INHIBITORY EFFECTS OF α-AMANITIN ON RNA SYNTHESIS AND INDUCTION OF DOPA-DECARBOXYLASE BY β-ECDYSONE

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1. Introduction

DOPA-decarboxylase, an enzyme present in the integument of many insects [1, 2] is involved in the tanning process of the cuticle [3], by producing dopamine from DOPA. The *N*-acetyl derivative of dopamine has been shown to be the sclerotizing agent in a wide variety of insects [1–3]. We have previously shown in old Calliphora larvae that DOPA-decarboxylase is induced by ecdysone [4]. Puparium formation, and the induction of DOPA-decarboxylase by ecdysone can be abolished by inhibitors of RNA synthesis such as actinomycin D [5]. The demonstration that actinomycin D may have other actions, independent of the effects on transcription [6–8] made it desirable to confirm these observations by other inhibitors of RNA synthesis.

α-Amanitin, a polypeptide product of the toadstool Amanita phalloides [9], inhibits RNA synthesis in mammalian tissues [10–13] and in yeast [14] by inactivating DNA-dependent RNA polymerase. This finding would permit the reevaluation of the relation between transcription and enzyme induction, provided that α-amanitin inhibits RNA synthesis in insects.

2. Material and methods

Calliphora erythrocephala Meig. larvae were reared on horse meat at 22° and a relative humidity of 50%.

Nuclei were isolated from the integument by homogenization in 0.25 M sucrose, containing 0.0025 M KCl, 0.02 M $MgCl_2$ and 0.065 M Tris-HCl, pH 7.55, filtration through 8 layers of cheese cloth and centrifugation for 5 min at 1200 *g*. The sediment was taken up in 2 ml of the same buffer and layered on 20 ml of 10% (w/v) Ficoll in the same buffer. The sediment obtained after centrifugation at 1200 *g*, 20 min was taken up in 0.065 M Tris-HCl, pH 7.9 and used as nuclear preparation.

The RNA synthesizing mixture consisted of 1 mM each of ATP, GTP, CTP; 0.1 μCi ^{14}C-UTP (55 mCi/mM); 10 μg creatine phosphokinase, 10 μM creatine phosphate; 3.0 μM mercaptoethanol; 0.5 μM $MnSO_4$ or 3 μM $MgSO_4$; NH_4SO_4 0.4 ionic strength; 60 Iu penicillin G; 20 μM Tris-HCl pH 7.9 α-amanitin was added in a dose of 2 μg/ml.

3. Results and discussion

We first tested the effect of α-amanitin on *in vivo* and *in vitro* RNA synthesis in Calliphora erythrocephala larvae epidermis, which is the responsible tissue for the synthesis of DOPA-decarboxylase [5].

For the *in vitro* studies nuclei were isolated from epidermis and incubated with 2 μg/ml α-amanitin in a standard RNA synthesizing system under different ionic conditions. As shown previously, epidermal nuclei *in vitro* show intense incorporation of RNA precursors into RNA [15]. As seen in table 1 a significant inhibition of RNA synthesis by α-amanitin can be seen under all ionic conditions tested especially in the presence of ammonium sulfate. The

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Table 1
Effect of α-amanitin on RNA synthesis by isolated nuclei from epidermis of Calliphora larvae.

Ionic condition	Total cpm incorporation in nuclear RNA				% Inhibition
	Control		α-Amanitin		
Mn^{2+}	137 107	122	71 65	68	44
$Mn^{2+} + (NH_4)_2SO_4$	408 413	410	174 180	177	57
Mg^{2+}	103 82	92	45 69	57	40
$Mg^{2+} + (NH_4)_2SO_4$	279 278	279	98 118	108	61

Incubation was performed for 10 min at 37° in the RNA synthesizing mixture described in Materials and methods. The measurement of incorporated radioactivity was made by precipitating aliquots on filter paper as described in [16]. The values are the average of duplicate experiments.

Table 2
Effect of α-amanitin on RNA synthesis by epidermis of Calliphora larvae.

Duration of pulse (min)	Duration of application of α-amanitin (hr)	% Inhibition
30	1	3
30	2	13
30	3.5	52
120	5	88

α-Amanitin (1 μg per animal) and ^{3}H-uridine (1.3 μCi per animal (Sp. Act. 19.4 Ci/mM) each dissolved in 5 μl insect Ringer were injected into the posterior part of ligated Calliphora larvae 24 hr after the pupation of head. At the appropriate time periods the integuments were prepared and homogenized in a buffer consisting of 0.14 M NaCl, 0.05 M Na citrate, containing 15 μg/ml polyvinyl sulfate and 4 mg/ml bentonite, and then shaken at 65° for 10 min with equal volumes of 80% phenol. The RNA was isolated from the water phase as described in [18]. Radioactivity measurements were performed on filter papers in a liquid scintillation counter as described previously [16].

degree of inhibition is fairly similar to that observed with isolated rat liver nuclei [10]. About 60% inhibition is seen with a dose of 2 μg/ml.

As is the case for RNA polymerases from other species, extraction and purification on DEAE-cellulose of the enzyme from the integument of Calliphora larvae revealed two main peaks of activity (C.E.S. unpublished results). The peak eluting at higher ionic strength was inhibited by α-amanitin whereas the first peak ascribed to the nucleolar enzyme was resistant to the toxin.

For the *in vivo* experiments 1 μg α-amanitin (20 mg/g) was injected into the posterior part of ligated larvae 24 hr after pupation of the head. One to six hours later ^{3}H-uridine was injected followed 30 min later by sacrifice. The integument was then prepared and the RNA of the epidermis was isolated by the hot phenol method as described previously [17]; base analysis and MAK column chromatography has shown that most of the labelled RNA is DNA-like [17]. Amanitin inhibits RNA synthesis *in vivo* as seen from table 2; 52% inhibition was observed 3.5 hr after application of the toxin. To study the effects of amanitin on the synthesis of ribosomal RNA, we increased the exposure to ^{3}H-uridine to a 2-hr period [17]. α-Amanitin application for 6 hr inhibits RNA synthesis under these conditions to 88% (see table 2). Analysis of the labelled RNA by sucrose gradient centrifugation [18] and by acrylamide gel electrophoresis [19] revealed that the biosynthesis of the ribosomal precursor RNA is almost completely blocked by α-amanitin. Fig. 1 shows the effect of the toxin on the labelling of the RNA isolated from integument microsomes. With the exception of the tRNA an almost complete inhibition of the labelling of the extracted RNA is evident.

We then studied the induction of DOPA-decarboxylase by β-ecdysone in the presence of α-amanitin. We had previously shown that 6–8 hr after intraabdominal injection of α-ecdysone to ligated head-pupated Calliphora larvae, a significant increase in DOPA-decarboxylase activity can be seen. With the more potent β-ecdysone, a more rapid increase in enzyme activity is observed (see table 3 and [20]). Two series of experiments were conducted. In the first amanitin was given 3 hr prior to the injection of β-ecdysone, in the second 3 hr after the hormone. In all cases the enzyme assay was performed 5 hr after ecdysone injection. As seen from the results in table 3, amanitin inhibits the induction of DOPA-decarboxylase if given prior to β-exdysone, but has no

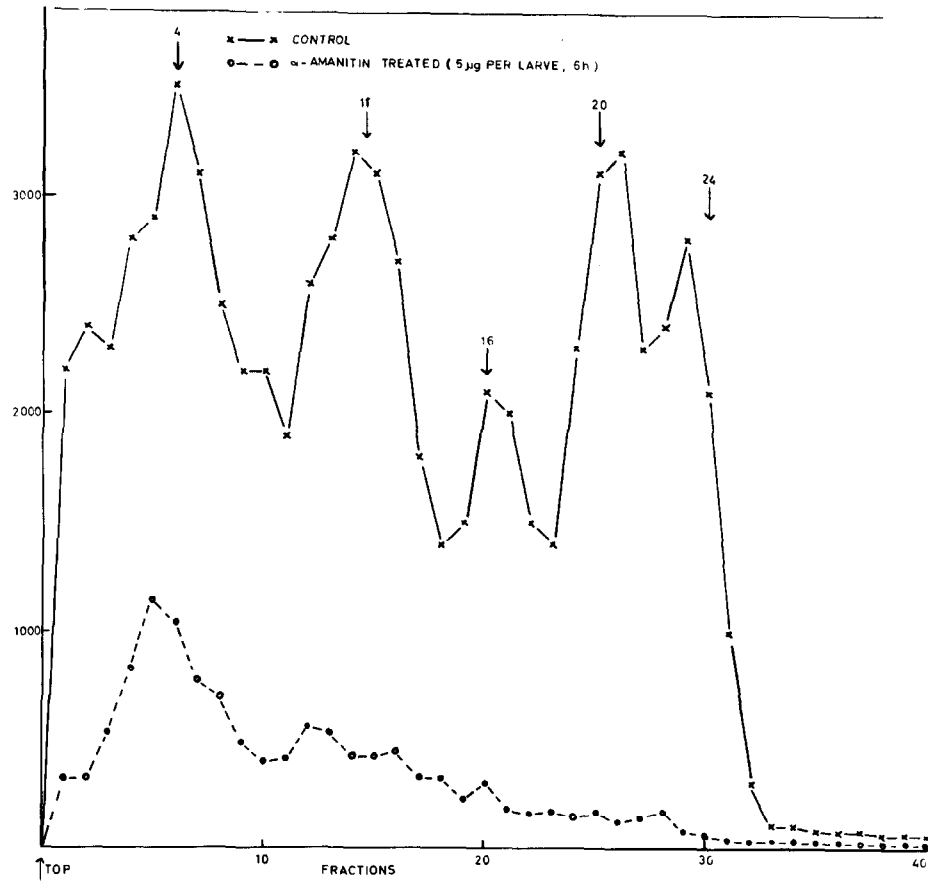

Fig. 1. Sucrose gradient centrifugation of RNA isolated from the microsomal fraction of the integument of control and α-amanitin treated Calliphora larvae. 5 µg amanitin per animal were given at 6 and 3 hr before sacrifice and a ^{3}H-orotic acid pulse for 3 hr. Microsomes were isolated in principle as described in [18]. Centrifugation for 14 hr, at 24,000 rpm through a 15–30% sucrose gradient in a SW 41 Rotor. ↓ S-values; X——X control; o- - -o α-amanitin treated.

effect on the induction process if administered after the hormone. In these experiments amanitin was injected three hours before the hormone in order to have maximal RNA inhibition at the time the hormone starts to act on RNA synthesis of the epidermis [21, 22]. These results point to the involvement of transcription in the induction process.

Similar results have been recently obtained with regard to the induction of tyrosine transaminase of rat liver by cortisol [23]. In that case, as described here for the DOPA-decarboxylase, enzyme induction by cortisol is completely inhibited by the previous administration of amanitin. Due to the fact that amanitin not only inhibits the biosynthesis of DNA-like RNA, but also of ribosomal RNA, (see also [24]) we cannot definitely ascribe its inhibitory action to the inhibition of the synthesis

Table 3
Effects of α-amanitin on the induction of DOPA-decarboxylase by β-ecdysone.

	mμM Dopamine formed/mg protein amanitin injected	
	3 Hr after ecdysone I	3 Hr prior to ecdysone II
Control	34.8	68.8
Ecdysone	119.3	127.3
Amanitin alone	–	53.5
Amanitin + ecdysone	108.7	88.3

Posterior parts of ligated Calliphora larvae, 24 hr after pupation of the head were used. α-Amanitin (1 μg/5 μl insect Ringer) were given: I: ecdysone for 5 hr and amanitin for the last 2 hr; II: amanitin for 8 hr and ecdysone for the last 5 hr. Groups of 15 animals were used for each experiment. Enzyme determination was performed on the basis of transformation of ^{14}C-DOPA to ^{14}C-dopamine which was separated by paper electrophoresis and measured by scintillation counting [3].

of the mRNA's needed to code for the respective enzymes. Since most of the newly synthesized RNA found in amanitin treated animals was tRNA, we feel justified in ascribing the inhibitory action of α-amanitin to the blockage of new genetic information in the nucleus, which is essential for protein synthesis.

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