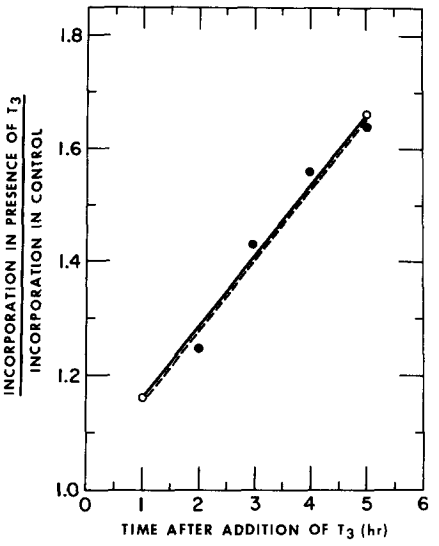

Figure 3. Effect of α-amanitin on stimulation of proteoglycan synthesis by T_3. α-Amanitin (20 μg/ml) was added at the beginning of the incubation period. After 3 hr T_3 (4 nM) was added and at intervals groups of 5 cartilages were labeled with $^{35}SO_4^{2-}$ (10 mCi/mmol) for 60 min. Results are expressed as incorporation of $^{35}SO_4^{2-}$ into glycosaminoglycans from cartilages treated with T_3, divided by incorporation into glycosaminoglycans from cartilages not exposed to T_3.

was inhibited by 88% and that into the unbound RNA by 73%; and an additional 2 hr of exposure produced a further small increase in the inhibition. We conclude that synthesis of mRNA by RNA polymerase II, as measured by incorporation of ^{32}P into poly(A)-rich RNA, is strongly inhibited by α-amanitin in these incubations.

To determine the effect of α-amanitin on synthesis of chondroitin sulfate and its stimulation by T_3, sterna were preincubated for 3 hr with or without the addition of α-amanitin (20 μg/ml). T_3 was then added to some of the cartilages and incorporation of $^{35}SO_4^{2-}$ into glycosaminoglycans was measured at intervals by pulse-labeling with $^{35}SO_4^{2-}$. There was a small (< 20%) reduction in chondrotin sulfate synthesis in cartilages that had been exposed to α-amanitin, as compared to those that were not. Figure 3 shows the rate at which T_3 stimulated sulfation in untreated cartilages or in cartilages that had been pretreated with α-amanitin; the results are presented as incoporation of $^{35}SO_4^{2-}$ into chondroitin

sulfate in T_3-treated cartilages divided by the incorporation in cartilages that were not exposed to T_3, but were otherwise treated identically. It is apparent that the rate of stimulation of proteoglycan synthesis by T_3 was the same in sterna that were incubated with α-amanitin, as it was in sterna that were not exposed to the inhibitor. This result contrasts with, for instance, the induction of liver tyrosine amino-transferase by cortisol, which is considered to involve de novo synthesis of mRNA (16,17) and is prevented by prior administration of α-amanitin (18). We conclude that while there is a requirement for de novo synthesis of some species of RNA for the stimulation by T_3, this RNA does not seem to be synthesized by polymerase II and thus does not satisfy one of the currently accepted characteristics of mRNA.

Recently, Samuels and Shapiro (19) described a cell system similar to the one described here in its reaction to T_3. GH_1 cells in tissue culture produce increased amounts of growth hormone when T_3 is added to the medium at physiological concentrations. This stimulation of growth hormone synthesis in GH_1 cells, like the stimulation of proteoglycan synthesis in our system, appears to require de novo synthesis of a stable species of RNA at an early stage. The early stage of T3 action does not seem to involve protein synthesis, since it is not inhibited by cycloheximide in either GH_1 cells (19) or chick embryo cartilage (our unpublished experiments).

Involvement of RNA synthesis in the early stages of thyroid hormone action has been suggested in other tissues and in vivo (20-22), but none of these studies offered any insight into the type of RNA that might be implicated. The experiments described here appear at first sight to exclude any of the three major classes of cytoplasmic RNA as being involved in stimulation of proteoglycan synthesis by T_3. Thus, RNA synthesized by RNA polymerase I seems to be ruled out because increased synthesis of rRNA, which is produced by this enzyme, would be expected to lead to a general increase in protein synthesis rather than the specific effect on proteoglycan synthesis that is observed. Messenger RNA formed by RNA polymerase II is also unlikely, since α-amanitin, which is a

strong inhibitor of this enzyme, does not affect stimulation by T_3. Finally, low molecular weight RNA produced by polymerase III can apparently be excluded since camptothecin probably has little effect on synthesis of this class of RNA, yet it completely inhibits the stimulation of proteoglycan synthesis. It is possible that the RNA involved is a mRNA whose synthesis is not sensitive to α-amanitin, or a species of tRNA whose synthesis is sensitive to camptothecin; alternatively it might be an unknown type of RNA with a regulatory function. While recognizing that these speculations are based on experiments with metabolic inhibitors, whose modes of action may not be entirely clear, we believe that our results point to a novel mode of action for thyroid hormones in chick embryo cartilage.

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