Effect of the Exotoxin of *Bacillus thuringiensis* on Normal and Ecdysone-Stimulated Ribonucleic Acid Polymerase Activity in Intact Nuclei from the Fat-Body of *Sarcophaga bullata* Larvae

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A nuclear preparation from the fat-body of *Sarcophaga bullata* was obtained which incorporates nucleotides at a steady rate. Two activities, differing in their response to α -amanitin, are present. The activities are not separated by changes in the concentration of $(\text{NH}_4)_2\text{SO}_4$ as effectively as in mammalian nuclei. The activity resistant to α -amanitin is stimulated by ecdysone, and both normal and the ecdysone-stimulated activities are inhibited by the exotoxin. The amanitin-sensitive enzyme is also inhibited by exotoxin, but higher concentrations are required.

The exotoxin of *Bacillus thuringiensis* is an insecticidal material structurally related to ATP (Bond *et al.*, 1969b; Farkas *et al.*, 1969), which is excreted by the bacterium into the culture medium. It has a wide spectrum of activity and is particularly effective against holometabolous insects between instars and during pupation (Bond *et al.*, 1971). Adult insects are much less susceptible (Bond *et al.*, 1971). The exotoxin is also toxic to mice (de Barjac & Riou, 1969; Sebesta *et al.*, 1969; Bond *et al.*, 1969a). For these animals it has been shown (Sebesta *et al.*, 1969) that exotoxin inhibits the synthesis of RNA *in vivo* and we have shown that DNA-dependent RNA polymerase activities, in intact nuclei and in soluble form from rat liver, are inhibited by the compound (Beebee *et al.*, 1972). The inhibition is reversible and, as is true also for the enzyme from *Escherichia coli*, is competitive with ATP. The nucleolar enzyme is more susceptible than the nucleoplasmic one, especially in intact nuclei.

A satisfactory synthesis of the concept of exotoxin as an inhibitor of RNA polymerase and the involvement of RNA synthesis, stimulated by the moulting hormones (Sekeris, 1965), in the processes of metamorphosis would help to explain the puzzling and apparently insect-specific symptomatology of exotoxin poisoning (Bond *et al.*, 1971).

It is the purpose of the present work to investigate the effect of the exotoxin on normal and ecdysone-stimulated RNA polymerase activity in intact nuclei from insect larvae.

Experimental

Materials

Exotoxin of *Bacillus thuringiensis* was purified as described by Bond *et al.* (1969b) and used as the

sodium salt. A standard solution of exotoxin was kept frozen at -20°C .

Unlabelled nucleoside triphosphates were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; [^3H]UTP was from The Radiochemical Centre, Amersham, Bucks., U.K.; α -amanitin was a gift from Professor T. Wieland, Heidelberg, Germany; ecdysone was a gift from Hoffmann-La Roche Ltd., Basle, Switzerland. The hormone was dissolved in ethanol and stored at 4°C .

Methods

Preparation of fat-body nuclei. Nuclei were prepared by a modification of the method of Congote *et al.* (1969). Larvae (45) of *Sarcophaga bullata* were washed in ethanol and water and decapitated under a buffer containing 0.25 M-sucrose, 0.05 M-Tris-HCl, pH 7.6, 0.02 M-magnesium acetate, 0.025 M-KCl. All operations were conducted at 0°C . Fat-body tissue was carefully removed and homogenized in 15 ml of the same buffer by using ten strokes of a loose-fitting Potter homogenizer. The homogenate was filtered through four layers of muslin and layered over an equal volume of 10% (v/v) Ficoll in the same buffer. After centrifugation at 1200g for 30 min the supernatant was discarded and the nuclear pellet taken up in 0.05 M-Tris-HCl, (pH 7.9)–0.01 M- β -mercaptoethanol. This suspension was used in polymerase assays.

Measurement of the synthetic activity of RNA polymerase *in vitro*. Enzyme activity in the nuclei was assayed by measuring the incorporation of [^3H]UTP into products insoluble in trichloroacetic acid.

Two procedures, referred to elsewhere in the text as assays at high and low ionic strength, were used, and required, in a total volume of 0.25 ml: (a) at low

ionic strength, $0.75\mu\text{mol}$ of MgCl_2 ; (b) at high ionic strength, $0.5\mu\text{mol}$ of MnCl_2 and $62.5\mu\text{mol}$ of $(\text{NH}_4)_2\text{SO}_4$.

All solutions also contained $12.5\mu\text{mol}$ of Tris-HCl, pH 7.9, $2.5\mu\text{mol}$ of β -mercaptoethanol, 125nmol each of ATP, GTP and CTP, and $1\mu\text{Ci}$ ($\equiv 1\text{nmol}$) of $[^3\text{H}]\text{UTP}$. The enzyme was added as a nuclear suspension containing either $2.5\mu\text{g}$ or $5\mu\text{g}$ of DNA.

The solutions, in duplicate, were incubated for 10min at 30°C and then cooled in ice. Carrier RNA ($100\mu\text{g}$) and 3ml of 5% (w/v) trichloroacetic acid were immediately added and the solutions were then allowed to deposit a precipitate over 15min at 0°C . The solutions were filtered on Oxoid MF60 membranes ($0.45\mu\text{m}$ pore size) and these were subsequently washed with 15ml of the trichloroacetic acid solution. Each membrane was immersed in 1.0ml of 0.3M-KOH and incubated for 1h at 37°C . Portions (0.5ml) of the hydrolysate were added to 5ml of scintillation mixture [33% (v/v) Triton X-100, 67% (v/v) toluene, containing 5g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre]. The radioactivity of the hydrolysates was measured on an Intertechnique liquid-scintillation spectrometer after the decay of alkaline chemiluminescence. Counting efficiency, determined by

using alkaline solutions with accurately known amounts of $[^3\text{H}]\text{UTP}$, was 25%.

Exotoxin and hormone were added to individual experiments in the amounts described in the text.

Assay of polynucleotide phosphorylase (EC 2.7.7.8). This enzyme was assayed by repeating the procedure described above, with the solutions made 0.5M in ADP (Beebee *et al.*, 1972).

Assay of ribonuclease (EC 2.7.7.16). Polymerase activity was measured as detailed above, except that after 10min unlabelled UTP was added to a final concentration of 1mM. After incubation for 20min more, the solutions were treated in the usual way and the radioactivity of the precipitate was compared with that of controls incubated for the first 10min only.

Determination of protein and DNA. Protein was measured by the method of Lowry *et al.* (1951) and DNA by the method of Burton (1956).

Results

The incorporation of $[^3\text{H}]\text{UTP}$ into material precipitated by trichloroacetic acid is dependent on three of the four nucleoside triphosphates, the exception being CTP, and is sensitive to deoxyribonuclease, ribonuclease and actinomycin D. There is a slight

Fig. 1. Bivalent-cation requirements of larval RNA polymerases

The assay solutions were made up as described in the Experimental section, except that the MgCl_2 and MnCl_2 concentrations were varied as specified on the abscissa of the two graphs. All assay solutions contained $2.5\mu\text{g}$ of nuclear DNA. (a) Activity at low ionic strength; (b) activity at high ionic strength. ●, Mg^{2+} -stimulated activity; ○, Mn^{2+} -stimulated activity.

Fig. 2. Stimulation of larval RNA polymerases by ionic strength

Each assay solution contained, in a total volume of 0.25ml; $12.5\mu\text{mol}$ of Tris-HCl, pH 7.9; $2.5\mu\text{mol}$ of β -mercaptoethanol; 125nmol each of ATP, GTP and CTP; $1\mu\text{Ci}$ ($\equiv 1\text{nmol}$) of $[^3\text{H}]\text{UTP}$; $5\mu\text{g}$ of nuclear DNA. The $(\text{NH}_4)_2\text{SO}_4$ concentration was varied over the range shown on the abscissa and each solution had, in addition, $0.75\mu\text{mol}$ of MgCl_2 or $0.5\mu\text{mol}$ of MnCl_2 . ●, With 3mM- Mg^{2+} ; ○, with 2mM- Mn^{2+} .

endogenous ribonuclease activity manifest at high ionic strength and a polynucleotide phosphorylase activity present under the same conditions (Beebee, 1972).

The data in Figs. 1–3 and in Beebee (1972) show that the properties of the RNA polymerase are similar to those observed in nuclei isolated from a wide range of other species (Widnell & Tata, 1964; Roeder & Rutter, 1969; Horgen & Griffin, 1971), including a related insect (Sekeris *et al.*, 1965; Congote *et al.*, 1969; Shaaya & Sekeris, 1971). The pattern of inhibition of the polymerase activities by α -amanitin, recorded in Table 1, also is similar to that noted by

Fig. 3. Time-course of larval RNA polymerase activities
Assays were done as described in the Experimental section, except that the time of incubation was varied as shown. ●, Incorporation at low ionic strength; ○, incorporation at high ionic strength.

Table 2. Effect of exotoxin on α -amanitin-sensitive and α -amanitin-resistant RNA polymerase in intact nuclei
The values in this table are calculated from the results presented in Fig. 5 by making the assumptions noted.

	At low ionic strength	At low ionic strength in the presence of ecdysone
Control without exotoxin	2000 c.p.m.	2900* c.p.m.
Enzyme sensitive to α -amanitin	740† c.p.m.	1070† c.p.m.
Enzyme resistant to α -amanitin at 50% of its total activity	1370 c.p.m.	2435‡ c.p.m.
Exotoxin concentration required to produce this activity	10 nM	50 nM
α -Amanitin-sensitive enzyme at 50% activity	370 c.p.m.	535 c.p.m.
Exotoxin concentration required to produce this activity	5 μ M	80 μ M

* Control value in the absence of ecdysone.
† Assuming, from Table 1, that the α -amanitin-sensitive enzyme represents 37% of the total.
‡ This assumes that ecdysone does not alter the α -amanitin-sensitive activity (see Fig. 4).

Shaaya & Sekeris (1971) for nuclei from the epidermis of *Calliphora erythrocephala*, and is qualitatively comparable with the pattern observed in nuclei from other species. The main point of difference and, as shown below, it is an important one, is that the conditions of ionic strength do not separate the two polymerase activities so efficiently as is the case for mammalian nuclei. This fact makes it difficult to interpret the very characteristic time-courses, which are shown in Fig. 3, and which are quite different from those found for RNA polymerase activity in mammalian nuclei. The incorporation of label at low ionic strength continues in a linear manner for at least 1 h and the incorporation at high ionic strength is biphasic, with a rapid incorporation for the first 10 min and a slower rate between 10 min and 1 h. In mammalian nuclei, the incorporation at low ionic strength ceases after approx. 10 min (Widnell & Tata, 1964).

Thus the substrate requirements, cation dependence and sensitivity to actinomycin and amanitin follow closely the patterns recorded for RNA polymerase activities in other eukaryotic systems (see Table 3), but the incorporation of nucleotides as a function of

Table 1. Effects of α -amanitin on fat-body RNA polymerase

Assay conditions were those described in the text with the additions noted.

Assay conditions	RNA polymerase activity (c.p.m.)	
	Low ionic strength	High ionic strength
Normal	1359	2497
With 0.1 μ M- α -amanitin	1386	1394
With 1 μ M- α -amanitin	856	1082
With 10 μ M- α -amanitin	901	1156

Table 3. General characteristics of DNA-dependent RNA polymerases from eukaryotic organisms

Property	Enzymes		References
	I	II	
Order of elution from DEAE-cellulose or DEAE-Sephadex			Roeder & Rutter (1969)
Effect of ionic strength on activity	Active at low ionic strength	Active at high ionic strength	Widnell & Tata (1966)
Metal ion required for optimum activity	Mg ²⁺	Mn ²⁺	Widnell & Tata (1966)
Effect of α -amanitin	Uninhibited	Inhibited	Horgen & Griffin (1971); Stirpe & Fiume (1967); Novello & Stirpe (1970)
Sub-nuclear location	Nucleolus	Nucleoplasm	Roeder & Rutter (1970)
Effect of exotoxin	Sensitive to exotoxin	Relatively insensitive to exotoxin	Beebee <i>et al.</i> (1972)
Product	Ribosomal RNA	DNA-like RNA	Widnell & Tata (1966); Pogo <i>et al.</i> (1967)

Fig. 4. Effects of ecdysone on larval RNA polymerases

The assay solutions were constituted as described in the Experimental section for low ionic strength and contained 5 μg of nuclear DNA. Ecdysone was dissolved in ethanol and added to assays in a volume that never exceeded 1 μl. Control incubations always contained an equivalent amount of ethanol. (a) Effects at low ionic strength. (b) Effects at high ionic strength. ●, α -Amanitin-resistant activity; ○, α -amanitin-sensitive activity.

time and the effect of changing the ionic strength do not.

At low ionic strength, ecdysone produces a stimulation of the amanitin-resistant activity and has little

Fig. 5. Exotoxin inhibition of larval RNA polymerases at low ionic strength

The assay solutions were constituted as described in the Experimental section for low ionic strength and contained 5 μg of nuclear DNA. Ecdysone was added in the manner described for Fig. 6 and the exotoxin was added to make up the concentrations noted on the abscissa. ●, Incorporation at normal low ionic strength (Expt. 1); ○, incorporation at low ionic strength stimulated by 1.5 μg of ecdysone/ml (Expt. 2). The symbol ○ indicated by an arrow shows the unstimulated control value for Expt. 2.

effect on the amanitin-sensitive activity (Fig. 4). At high ionic strength, the effect is reversed and smaller. Hormone-mediated effects on RNA polymerase have been observed in other systems and the magnitude of the effect that we observe here compares favourably with those other cases. It is likely, however, that the effects observed *in vitro* are much less dramatic

than the effects that occur on hormone stimulation *in vivo* (Sahota & Mansingh, 1970), because other, as yet unidentified, factors are probably necessary to optimize hormone action (Williams & Kambysellis, 1969; Richman & Oberlander, 1971).

The dose-response curves for inhibition of nuclear polymerases by exotoxin at low ionic strength (Fig. 5) are more complex than those for inhibition of rat liver nuclear polymerase (Beebee *et al.*, 1972). Each curve takes the form of two dose-response curves superimposed one on the other. We interpret this to be a consequence of the inability of changes in ionic strength to separate the nucleolar and nucleoplasmic polymerase activities so efficiently, as is the case for rat liver nuclei. The first part of the dose-response curve at low ionic strength demonstrates the effect of exotoxin on the amanitin-resistant (see Table 3) enzyme and the second part on the amanitin-sensitive enzyme. This order of response to exotoxin is made probable by the magnitude of the inhibition in the two parts of the curve, the first part corresponding to about 60% and the second part to about 40% of the total activity, and these values correspond to the

amounts of amanitin-resistant and amanitin-sensitive enzyme respectively, found for low-ionic-strength conditions and given in Table 1. The results in Table 1 can be used to calculate values for the effect of exotoxin on each enzyme and the results of such calculations are presented in Table 2. The values are approximate and they indicate the insect enzymes to be more sensitive to exotoxin than the enzymes of mammalian nuclei by a factor of about 10 and to differ from each other in their response to exotoxin. The difference in the response of the two enzymes is enhanced by ecdysone. That the biphasic dose-response curves for exotoxin are indeed a consequence of a differential

Fig. 6. Exotoxin inhibition of larval RNA polymerases at high ionic strength

Assay solutions were constituted as described in the Experimental section for high ionic strength. Exotoxin was added to the concentrations noted on the abscissa. The amanitin-sensitive curve is obtained by subtraction of the activity surviving in the presence of α -amanitin from that observed in its absence. ●, Assays at normal high ionic strength; ○, assays at high ionic strength with $1\mu\text{M}$ - α -amanitin; Δ, α -amanitin-sensitive activity. Assays contained $2.5\mu\text{g}$ of nuclear DNA.

Fig. 7. Elution profile of larval RNA polymerases from DEAE-cellulose

Larvae (100) were homogenized in 100ml of 0.05M-Tris-HCl, pH 7.9, 25% (v/v) glycerol, 5mM-magnesium acetate, 0.1mM-EDTA, 1mM-dithiothreitol (TGMED medium), containing 1M-(NH_4) $_2$ SO $_4$, by using a Polytron homogenizer at 0°C. The homogenate was stirred at 4°C for 1 h and precipitated proteins were removed by centrifugation at 30000g for 20min. The concentration of (NH_4) $_2$ SO $_4$ was raised to 3M by addition of solid, and stirring continued for 3h. Precipitated protein was collected by centrifugation and resuspended in 12ml of TGMED medium containing 0.05M-(NH_4) $_2$ SO $_4$; the suspension was extensively dialysed against this same medium, after which the solution was cleared by centrifugation at 30000g for 20min. A portion (6ml) of the supernatant was applied to a column (1cm $\times$ 12cm) of DE42 DEAE-cellulose previously equilibrated with TGMED-medium, and polymerase was eluted by raising the concentration of (NH_4) $_2$ SO $_4$ as shown in the figure. Fractions (1ml) were collected and 0.1 ml portions assayed for polymerase activity, under conditions similar to those used with soluble rat liver RNA polymerase (Beebee *et al.*, 1972). Each assay mixture contained $25\mu\text{g}$ of calf thymus DNA. ○, RNA polymerase activity; ---, concentration of (NH_4) $_2$ SO $_4$ (M).

behaviour against the two enzymes is clearly shown in Fig. 6 for the system of high ionic strength, where amanitin has separated the two activities clearly. The higher ionic strength has lowered the sensitivity of both enzymes to exotoxin.

The elution profile from DEAE-cellulose of the RNA polymerases solubilized from total tissue of *Sarcophaga bullata*, shown in Fig. 7, is similar to that obtained for polymerases solubilized from mammalian nuclear systems by Roeder & Rutter (1969) and from insect larval epidermis by Doenicke *et al.* (1972).

Discussion

Although exotoxin is toxic to insects and to mice, there are small quantitative differences in the toxicity and striking differences in the symptoms associated with intoxication. Results in the present paper, when taken together with the work of Sekeris, Karlson and their colleagues (see Sekeris, 1965), on the mechanisms by which insect metabolism is controlled through the different stages of larval development, offer some explanation for these differences.

To take first of all the quantitative differences that exist between the toxicity of exotoxin to insects and that to mice, the LD₅₀ to insects by injection is approx. 0.1–0.5 µg/g and to mice 18 µg/g (Bond *et al.*, 1969a; Sebesta *et al.*, 1969).

There is therefore a difference of roughly two orders of magnitude in the response. The concentration of exotoxin required to inhibit the amanitin-resistant enzyme in insect larval nuclei is 10 nM (Table 2) and in rat liver nuclei is 0.8 µM. The equivalent values for the amanitin-sensitive enzymes are 5 µM and 0.1 mM respectively. Sebesta and his colleagues have reported (Sebesta *et al.*, 1969) that injection of one-third of the LD₅₀ dose into mice results in a 60% inhibition of total liver RNA synthesis *in vivo*. If we suppose that 80% of the weight of a mouse is due to water and that all of the exotoxin is present in the aqueous phase (a reasonable assumption, since exotoxin is insoluble in all the common organic solvents and is a very polar molecule) then at 0.33 × LD₅₀ the exotoxin concentration would be approx. 8 µM. If the quantitative responses of mouse liver are similar to those of rat liver, this concentration would inhibit all RNA synthesis by the nucleolar enzyme and 5–10% of the activity of the nucleoplasmic enzyme (Beebee *et al.*, 1972).

The correlation between enzyme inhibition and toxicity in mammals appears therefore to be a simple one requiring reasonable and simple assumptions and, as the difference in enzyme response between insects and mammals is of the same order of magnitude as the difference in LD₅₀ values, these same simple assumptions apply to the larvae of *S. bullata* and perhaps to the larvae of holometabolous insects in general. Certainly, the conclusions that we draw from

this work, that nuclear RNA syntheses in rat liver and in insect fat-body are similar in their response to toxins, are supported by the work of Hadjiolov & Mackedonski (1972) and of Shaaya & Clever (1972). The similarity of their findings about the effect of α-amanitin on mammals and insects *in vivo* is in very good agreement with our results *in vitro*.

If the effect of inhibition by exotoxin of nucleolar RNA synthesis, including that synthesis stimulated by ecdysone, which we have shown to occur *in vitro*, corresponds to an inhibition of ribosomal RNA synthesis and ecdysone-stimulated RNA synthesis *in vivo*, then an explanation of the symptoms of exotoxin intoxication immediately presents itself. Sekeris (1965) has shown that when ecdysone is injected into ligated *Calliphora erythrocephala* there is a stimulation of ³²P incorporation into both mRNA and rRNA. He has also shown that the mRNA so stimulated is capable of directing the synthesis of dopa (dihydroxyphenylalanine) decarboxylase *in vitro* and that stimulation of the synthesis of that enzyme is a consequence of both natural ecdysone production and injection of ecdysone into ligated insects. Dopa decarboxylase controls sclerotization of the larval cuticle in the first stages of pupation. This sequence of events (synthesis of RNA, synthesis of enzyme, sclerotization, pupation) explains why holometabolous insects, when exposed to high doses of exotoxin, either fail to pupate or, at slightly lower doses, die as deformed pupae (Bond *et al.*, 1971), which often are only partially sclerotized. Symptoms involving morphological effects on pupae are manifest at relatively high exotoxin doses, presumably because it is mRNA rather than rRNA that is involved in the causal chain described above.

We have no direct evidence on the nature of the inhibition in the insect larval system. By analogy with the effect of exotoxin on the RNA polymerase from *E. coli* (Sebesta *et al.*, 1968) and the RNA polymerase in rat liver nuclei (Beebee *et al.*, 1972), it is likely that the inhibition is competitive with ATP. If so, the sensitivity of insects during (as distinct from the onset of) pupation may be increased by the energy demands of and the number of alternative catabolic fates for ATP in the puparium.

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