Effect of an Exotoxin from *Bacillus thuringiensis* on Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase in Nuclei from Adult *Sarcophaga bullata*

UNUSUAL BEHAVIOUR OF EUKARYOTIC POLYMERASES TO INHIBITORS

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The DNA-dependent RNA polymerase activities in nuclei isolated from adult *Sarcophaga bullata* are unusual in their responses to metal ions, ionic strength and inhibitors. There is an activity that is sensitive both to rifamycin and to α -amanitin. The activity is less sensitive to *Bacillus thuringiensis* exotoxin than is larval polymerase, and low concentration of exotoxin provoke a slight stimulation.

We have tried to show in the preceding paper (Beebee & Bond, 1973) that many of the symptoms of poisoning in insects caused by the exotoxin of *Bacillus thuringiensis* are to be expected on the basis of our observations on its effect on the RNA polymerase of nuclei from *Sarcophaga bullata* larvae and the observations of Sekeris and his colleagues (Sekeris, 1965) on the effect that ecdysone-stimulated RNA synthesis has on puparium formation.

The profound but symptomatologically very different toxicity to mice (for a review see Bond *et al.*, 1971) would be the result of different control mechanisms for RNA synthesis in mammals compared with those in holometabolous insects before and during metamorphosis.

It remains to explain the curious insensitivity of adult insects to the exotoxin. We have found that injection of adult houseflies with 1 μ g of exotoxin/animal did not result in a significant kill (P. D. McFarlane & J. D. Tipton, unpublished work). The LD₅₀ to larval insects by injection is approx. 0.01 μ g/animal. So much of the toxic behaviour of exotoxin is explained by the patterns of its inhibition of RNA polymerase in intact nuclei (Beebee *et al.*, 1972; Beebee & Bond, 1973) that it seemed possible that the anomalous toxicity to adult diptera would reflect an anomalous response of the RNA polymerase(s) occurring in the nuclei of such insects.

Experimental

Materials

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

Unlabelled nucleoside triphosphates were 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; actinomycin D and rifamycin SV were from Calbiochem Ltd., London W.1, U.K.

Methods

Preparation of nuclei. Adult *Sarcophaga bullata* were cooled to 4°C, at which temperature they were inactive and easily handled. Insects (60) were floated on 50 ml of 0.25M-sucrose in a buffer medium consisting of 0.05M-Tris-HCl, pH 7.5, 0.025M-KCl and 0.02M-MgCl₂ (subsequently referred to as TKM buffer) and homogenized for 2 min at 0°C, by using a Polytron PT OD 10 instrument with a large head, at a speed setting of 3. All subsequent operations were at 0–4°C. The crude homogenate was filtered through eight layers of muslin and further homogenized with five strokes of a tight-fitting Potter homogenizer.

This homogenate was layered over 10 ml of 1.9M-sucrose in TKM buffer and centrifuged for 30 min at 100000g. The pellets were resuspended by gentle homogenization in a total of 10 ml of 2.2M-sucrose in TKM buffer and centrifuged for 10 min at 500g. The supernatant was carefully decanted, leaving a sediment of fine chitin particles, and diluted with an equal volume of 0.25M-sucrose in TKM buffer. The suspension was then centrifuged for 15 min at 2500g. The nuclear pellets were resuspended in 2 ml of 0.05M-Tris-HCl buffer, pH 7.9, containing 0.01M- β -mercaptoethanol, and used in RNA polymerase assays.

Assay for RNA polymerase activity. Assay conditions at low and high ionic strength were the same as those described for larval fat-body nuclear RNA synthesis in the preceding paper (Beebee & Bond, 1973), except that, for low ionic strength, $1.5\ \mu\text{mol}$ of MgCl_2 was added.

Determination of protein and DNA. Protein was measured by the method of Lowry *et al.* (1951) and DNA by the method of Burton (1968). Bovine serum albumin and calf thymus DNA were used as standards.

Assay of polynucleotide phosphorylase (EC 2.7.7.8). The RNA polymerase assays were repeated, as described above, with the addition of $0.5\ \text{mM}$ -ADP. The nucleoside diphosphates are the substrates for polynucleotide phosphorylase and the principle of this method of assaying for polynucleotide phosphorylase has been outlined by Beebee *et al.* (1972).

Assay of ribonuclease (EC 2.7.7.16). Polymerase activity was measured as detailed above, except that after 10 min unlabelled UTP was added to a final concentration of $1\ \text{mM}$. After incubation for 15 min 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 10 min only.

Results

Incorporation of $[^3\text{H}]\text{UTP}$ into trichloroacetic acid-insoluble products by the nuclear preparation from adult *Sarcophaga bullata* requires the three unlabelled mononucleotides and is inhibited by actinomycin D. The dependence on CTP is much sharper than was the case with the larval nuclei (Beebee & Bond, 1973), whereas the dependence on GTP is less marked.

The preparation is free of polynucleotide phosphorylase activity; ribonuclease activity is small and limited to conditions of low ionic strength. This is reminiscent of the behaviour of ribonuclease in mammalian nuclear preparations, but in insect larval systems ribonuclease is more evident at high ionic strength (Beebee & Bond, 1973). The response of the polymerase activity to ionic strength, outlined in Fig. 1, is unusual in that the Mg^{2+} -activated incorporation goes through a peak at an $(\text{NH}_4)_2\text{SO}_4$ concentration of approx. $0.2\ \text{M}$, whereas the Mn^{2+} -activated incorporation has no very obvious maximum and, indeed, its activity changes little through the range of $(\text{NH}_4)_2\text{SO}_4$ concentration studied. In Figs. 2 and 3 the response to various concentrations of Mg^{2+} and Mn^{2+} is shown. At low ionic strength (Fig. 2) there is no well-defined optimum for either cation. Both show optima but, when compared with the identical situation for mammalian nuclei or insect larval nuclei (Beebee, 1972), these are less sharp and less well defined. The results of Fig. 3 for the Mn^{2+} -activated incorporation are not unlike those observed for mam-

malian and insect larval nuclei, but the activation brought about by Mg^{2+} at high ionic strength has no parallel in these systems.

Response to inhibitors

With these effects of metal ion and ionic strength on the enzyme activity in mind, we examined the effects of inhibitors under the three sets of conditions indicated in Table 1. The enzyme activated by Mn^{2+} at high ionic strength is distinct in being completely resistant to rifamycin SV. The incorporation of label activated by Mg^{2+} , at both high and low ionic strength, is inhibited by α -amanitin and rifamycin (Table 1 and Figs. 3 and 4). Inhibition by rifamycin is complete in both cases, but the responses to α -amanitin are quantitatively distinct, that at low ionic strength being less sensitive. In both cases 30–40% of the total activity is sensitive to rifamycin but resistant to α -amanitin. This is the behaviour to be expected of prokaryotic RNA polymerases and the possibility that this proportion of the overall activity is a consequence of bacterial contamination cannot be ruled out. However, we are not aware of a nuclear RNA polymerase activity that is sensitive to rifamycin and α -amanitin.

Fig. 1. Effects of ionic strength on RNA polymerase activity

Assay solutions were constituted as described in the Experimental section with the $(\text{NH}_4)_2\text{SO}_4$ concentration varied as shown. The Mg^{2+} and Mn^{2+} concentrations employed, 6 and $4\ \text{mM}$ respectively, were those found to have an optimum effect at low ionic strength. All assay solutions contained $5\ \mu\text{g}$ of nuclear DNA. ○, Polymerase activated by Mn^{2+} ; ●, polymerase activated by Mg^{2+} .

The effect of exotoxin on the adult polymerases (Fig. 5) reveals a small but reproducible stimulation, which we have not observed in any previous system. The enzymes activated by Mg^{2+} and Mn^{2+} differ in their responses to exotoxin in much the same way as we have found for mammalian and insect larval systems (Beebee *et al.*, 1972; Beebee & Bond, 1973). The concentrations required to inhibit activity by 50% are $5\mu M$ for the Mg^{2+} -activated enzyme and 0.1 mM for the Mn^{2+} -activated enzyme. The observed effects of exotoxin would not be in quantitative accord with

a bacterial source for any part of the incorporation activated by Mg^{2+} . The exotoxin/ATP ratio at $5\mu M$ -exotoxin, which brings about a 50% inhibition of the Mg^{2+} -activated enzyme at high ionic strength (Fig. 5), is 1:100, a value that would not produce an observable inhibition of RNA polymerase from *Escherichia coli* (Sebesta & Horska, 1968).

Fig. 2. Bivalent-cation requirements of RNA polymerase activity at low ionic strength

Assay solutions were constituted as described in the Experimental section for assays at low ionic strength with the two bivalent metal ions added in the amounts indicated. Each solution contained $5\mu g$ of nuclear DNA. $\circ$, Polymerase activated by Mn^{2+} ; $\bullet$, polymerase activated by Mg^{2+} .

Fig. 3. Bivalent-cation requirements of RNA polymerase activity at high ionic strength

Assay solutions were constituted as described in the Experimental section for high ionic strength with the bivalent metal ions added in the amounts indicated. Each solution contained $2.5\mu g$ of DNA. $\circ$, Polymerase activated by Mn^{2+} ; $\bullet$, polymerase activated by Mg^{2+} .

Table 1. Effects of inhibitors on RNA polymerase activities

Normal assay conditions for 'low ionic strength' (6 mM-Mg^{2+}) and 'high ionic strength' (2 mM-Mn^{2+}) refer to those described in the text. For 'high ionic strength (7 mM-Mg^{2+})', $62.5\mu\text{mol}$ of $(\text{NH}_4)_2\text{SO}_4$ was used in 0.25 ml with $1.75\mu\text{mol}$ of MgCl_2 , with all the other constituents remaining the same.

Assay conditions	RNA polymerase activity (c.p.m.)		
	Low ionic strength (6mM-Mg ²⁺)	High ionic strength (2mM-Mn ²⁺)	High ionic strength (7mM-Mg ²⁺)
Normal	1280	1970	1523
With 1 μM-α-amanitin	1068	1397	562
With 10 μM-α-amanitin	407	870	595
With 5 μg of actinomycin D/ml	—	908	208
With 5 μg of rifamycin SV/ml	—	2201	78

Fig. 4. Time-course of RNA polymerase activities

Each assay solution contained, in a total volume of 0.6ml, 0.05M-Tris-HCl, pH 7.9, 10mM- β -mercaptoethanol, 0.5mM-ATP, GTP and CTP, 4nM-[3 H]UTP (sp. radioactivity 1Ci/mmol) and 30 μ g of nuclear DNA with the following additions: Δ , 6mM-MgCl₂, 10 μ M- α -amanitin and 3 μ g of rifamycin SV; $\circ$, 0.25M-(NH₄)₂SO₄, 2mM-MnCl₂ and 3 μ g of rifamycin SV; $\bullet$, 0.25M-(NH₄)₂SO₄ and 7mM-MgCl₂. Duplicate 50 μ l samples were withdrawn at the times indicated and absorbed on Whatman no. 1 filter papers (2cm). The papers were immediately immersed in a large excess of 5% (w/v) trichloroacetic acid at 0°C for a period of 15min. The papers were washed six times with fresh trichloroacetic acid and then dried at 60°C. Each filter paper was placed in a scintillation vial and 3ml of scintillation 'cocktail' (3.5g of 2,5-diphenyloxazole and 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene in 1 litre of toluene) added. The vials were counted in a Beckman or an Intertechnique liquid-scintillation spectrometer.

Discussion

Probing similar enzyme activities in intact organelles is not a simple task and it is easy to ascribe undue importance to small changes in the response of such systems to experimental conditions. It would be unwise, for example, to read too much into the failure of the generally accepted condition of low and high salt concentration to separate the polymerase enzyme activities examined here into two recognizably distinct activities, as they have for several other nuclear preparations. Nor are the responses to metal-ion activation likely to provide any firmer a basis for precise separation of enzyme activities. It is known that solubilization can and does alter the susceptibility of the two main eukaryotic nuclear polymerase enzymes, both to stimulation by ionic strength and to which metal ion (Mg²⁺ and Mn²⁺) is the more effective activator (Roeder & Rutter, 1969).

Fig. 5. Inhibition of RNA polymerase activities in nuclei from adult *S. bullata* by exotoxin

Assay solutions were constituted as described in the Experimental section for high ionic strength and contained 5 μ g of nuclear DNA. Enzyme activity was measured in the presence of $\circ$, 2mM-MnCl₂ and $\bullet$, 7mM-MgCl₂.

The response of the nuclear enzymes to α -amanitin and rifamycin is another matter. Experimental documentation that α -amanitin inhibits the nucleoplasmic Mn²⁺-activated, high-ionic-strength-stimulated polymerase II in intact nuclei and after solubilization, and has no effect on polymerase I in either case, is now extensive (see Table 3 in Beebee & Bond, 1973). There is equally impressive evidence that rifamycin inhibits prokaryotic polymerases but not eukaryotic ones (Meilhac *et al.*, 1972).

Of the several anomalies thrown up by the present results, the most puzzling and the most unequivocal can be stated simply. There is a polymerase enzyme that is sensitive to α -amanitin and to rifamycin.

In a detailed analysis of polymerase enzymes from coconut nuclei, Mondal *et al.* (1972) have shown that the insensitivity of these polymerases to rifamycins is due to the association of a specific protein unit with the enzymes. Further, Chesterton & Butterworth (1971a,b,c) have shown that the properties of the soluble polymerase(s) isolated from nuclei depend in part on the ionic strength of the media during sonication, and with Keding *et al.* (1971) they postulate that amanitin-sensitivity is conferred by a specific subunit.

Our results therefore suggest that in adult insects the RNA polymerase enzymes exist in a form that is operationally unusual but needs to involve little more than the rearrangement of subunits from what has come to be regarded as the normal pattern.

There is as yet insufficient accumulated experimental information on the effect of exotoxin on eukaryotic RNA polymerases to use the compound as a diagnostic tool. The present results are, however, encouraging both for possible diagnostic use of the

exotoxin and to explain its toxicology. Thus the changes in the RNA polymerases of adult *S. bullata* relative to the larval form (and to other eukaryotes), whatever they may be, have resulted in a change in the pattern of, and quantitative response to, exotoxin inhibition. As a diagnostic tool, exotoxin is therefore responsive to such changes.

Toxicologically, the decrease in the inhibitory effect affords an explanation for the relative resistance of the adult insect. It is unlikely that the connexion is as simple as it seems, but the relationship between the potency of exotoxin as an inhibitor of RNA polymerase in intact nuclei and as a physiological poison in the three systems that we have so far examined appears to be direct.

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