Differential Inhibition of Mammalian Ribonucleic Acid Polymerases by an Exotoxin from *Bacillus thuringiensis*

THE DIRECT OBSERVATION OF NUCLEOPLASMIC RIBONUCLEIC ACID POLYMERASE ACTIVITY IN INTACT NUCLEI

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The effects of the exotoxin from *Bacillus thuringiensis* on DNA-dependent RNA polymerases from rat liver were examined. The exotoxin inhibits all RNA polymerase activity at both low and high ionic strength in intact nuclei, and soluble enzymes are similarly affected. This inhibition is relieved by ATP. Dephosphorylated exotoxin did not inhibit the soluble enzymes. Nucleolar and nucleoplasmic RNA polymerases respond to different concentration ranges of exotoxin, and the compound can be used in intact nuclei to isolate the nucleoplasmic activity.

Despite its initial promise as a specifically insecticidal compound (for a review, see Bond *et al.*, 1971), it is now established that the exotoxin of *Bacillus thuringiensis* is also profoundly toxic to mammals (de Barjac & Riou, 1969; Sebesta *et al.*, 1969; Bond *et al.*, 1969a). Sebesta and his colleagues have shown that the exotoxin depresses RNA synthesis in mice *in vivo* (Sebesta *et al.*, 1969) and inhibits the RNA polymerase from *Escherichia coli* *in vitro* (Sebesta & Horska, 1968) in such a way that the inhibition can be reversed by ATP. The inhibition of the *E. coli* enzyme is not dramatic enough to provide a convincing explanation for the toxicity of the compound in general and for its ability to depress RNA synthesis in mice in particular. It seemed likely that the response of mammalian polymerases to exotoxin would differ from the response of bacterial polymerases, to reflect the fact that the compound is toxic to mice but not to bacteria. We examine here the effects of exotoxin *in vitro* on RNA polymerase systems from a mammalian source. The studies were designed to establish if (1) the effect of exotoxin on mammalian RNA polymerase differed quantitatively from its effect on the bacterial enzyme, and (2) the effect of exotoxin on the enzyme was the same with intact nuclei as with the purified soluble enzymes.

Materials and Methods

Materials

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

sodium salt. A standard solution of exotoxin was kept frozen at -20°C . Dephosphorylated exotoxin was prepared as described by Bond *et al.* (1969b). Both were stored as stock solutions.

Unlabelled nucleoside triphosphates, yeast RNA and calf thymus DNA were obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; [^3H]UTP was from The Radiochemical Centre, Amersham, Bucks., U.K. α -Amanitin was a gift from Dr. S. Pilgis, Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tenn., U.S.A.

Preparation of RNA polymerase

Preparation of nuclei. Nuclei were prepared by the method of Blobel & Potter (1966), by using livers from 350g male Wistar rats. The nuclei were suspended in 0.34M-sucrose and used in assays. RNA polymerase activity was dependent on all four nucleoside triphosphates and could be separated into an activity manifest in buffers of low ionic strength, requiring Mg^{2+} and insensitive to α -amanitin, and an activity requiring buffers of high ionic strength, which was Mn^{2+} -dependent and sensitive to α -amanitin.

Separation of enzyme activities. Polymerase activities evident in buffers of low and high ionic strength were shown to be restricted to nucleolus and nucleoplasm respectively. These nuclear components were separated by the method of Busch *et al.* (1967). Nucleoli synthesized RNA only in buffers of low ionic strength and were unaffected by α -amanitin.

Solubilization of activity and separation of the

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enzymes. RNA polymerase activity from nuclei prepared as described above was solubilized by the method of Roeder & Rutter (1970). Activity was assayed as 'mixed soluble polymerase' (fraction 4 of Roeder & Rutter, 1970) and was separated on DEAE-Sephadex into polymerases I and II. Mixed soluble RNA polymerase was completely dependent on added DNA and all four nucleoside triphosphates, and was partially inhibited by α -amanitin. Polymerases I and II were also dependent on DNA and nucleotides; polymerase I was unaffected by $1\mu\text{M}$ - α -amanitin, but polymerase II activity was completely inhibited by this concentration of α -amanitin. Mixed soluble RNA polymerase had a specific activity of 0.41 nmol of UMP incorporated/h per mg of protein and lost 30% of its activity at -78°C in 7 days. Polymerase I had a specific activity of 9.4 nmol of UMP incorporated/h per mg of protein, an ionic-strength optimum at 25mM -(NH_4)₂SO₄ and a half-life of 2 h at -78°C . Polymerase II had an activity of 37.6 nmol of UMP incorporated/h per mg of protein, an ionic strength optimum at 75mM -(NH_4)₂SO₄ and a half-life of 2 h at -78°C .

Mixed soluble RNA polymerases were separated by the method of Burgess (1969) into at least 20 distinct or semi-distinct bands on polyacrylamide gels in 0.1% (w/v) sodium dodecyl sulphate.

All enzyme preparations were free of polynucleotide phosphorylase activity, and ribonuclease activity was restricted to intact nuclei at low ionic strength.

Assays for RNA polymerase

Intact nuclei. Unless otherwise stated, each assay contained in a final volume of 0.25 ml: (a) for nucleolar activity; $25\mu\text{mol}$ of tris-HCl buffer, pH 8.1; $2.5\mu\text{mol}$ of β -mercaptoethanol; $1.25\mu\text{mol}$ of magnesium acetate; $0.125\mu\text{mol}$ each of ATP, GTP and CTP; $1\mu\text{Ci}$ of [³H]UTP of specific radioactivity 1 Ci/mmol; 0.1 ml of nuclear suspension containing $300\mu\text{g}$ of DNA; (b) for nucleoplasmic activity; $25\mu\text{mol}$ of tris-HCl buffer, pH 8.1; $2.5\mu\text{mol}$ of β -mercaptoethanol; $0.75\mu\text{mol}$ of MnCl₂; $50\mu\text{mol}$ of (NH_4)₂SO₄; nucleotides and nuclear suspension as in (a).

Exotoxin was added to individual experiments in the amounts and at the time described in the text. Assay mixtures, all in triplicate, were incubated for 10 min at 37°C . The reaction was stopped by cooling the mixtures in ice and adding 4 ml of 10% (w/v) trichloroacetic acid containing 0.02 M-sodium pyrophosphate. After 10 min the mixtures were filtered on Oxoid MF60 membranes, then the filters were washed twice with 5% (w/v) trichloroacetic acid and once with water, before being dried at 70°C for 1 h. Then 3 ml of toluene scintillator [8.75 g of 2,5-diphenyloxazole and 0.125 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 2.5 litres of toluene] was added and radioactivity was measured in a Beckman liquid-scintillation counter. The counting efficiency was 7%,

and was determined by the use of filters with known amounts of [³H]UTP and nuclear suspension. Variation about the mean of triplicate assays was 17% at 400 c.p.m. and 6% at 20000 c.p.m.

Soluble enzymes. Assays, in a final volume of 0.125 ml, contained: $9\mu\text{mol}$ of tris-HCl buffer, pH 7.9; $0.625\mu\text{mol}$ of magnesium acetate; $0.2\mu\text{mol}$ of MnCl₂; $0.25\mu\text{mol}$ of β -mercaptoethanol; $1.25\mu\text{mol}$ of KCl; $25\mu\text{g}$ of calf thymus DNA; 95nmol each of ATP, GTP and CTP; $0.5\mu\text{Ci}$ of [³H]UTP (specific radioactivity 1 Ci/mmol). In addition, assays for polymerase I contained $2.5\mu\text{mol}$ of (NH_4)₂SO₄ and $12\mu\text{g}$ of protein; for polymerase II, assays contained $9.5\mu\text{mol}$ of (NH_4)₂SO₄ and $3\mu\text{g}$ of protein; assays for mixed soluble polymerase contained $300\mu\text{g}$ of protein. Exotoxin was added as described in the text. α -Amanitin, when used, was $1\mu\text{M}$.

Assays were incubated at 30°C for 10 min and were stopped by cooling them in ice. Carrier RNA (0.1 ml of a 1 mg/ml solution) was then added, followed by 0.1 ml of 5% (w/v) sodium dodecyl sulphate, and while each assay mixture was vigorously shaken, 2 ml of 10% trichloroacetic acid containing 0.04 M-sodium pyrophosphate was added. The mixtures were left for 1 h at 0°C , then the precipitated RNA was filtered on Oxoid membranes and its radioactivity was measured as described above. The counting efficiency was 10%.

Polynucleotide phosphorylase. We never observed any effect on enzyme activity when 0.5 mM-ADP was added to our assays. Nucleoside diphosphates are the substrates for polynucleotide phosphorylase and the addition of one would be expected to decrease the incorporation of any other. This principle has been used, in a similar assay procedure, by Anthony *et al.* (1969) and the relevant properties of the enzyme have been recorded by Basilio & Ochoa (1963).

Ribonuclease. Polymerase assays were done as described above, but after 10 min, actinomycin D was added to a final concentration of $10\mu\text{g}/\text{ml}$ together with unlabelled UTP at a final concentration of 0.5 mM. After a further incubation of 10 min, assay mixtures were treated as described above and the loss in radioactivity from the material precipitated with trichloroacetic acid, relative to that in controls incubated for the first 10 min only, was used as a measure of ribonuclease activity.

Determination of protein and DNA. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard and DNA by the method of Burton (1956).

Results

Inhibition of RNA polymerase in intact nuclei

The extent of inhibition of polymerase activity is dependent on the concentration of exotoxin in the

Fig. 1. Effect of exotoxin concentration on the nucleolar and nucleoplasmic RNA polymerase activities in intact nuclei

(a) For nucleolar enzyme activity, each assay contained, in a total volume of 0.25 ml: 25 μmol of tris-HCl buffer, pH 8.1; 2.5 μmol of β-mercaptoethanol; 1.25 μmol of magnesium acetate; 0.125 μmol each of ATP, GTP and CTP; 1 μCi of [³H]UTP (specific radioactivity 1 Ci/mmol); 0.1 ml of nuclear suspension containing 300 μg of DNA; 0.25 nmol of α-amanitin; exotoxin as indicated on the graph. (b) The activity due to the nucleoplasmic enzyme was obtained for each concentration of exotoxin from two separate assays. In the first, the mixture is the same as in (a) above, but 0.75 μmol of MnCl₂ replaces the 1.25 μmol of magnesium acetate and there is, in addition, 50 μmol of (NH₄)₂SO₄. In the second, the α-amanitin is omitted. When the first assay records a finite activity, this is subtracted from the second to obtain the value given on the graph.

Table 1. Effect of different concentrations of ATP and GTP on the inhibition by exotoxin of RNA polymerase activities in intact nuclei

The recorded incorporations are after subtraction of the background radioactivity (688 c.p.m.). Assays were done as described in the text. Nucleolar assays were always with 1 μM-α-amanitin. Normal ATP concentration was 0.5 mM; the elevated concentration was 1.5 mM; exotoxin was always at 5 μM.

	Nucleolar enzyme activity (c.p.m. incorporated)	Nucleoplasmic enzyme activity (c.p.m. incorporated)
Normal incorporation	5028	12485
Incorporation with 5 μM-exotoxin	811	8467
Incorporation with toxin and 1.5 mM-ATP	1123	11950
Incorporation with toxin and 1.5 mM-GTP	557	7790

assay mixtures. Dose-response curves for inhibition by exotoxin are shown in Fig. 1. They were determined for nucleolar activity in the presence of α-amanitin, and for nucleoplasmic activity with exotoxin present from the beginning of the incubation in both cases.

If it is assumed that the inhibition is competitive with ATP (see below), the ratio of K_m/K_i is given by the slope of the line obtained when V/v is plotted against $[I]/[S]$ where V is the uninhibited incorporation,

v the inhibited incorporation, $[I]$ the exotoxin concentration and $[S]$ the ATP concentration. For the nucleolar activity, this value is 250 and for the nucleoplasmic activity, 10.

Reversal of inhibition of intact nuclei

Table 1 shows the degree of inhibition produced by 5 μM-exotoxin on the nucleolar and nucleoplasmic activities at an ATP concentration of 0.5 mM and the

Fig. 2. Reversal of exotoxin inhibition by dilution

Nuclei were preincubated alone or with 5 μ M-exotoxin for 5 min at room temperature. Samples of equal volume were then used in polymerase assays containing in (a) the normal concentrations (given in the text) of all components, but in the different volumes shown. In (b) the procedure was the same except that the concentration of ATP was altered to keep the ATP/exotoxin molar ratio constant at 250. ●, Control, high ionic strength [○ in (b)]; ○, control, low ionic strength [● in (b)]; ▲, inhibited, high ionic strength; △, inhibited, low ionic strength. All low-ionic-strength assays contained 1 μ M- α -amanitin.

Fig. 3. Effect of exotoxin concentration on the solubilized and separated RNA polymerases at two ATP concentrations

Each assay contained, in a final volume of 0.125 ml: 9 μ mol of tris-HCl buffer, pH 7.9; 0.625 μ mol of magnesium acetate; 0.2 μ mol of $MnCl_2$; 0.25 μ mol of β -mercaptoethanol; 1.25 μ mol of KCl; 25 μ g of calf thymus DNA; 95 nmol of GTP and CTP and either 95 or 190 nmol of ATP; 0.5 μ Ci of [³H]UTP (specific radioactivity 1 Ci/mmol); exotoxin as indicated on the graphs. The assays also contained: for polymerase I (Fig. 3a) 2.5 μ mol of $(NH_4)_2SO_4$ and 12 μ g of protein; for polymerase II (Fig. 3b) 7.5 μ mol of $(NH_4)_2SO_4$ and 3 μ g of protein. (a) Polymerase I: ●, 1 mM-ATP; ○, 0.5 mM-ATP. (b) Polymerase II: ▲, 1 mM-ATP; △, 0.5 mM-ATP.

effect on that inhibition of increasing the ATP concentration to 1.5 mM. The nucleoplasmic activity is less profoundly affected by exotoxin, retaining 68%

of its activity, and recovers almost its full activity at the high ATP concentration. At the concentration of exotoxin used the nucleolar activity is more pro-

Fig. 4. Double-reciprocal plots of the inhibition of (a) polymerase I and (b) polymerase II by exotoxin

The assay systems were those described for Fig. 3 with the ATP and exotoxin concentration adjusted to the values noted on the graphs. v is c.p.m. incorporated into material precipitated by trichloroacetic acid after 5 min of reaction. ●, Uninhibited enzyme; ○, with 3 μ M-exotoxin; △, with 6 μ M-exotoxin; ▲, with 9 μ M-exotoxin.

foundly inhibited and is, from Fig. 1(a), relatively insensitive to changes in exotoxin concentration. If the inhibition is competitive, the nucleolar activity should be unresponsive also to changes in the concentration of the substrate involved in the competition provided that $K_m \neq K_i$ ($K_i < K_m$). In agreement with this, and as would be predicted from the results of Fig. 1, the observed effect of ATP concentration on the inhibition of nucleolar activity is less than that for the nucleoplasmic activity.

Inhibition is relieved at both high and low ionic strength when the nuclei preincubated with exotoxin are diluted in various volumes of reaction medium. The inhibition of nucleoplasmic and nucleolar activities is not relieved if during this procedure the exotoxin/ATP ratio is kept constant. These effects are shown in Fig. 2.

Inhibition of soluble RNA polymerase

Polymerases I and II were tested for dose response to exotoxin at two concentrations of ATP. The results (Fig. 3) show that soluble enzymes respond to exotoxin concentrations in the same way as intact nuclei do. Polymerase II is more sensitive to exotoxin than is the nucleoplasmic activity and this is reflected in the K_m/K_i values which, calculated as described above, are 480 for polymerase I and 220 for polymerase II.

Reversal of inhibition in the soluble enzymes

ATP relieved inhibition of both soluble enzymes. These were assayed separately at several ATP and

Table 2. Effect of dephosphorylated exotoxin on RNA polymerase activity in mixed soluble polymerase

The background radioactivity (259 c.p.m.) has been subtracted from the incorporations shown. Assays, in a final volume of 0.125 ml, contained: 9 μ mol of tris-HCl buffer, pH 7.9; 0.625 μ mol of magnesium acetate; 0.2 μ mol of $MnCl_2$; 0.25 μ mol of β -mercaptoethanol; 1.25 μ mol of KCl; 25 μ g of calf thymus DNA; 95 nmol each of ATP, GTP and CTP; 0.5 μ Ci of [3H]UTP (specific radioactivity 1 Ci/mmol); 2.5 μ mol of $(NH_4)_2SO_4$; 300 μ g of protein; 0.2 mM-dephosphorylated exotoxin.

	Total enzyme activity (c.p.m. incorporated)
Normal incorporation	4121
Incorporation in the presence of dephosphorylated exotoxin	4361

exotoxin concentrations (Fig. 4). The pattern of the double-reciprocal plots implies that inhibition is competitive in both cases.

That dephosphorylated exotoxin has no inhibitory effect on mixed soluble RNA polymerase at concentrations at which exotoxin would inhibit completely is shown in Table 2.

Discussion

The present results go some way toward explaining the toxicity of exotoxin to mammals. Both the polymerase activities examined here are much more

sensitive to exotoxin than is the polymerase from *E. coli*, in the case of the nucleolar activity by two orders of magnitude. Further, the character of the inhibition remains the same in intact nuclei and in soluble enzymes.

Other experiments have shown that exotoxin is cytotoxic to mammalian cells in tissue culture (N. Ho & P. Wildy, personal communication). Together, these observations establish that exotoxin can cross cell and nuclear membranes in mammalian systems. However, the quantitative differences between intact nuclei and the soluble enzymes that we observe and the increased response of the nucleoplasmic enzyme when nuclei are preincubated with exotoxin (Fig. 2) could mean that its movement is not completely unrestrained.

One puzzling feature remains. The results here confirm the observations with bacterial enzyme (Sebesta & Horska, 1968) that the inhibition of RNA polymerase by exotoxin is competitive with ATP. In the present work this is as true for intact nuclei as for the soluble enzymes.

That inhibition of the (supposed) target enzyme in a way that is fully competitive with a ubiquitous metabolite could result in so high a toxicity is unusual, if not unique. It requires either that the cell is incapable of presenting ATP to the polymerase at high concentration (or at least of instituting this response to inhibition) or that the exotoxin interferes with an additional enzyme involved in ATP synthesis.

The mammalian enzymes are, like the *E. coli* enzyme, unaffected by dephosphorylated exotoxin. One can postulate that the low toxicity of orally administered exotoxin (de Barjac & Riou, 1969; V. K. Brown, C. M. C. Muir & R. P. M. Bond, unpublished work) depends on a digestive dephosphorylation. Other enzymes, which we have found to be inhibited by exotoxin, are similarly unaffected by the dephosphorylated material (R. P. M. Bond & D. W. Eaton, unpublished work).

The very marked difference in sensitivity to exotoxin between the two mammalian polymerases examined here makes exotoxin an excellent complement to α -amanitin for the separation and independent observation of nucleolar and nucleoplasmic activities in intact nuclei (Kedinger *et al.*, 1970; Stirpe & Fiume, 1967; Novello & Stirpe, 1969; Novello *et al.*, 1970). At an ATP/exotoxin molar ratio of 25 and provided that exotoxin is not preincubated with the nuclei, the nucleolar activity is

fully inhibited whereas 75% of the nucleoplasmic activity survives. It will be of interest to see if this utility proves general for eukaryotic organisms as has been the case for α -amanitin.

Note added in proof

The results with intact nuclei *in vitro* reported in this paper are in very good agreement with the effects on RNA synthesis *in vivo* observed by Mackedonski *et al.* (1971).

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