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Inhibition of Hepatic Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerases by the Exotoxin of *Bacillus thuringiensis* in Comparison with the Effects of α-Amanitin and Cordycepin

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The action of *Bacillus thuringiensis* exotoxin, a structural analogue of ATP, on mouse liver DNA-dependent RNA polymerases was studied and its effects were compared with those of α-amanitin and cordycepin. (1) Administration of exotoxin *in vivo* caused a marked decrease in RNA polymerase activity of isolated nuclei at various concentrations of Mg^{2+}, Mn^{2+} and $(NH_4)_2SO_4$. A similar action was recorded after addition of exotoxin to isolated nuclei from control or exotoxin-treated mice. (2) Chromatographic separation of nuclear RNA polymerases from mice treated *in vivo* with exotoxin showed a drastic decrease of the peak of nucleoplasmic RNA polymerase, whereas the peak of nucleolar RNA polymerase remained unaltered. The same effect was observed after administration of α-amanitin *in vivo*, but cordycepin did not alter the relative amounts of the two main RNA polymerase peaks. (3) Administration of exotoxin *in vivo* did not alter the template activity of isolated DNA or chromatin tested with different fractions of RNA polymerase from control or exotoxin-treated mice. (4) Addition of exotoxin to isolated liver RNA polymerases inhibited both enzyme fractions. However, the α-amanitin-sensitive RNA polymerase was also 50–100-fold more sensitive to exotoxin inhibition than was the α-amanitin-insensitive RNA polymerase. Kinetic analysis indicated the exotoxin produces a competitive inhibition with ATP on the nucleolar enzyme, but a mixed type of inhibition with nucleoplasmic enzyme. The results obtained indicate that the *B. thuringiensis* exotoxin inhibits liver RNA synthesis by affecting nuclear RNA polymerases, showing a preferential inhibition of the nucleoplasmic α-amanitin-sensitive RNA polymerase.

There exist different RNA polymerases in animal cell nuclei (Widnell & Tata, 1966; Roeder & Rutter, 1969; Blatti *et al.*, 1970; Chambon *et al.*, 1970), and these may be modulators in the selection of synthesis of the several RNA species (Smuckler & Tata, 1972; Chambon *et al.*, 1970). One means of analysis of the role these enzymes may play in the regulation of transcription can be derived from response to various inhibitors of specific RNA formation. α-Amanitin, an agent with a selective action on the nucleoplasmic RNA polymerase, has been most frequently used in this manner (Fiume & Wieland, 1970; Jacob *et al.*, 1970*a*,*b*).

Sebesta and co-workers (Sebesta *et al.*, 1969*a*,*b*; Farkas *et al.*, 1969) and others (de Barjac & Dedonder, 1968; Bond *et al.*, 1969) have isolated and chemically characterized an exotoxin of *Bacillus thuringiensis* var. Berlin and have shown that it is a structural analogue of ATP. The exotoxin is composed of adenosine, ether-linked at its 5′-C atom to D-glucose, which in turn is bound to phosphorylated allaric acid (Farkas *et al.*, 1969) [allumcic acid (Bond *et al.*, 1969)]. Administration of this exotoxin to mice *in vivo* inhibits liver RNA synthesis (Sebesta *et al.*, 1969*a*,*b*; Mackedonski *et al.*, 1970) and effects preferentially ribosomal RNA formation (Mackedonski *et al.*, 1970). No influence on the synthesis of DNA or proteins was noted. It was also shown that *in vitro* the exotoxin inhibits *Escherichia coli* RNA polymerase, acting as a competitive inhibitor of ATP (Sebesta & Horska, 1968, 1970).

The identification of the exotoxin as an ATP analogue and its relative selectivity for ribosomal RNA formation, prompted us to investigate its action on the several liver RNA polymerases. It was found that the exotoxin given *in vivo* inhibits RNA polymerase by affecting the enzyme molecule, whereas the template apparently remains unaltered. The nucleoplasmic (α-amanitin-sensitive) enzyme is much more strongly affected. Similar changes are caused by administration of α-amanitin *in vivo*, but not of cordycepin. Experiments *in vitro* with isolated RNA polymerases

show that the nucleolar (α-amanitin-insensitive) enzyme is more resistant to inhibition by the exotoxin as compared with the nucleoplasmic enzyme. These findings constitute the subject of the present paper.

Experimental

Animals

Inbred male CBA mice (reared at the National Institute for Medical Research) weighing 15–20g were used for studies both *in vivo* and *in vitro*. Male Sprague–Dawley rats weighing 150–200g were the source of liver for RNA polymerase isolation in some experiments.

Exotoxin and other drugs

B. thuringiensis exotoxin was kindly supplied by Dr. K. Sebesta, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. An estimated molecular weight of 702 was used in calculating the concentration of exotoxin. The material was kept at −20°C and solutions were made immediately before use. Unstarved mice received 20μg of exotoxin per g body wt. by intraperitoneal injection. Control animals received equivalent volumes of saline (0.9% NaCl). In all experiments *in vivo* mice were killed 2h after injection. α-Amanitin was a gift from Dr. Th. Wieland, Max-Planck Institut für Medizinische Forschung, Heidelberg, Germany. For studies *in vivo*, α-amanitin was dissolved in saline and given intraperitoneally to rats at a dose of 1μg/g body wt. Cordycepin was generously supplied by Dr. W. E. Gutteridge, National Institute for Medical Research, London NW7 1AA, U.K. This material was dissolved in saline and given to mice by intraperitoneal injection at a dose of 250μg/g body wt.

Preparation of nuclei

Livers, freed of adherent gall bladders and ductal tissue, were placed in tared beakers containing 0.25M-sucrose, 5mM-$MgCl_2$, 25mM-KCl, 50mM-tris–HCl buffer, pH7.5 (at 20°C), then were minced and homogenized in 2vol. of the same medium (Blobel & Potter, 1966). The resultant brei was filtered through Swiss nylon bolting-cloth (200mesh), and the filtrate was mixed with 2vol. of 2.3M-sucrose containing 5mM-$MgCl_2$, 25mM-KCl, 50mM-tris–HCl buffer, pH7.5. The diluted homogenate was layered on 0.25vol. of 2.3M-sucrose buffer and centrifuged at 2°C for 60min at 77000g (r_{av}. 11cm). The supernatant was aspirated from over the grey–white nuclear pellets, the tubes were inverted and drained, and the nuclei were processed further.

Isolation and fractionation of DNA-dependent RNA polymerases

An adaptation of the method of Roeder & Rutter (1969) was used as follows. Nuclei were suspended in 1.0M-sucrose, 25mM-$MgCl_2$, 5mM-dithiothreitol, 50mM-tris–HCl buffer, pH7.4. To this suspension 0.08ml of 4.0M-$(NH_4)_2SO_4$ (adjusted to pH7.9 with aq. NH_3) per ml was added with vigorous mixing. The viscous mixture was left to stand in ice for 10min, then sonicated with 10s bursts of a Branson sonicator with a power setting of 3, separated by 30s cooling periods, for a total sonication time of 60s. The suspension was immediately diluted with 2vol. of 0.05M-tris–HCl buffer, pH7.9, containing 5mM-$MgCl_2$, 0.1mM-EDTA, 0.5mM-dithiothreitol and 25% (v/v) glycerol. The resulting opalescent suspension was centrifuged at 78480g (r_{av}. 7.8cm) for 1h at 2°C.

The clear faint-yellow suspension was carefully aspirated and 0.42g of $(NH_4)_2SO_4$ per ml of solution was added to it during 30min with constant stirring at 4°C. After the addition of $(NH_4)_2SO_4$, stirring was continued for an additional 30min. The milky suspension was centrifuged at 78480g (r_{av}. 7.8cm) for 90min at 2°C, the tubes were drained, and the grey–tan pellet was suspended in a minimal amount of 50mM tris–HCl buffer, pH7.9, containing 50mM-$MgCl_2$, 0.5mM-dithiothreitol, 0.1mM-EDTA and 25% (v/v) glycerol. This suspension was dialysed against 1000vol. of the same buffer containing 0.05M-$(NH_4)_2SO_4$ for 12h. The non-diffusible material was centrifuged at 105000g (r_{av}. 5.9cm) for 60min at 2°C and the pellet was discarded. The supernatant, containing the enzyme, was immediately applied to a DEAE-Sephadex A-25 column (0.9cm×30cm), washed with 10ml of 0.05M-$(NH_4)_2SO_4$ in the above tris–glycerol buffer, and the enzymes were eluted with a linear gradient of 0.1–0.5M-$(NH_4)_2SO_4$ in the same buffer. Fractions of 0.7ml were collected. The peaks of enzyme activity were pooled and either frozen immediately in liquid N_2 or dialysed briefly against 0.05M-tris–HCl buffer, pH7.9, containing 0.5mM-$MgCl_2$, 0.1mM-dithiothreitol and 25% (v/v) glycerol and then frozen. In addition, conductivity (measured with a home-made electrode and a Phillips conductivity meter) and protein were determined on the column fractions.

Preparation of chromatin and DNA

Nuclei were suspended in 0.25M-sucrose, 5mM-$MgCl_2$, 25mM-KCl, 50mM-tris–HCl buffer, pH7.4, by Vortex-mixer agitation and were centrifuged at 1000g (r_{av}. 4.25cm) for 10min at 2°C. This washing procedure was repeated twice. The washed nuclear pellet was suspended in 1ml of the same sucrose buffer per g of liver, and 0.05vol. of 20% (v/v) Triton X-100

(in the same solution) was added with gentle mixing. This suspension was centrifuged at 1000*g* ($r_{av.}$ 4.25cm) for 10min at 2°C. The pellet was suspended in 5ml of 10mM-tris–HCl buffer, pH8.0, and 25ml of 75mM-NaCl with 25mM-Na_2EDTA added. The suspension was sheared for 90s with an Ultra-Turrax homogenizer at the 80V setting. The material was centrifuged at 2000*g* ($r_{av.}$ 4.25cm) for 15min at 2°C and the pellet was resuspended in 75mM-NaCl, 25mM-Na_2EDTA and recentrifuged twice. The sediment was suspended in 50mM-tris–HCl buffer, pH8.0, and centrifuged at 8000*g* ($r_{av.}$ 4.25cm) for 15min at 20°C. This pellet was suspended in 5ml of 50mM-tris–HCl buffer, pH8.0, and mixed with 18ml of 1.7M-sucrose in 50mM-tris–HCl buffer, pH8.0. The mixture was underlaid with 7ml of the same sucrose solution and centrifuged at 51000*g* ($r_{av.}$ 9.1cm) for 12h at 2°C. The opalescent pellet was suspended in 10mM-tris–HCl buffer, pH8.0, and dialysed against the same buffer for 16h in the cold. The non-diffusible material was sheared with the Ultra-Turrax homogenizer at the 100V setting for 90s and the suspension was agitated with a wrist-action shaker for 30min. The fluid was centrifuged at 2°C for 30min at 8000*g* ($r_{av.}$ 4.25cm) and the supernatant material constituted the chromatin fraction. It was kept at 0°C with 1mM-NaN_3 as preservative.

DNA was prepared from the chromatin fraction by the addition of solid CsCl to make a 4M solution. The solution was gently mixed, placed in centrifuge tubes, overlaid with 0.2ml of mineral oil and centrifuged at 74000*g* ($r_{av.}$ 7.3cm) for 13h at 2°C. The supernatant fluid and oil were aspirated carefully, the pellet was suspended in 10mM-tris–HCl buffer, pH8.0, and dialysed against 1000vol. of the same buffer three times for 12h each. The DNA solution was kept at 0°C with 1mM-NaN_3 as preservative.

Chemical analyses

Protein was determined either by the technique of Lowry *et al.* (1951) with chymotrypsinogen B as standard or by the filter-paper-disc method of Bramhall *et al.* (1969). RNA and DNA were determined by the Schmidt–Thannhauser technique as described by Webb & Levy (1958).

Preparation of DEAE-Sephadex

The resin was swollen for 72h in water, mixed gently with 0.5M-NH_3 for 30min, washed with water until the pH of the eluent was 8.0, then suspended in 0.5M-H_2SO_4 for 30min and washed with water to pH7.0. The gel was then washed twice with 3vol. of 0.5M-$(NH_4)_2SO_4$, three times with 0.05M-$(NH_4)_2SO_4$ and 50twice with 0.05M-$(NH_4)_2SO_4$ in mM-tris–HCl buffer, pH7.8, containing 5mM-$MgCl_2$, 0.1mM-Na_2EDTA, 0.5mM-dithiothreitol and 25% (v/v) glycerol (0.05M-TGMED solution). After pouring, the column was washed with 50 bed vol. of the 0.05M-TGMED solution before use.

Assay of nuclear RNA polymerase activity

Nuclei were suspended in 50mM-tris–HCl buffer, pH7.9, containing 25mM-KCl and the protein content of the suspension was adjusted to 1mg/ml. The incubation mixture contained: 0.25mg of nuclear protein/ml; 0–10mM-$MnCl_2$; 0–30mM-$MgCl_2$; 0–400mM-$(NH_4)_2SO_4$; 0.8mM-ATP, GTP and CTP; 7μM-[^{3}H]UTP (specific radioactivity 1mCi/μmol); 50mM-KCl; 25mM-β-mercaptoethanol; 75mM-tris–HCl buffer, pH7.9 (at 37°C), and 5% (v/v) glycerol, in a total vol. of 0.08ml. Incorporation was carried out for 20min at 37°C, samples of 0.05ml were pipetted on to Whatman no. 1 filter-paper discs (2.4cm), which were immediately dropped into ice-cold 10% (w/v) trichloroacetic acid containing 5% (w/v) Na_4PPi. After 20min the discs were washed three times in 5% trichloroacetic acid with 5% (v/v) Na_4PP_i and then in 95% ethanol, absolute ethanol, ethanol–diethyl ether (1:1) and ether and were then dried (Mans & Novelli, 1961). The dried discs were placed in 10ml of toluene containing 0.1% of 2,5-diphenyloxazole and 0.05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. Radioactivity was measured in a Beckman L-200 liquid scintillation spectrophotometer with an efficiency for ^{3}H in this disc system of about 7%.

Assay of DNA-dependent RNA polymerase activity

The activity of the enzymes from the column eluent, and of pooled enzymes, was measured in a 0.08ml incubation mixture containing: 0.8mM-ATP, GTP and CTP; 7μM-[^{3}H]UTP (specific radioactivity 1mCi/mol); 77mM-KCl; 0.02mM-Na_2EDTA; 0.14mM-dithiothreitol; 2mM-$MnCl_2$; 0–400mM-$(NH_4)_2SO_4$; 75mM-tris–HCl buffer, pH8.3 (at 37°C); 7% (v/v) glycerol; denatured calf thymus DNA, native or denatured mouse liver DNA or mouse liver chromatin equivalent to 0.1mg of DNA/ml. The salt optimum concentration was similar to that described by Roeder & Rutter (1969) and each enzyme was assayed at the ionic strength for optimum incorporation. Incubation was carried out at 37°C for 30min. At 0 and 30min, 0.05ml samples were pipetted on to Whatman no. 1 filter-paper discs and processed as described above.

Physical analyses

Absorption spectra for chromatin and DNA were recorded at 20°C in 0.02M-NaCl, 0.02M-tris–HCl buffer, pH7.4, in a Unicam SP.1800 spectrophotometer. 'Melting' curves for chromatin and DNA were measured with a Unicam SP.700 spectrophoto-

meter with a programmed heating block. Buoyant densities of chromatin and DNA were measured with self-forming CsCl gradients (initial density 1.7) in 0.02M-NaCl, 5mM-EDTA, pH8.0, after 20h centrifugation at 44000rev./min in a Spinco model E analytical ultracentrifuge equipped with u.v. optics with an An-D rotor and with the temperature at 20°C. The photographic records were prepared and analysed with a Joyce–Loebl densitometer. *Micrococcus* DNA was used as a density marker and the densities were determined by the method described by Schildkraut *et al.* (1962).

Reagents

Analytical-grade reagents were used throughout. ATP, GTP and CTP as the respective sodium salts and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). [^{3}H]UTP, as the ammonium salt, was a product of The Radiochemical Centre (Amersham, Bucks., U.K.) and had a specific radioactivity of 1mCi/μmol.

Results

RNA polymerase and treatment with exotoxin in vivo

At 2h after intraperitoneal administration of 20μg of *B. thuringiensis* exotoxin/g body wt., no difference in liver weight/body weight ratios or of gross liver morphology was noted (liver weight/body weight ratios were 0.067 in controls and 0.064 in exotoxin-treated mice). Histological examination revealed only subtle alterations in hepatocyte nuclear structure, with nucleolar collapse.

The yield of nuclei isolated from liver of control and treated animals was not significantly different. Determination of the composition of the isolated nuclei revealed that there were not significant differences in protein and DNA content, but the RNA content of liver nuclei from exotoxin-treated mice was markedly decreased (Table 1).

The activity of the RNA polymerase of whole nuclei from control and exotoxin-treated mice was measured at low and high ionic strength with Mn^{2+} and Mg^{2+}. The results of these studies (Table 2) revealed that exotoxin treatment significantly decreased [^{3}H]UTP incorporation in the presence of both Mn^{2+} or Mg^{2+} at low and high ionic strength. The most marked inhibition was observed in RNA polymerase activity measured at high ionic strength in the presence of Mn^{2+}. To assess the effect of assay conditions on the observed decreased activity, the optimum requirements for RNA polymerase activity in nuclei of control and exotoxin-treated mice were determined at both high (Fig. 1) and low (Fig. 2) ionic strength. No differences in the optimum requirements for

Table 1. *Hepatic nuclear composition after exotoxin treatment in vivo*

Each mouse received 20μg of exotoxin/g body wt. 2h before being killed. Nuclei were isolated and analysed as described in the text. The ratios are mg of protein/mg of DNA and mg of RNA/mg of DNA respectively and represent the mean±one S.D. The number in parentheses indicates the number of separate, independent assays, each performed on nuclei isolated from at least four mice. Analysis of significance of difference was measured by the Student's *t* test.

	Protein/DNA ratio	RNA/DNA ratio
Control mice	3.65±0.56 (3)	0.220±0.016 (5)
Exotoxin-treated mice	3.64±0.82 (3)	0.151±0.025 (5) $P<0.05$

Table 2. *RNA polymerase activity of nuclei from the liver of control and exotoxin-treated mice*

Incorporation of [^{3}H]UTP into trichloroacetic acid-insoluble material was for 20min at 37°C as described in the Experimental section. Each value represents the mean±one S.D., with the number in parentheses representing the number of independent assays. Each assay was carried out with nuclei pooled from a minimum of four mice. The nuclei from exotoxin-treated mice were isolated 2h after administration of 20μg of exotoxin/g body wt.

	Enzyme activity (c.p.m./μg of nuclear DNA)		
Assay conditions	Control mice	Exotoxin-treated mice	% of control
$MnCl_2$ (2.68mM)	162±27 (5)	76±14 (5)	47
$MnCl_2$ (2.68mM)+$(NH_4)_2SO_4$ (114mM)	760±28 (5)	186±51 (5)	25
$MgCl_2$ (7.6mM)	75±18 (5)	28± 1 (5)	37
$MgCl_2$ (7.6mM)+$(NH_4)_2SO_4$ (114mM)	386±94 (5)	146±39 (5)	37

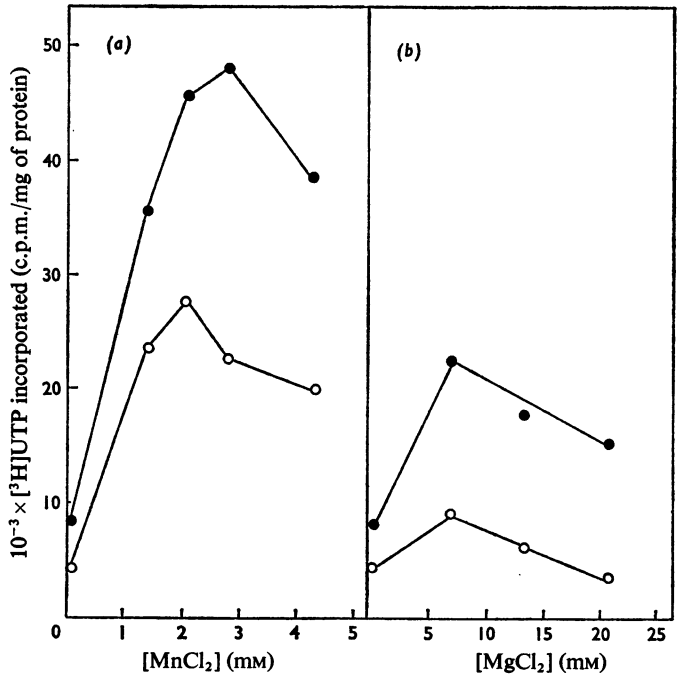

Fig. 1. *Effect of $MnCl_2$ and $MgCl_2$ concentration on [^3H]UTP incorporation by nuclei from control and exotoxin-treated mice at high ionic strength*

Exotoxin (20 μg/g body wt) was injected intraperitoneally and liver nuclei were isolated after 2h. The capacity of the isolated nuclei to incorporate [^{3}H]UTP was measured in a reconstituted cell-free system at a fixed $(NH_4)_2SO_4$ concentration of 114mM as described in the text. In (*a*) $MnCl_2$, and in (*b*) $MgCl_2$ concentrations were varied. Nuclei from the treated mice were less able to incorporate regardless of the bivalent metal concentration. ●, Control mice; o exotoxin-treated mice.

[^{3}H]UTP incorporation were apparent; the activity of nuclear RNA polymerases from exotoxin-treated mice was markedly lower than in controls at all ion concentrations tested.

Inhibition of RNA polymerase of isolated nuclei on incubation with exotoxin

To test the effect of the *B. thuringiensis* exotoxin *in vitro*, this agent was added to a suspension of liver nuclei isolated from control and exotoxin-treated mice (Fig. 3). Nuclei from control mice revealed a close to logarithmic response to increasing concentrations of exotoxin at optimum Mg^{2+} or Mn^{2+}, at both high and low ionic strength. A similar correlation was found when the exotoxin was added to suspensions of nuclei from the liver of mice treated *in vivo* with exotoxin and assayed at low ionic strength. The RNA polymerase activity of nuclei from mice treated *in vivo* with exotoxin showed a lack of response to added exotoxin, at high ionic strength. The absence of response to added low doses of exotoxin was most marked for Mn^{2+}/high-$(NH_4)_2SO_4$ RNA polymerase activity (Fig. 3*b*). These results suggested that a selective effect of exotoxin on some of the different nuclear RNA polymerases may underlie the observed differences.

Discrimination of exotoxin action on the components of the RNA polymerase complex

To obtain further insight into the action of exotoxin, DNA-dependent RNA polymerases were isolated from nuclei of control and exotoxin-treated mice, and fractionated by DEAE-Sephadex column chromatography. The elution pattern of enzymes from control livers showed two major (peaks A and B) and one minor (peak pre-A) peaks of RNA polymerase activity (Fig. 4). The first major peak (A) of activity was insensitive to α-amanitin, whereas the second peak (B) of activity was inhibited by more than 90% by concentrations of α-amanitin of 2 μg/ml. The first major peak, also called RNA polymerase A, corresponds to the nucleolar enzyme as described by

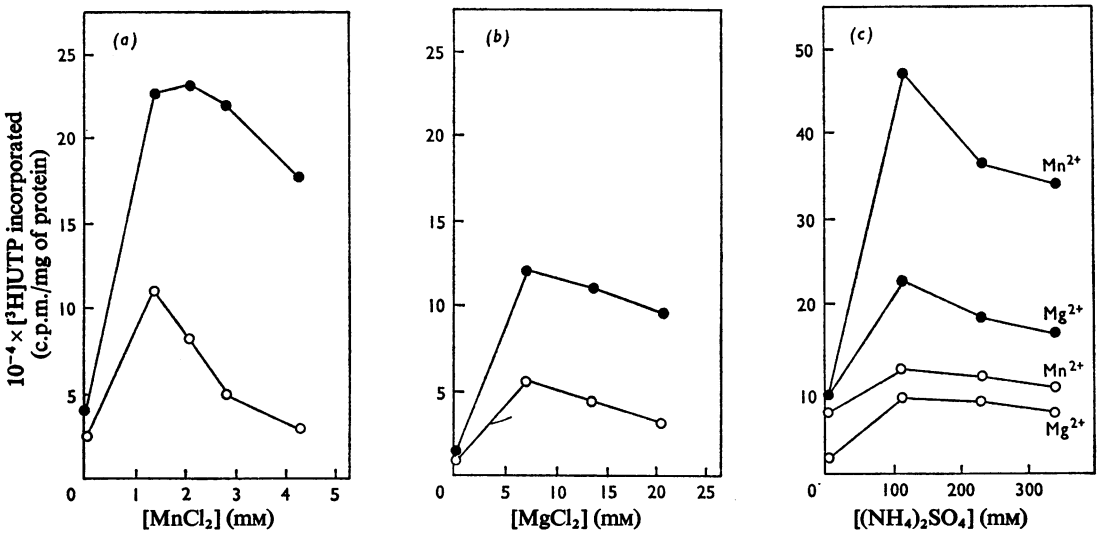

Fig. 2. *Effects of bivalent metal ion concentration and ionic strength on [^{3}H]UTP incorporation*

In (*a*) and (*b*), the bivalent metal ion concentration was varied in the absence of added $(NH_4)_2SO_4$. In (*c*) the bivalent metal concentration was fixed at 3mM-$MnCl_2$ and 6mM-$MgCl_2$ and the ionic strength was altered by changing the $(NH_4)_2SO_4$ concentration. ●, Control mice; ○, exotoxin-treated mice.

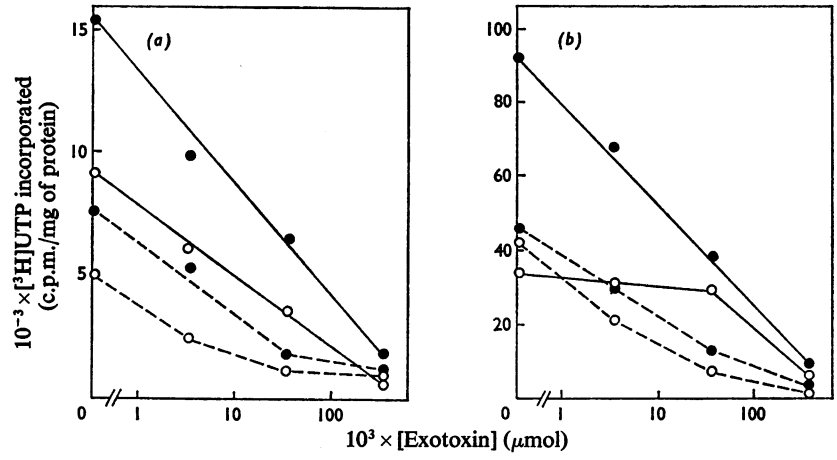

Fig. 3. *Effect of added exotoxin on* [^{3}H]*UTP incorporation by nuclei in vitro*

(*a*) Effect of added exotoxin at low ionic strength (that is without added $(NH_4)_2SO_4$). (*b*) Effect of added exotoxin at 114mM-$(NH_4)_2SO_4$. Nuclei isolated from: ●, control mice; ○, exotoxin-treated mice; ——, in the presence of 2mM-$MnCl_2$; – – – –, in the presence of 6mM-$MgCl_2$.

Roeder & Rutter (1970), whereas the second peak, called RNA polymerase B, corresponds to the nucleoplasmic enzyme (Roeder & Rutter, 1970). The relative amount of proteins was smaller in the peak of RNA polymerase B and hence had a higher specific activity.

Identical quantities of nuclei from treated and control mice were prepared and used to isolate the crude polymerase. The protein content of the crude solubilized enzyme was the same from these nuclei, comparing treated with control mice, and the crude

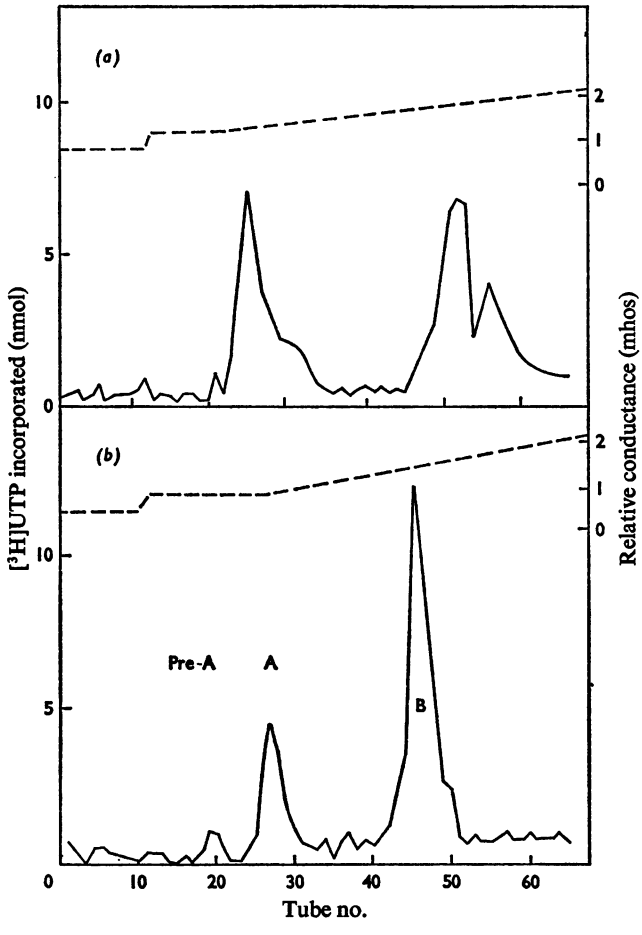

Fig. 4. *Elution profile on DEAE-Sephadex of RNA polymerase isolated from (b) control and (a) exotoxin-treated mouse liver*

DNA-dependent RNA polymerase was isolated and separated by column chromatography as described in the text. In the control, three peaks of activity are identified; peaks Pre-A and A are insensitive to α-amanitin and peak B is inhibited to 95% by 2 μg of α-amanitin/ml. In the exotoxin-treated profile, peak Pre-A is not apparent, peak A is larger and peak B smaller and seems to elute later in the column. Also, peak A is insensitive to α-amanitin and peak B is inhibited by this material. The salt gradient is shown by ----.

enzyme was applied to DEAE-Sephadex columns of very nearly identical geometry. Elution was carried out by as similar procedures as possible. Fractionation of the isolated RNA polymerase from the liver of mice treated *in vivo* with exotoxin revealed a decreased activity of the α-amanitin-sensitive RNA polymerase B, whereas the activity of the α-amanitin-insensitive RNA polymerase A appeared to be unaltered or even increased (Fig. 4). We do not know whether the delayed appearance of the peak of RNA polymerase B is real and significant, but the same pattern was observed in two independent experiments. The fractions showing enzymic activity of RNA polymerases A and B were pooled, the amount of proteins measured, and the specific activities of the two enzymes determined by using native mouse liver DNA (Table 3). The total yield of protein in peaks A and B was the same, comparing peaks A and B from control and treated mice, but the specific activity of RNA polymerase A increased and that of RNA polymerase B decreased. These results indicate that treatment of mice *in vivo* with exotoxin causes a selective decrease in the activity of RNA polymerase B.

To test for the specificity of the inhibition of RNA

Table 3. *Specific activities of pooled polymerase enzymes from livers of control and exotoxin-treated mice*

DNA was prepared from mouse liver nuclei by CsCl extraction as described in the Experimental section, then was dialysed and used without denaturation at a final concentration of 100 μg of DNA/ml. The fractions from chromatographic separation of polymerases A and B from control and exotoxin-treated mice were pooled and the protein was measured as described. Assay for specific activity of the pooled enzyme was carried out as indicated (see the Experimental section), except that the $(NH_4)_2SO_4$ concentration was adjusted to 0.1 M for both enzymes. Incubation was carried out at 37°C and each assay was repeated in triplicate. Zero-time assays and assays without added template had similar negligible amounts of incorporation (0.01 nmol of [^{3}H]UTP incorporated/mg of protein), which were subtracted from the values. The values are the means of three separate analyses.

	RNA polymerase activity (nmol of [^{3}H]UTP incorporated/30 min per mg of protein)			
	Control mice		Exotoxin-treated mice	
	Peak A	Peak B	Peak A	Peak B
DNA from control mice	2.62	15.1	5.15	9.35
DNA from exotoxin-treated mice	2.91	15.1	4.76	8.90

polymerases *in vivo* by exotoxin, similar experiments were carried out with α-amanitin and cordycepin, both of which inhibit RNA formation *in vivo*. α-Amanitin is known to interact with the enzyme molecule (Chambon *et al.*, 1970; Fiume & Wieland, 1970), whereas cordycepin, after phosphorylation to cordycepin 5′-triphosphate, is incorporated into the growing RNA chain and results in termination of transcription (Siev *et al.*, 1969). The elution pattern after DEAE-Sephadex chromatography for RNA polymerase extracted from the livers of rats treated *in vivo* with α-amanitin is shown in Fig. 5. The elution pattern of control rat liver RNA polymerases is similar to that of the mouse liver enzyme and similar to previous findings (Roeder & Rutter, 1969). The enzymic activity of peak A is insensitive to addition of α-amanitin, whereas the activity of peak B is almost completely abolished. These results are entirely consistent with the addition *in vitro* of α-amanitin to the enzyme preparation (Lindell *et al.*, 1970). Treatment of rats *in vivo* with α-amanitin causes a marked decrease of the RNA polymerase B peak activity, whereas RNA polymerase A activity remains unaltered. The markedly higher activity of peak pre-A in rat liver has also been noted after hormone treatment of hypophysectomized rats (Smuckler & Tata, 1971). That this peak is not due to simple altered elution of peak A is shown by the differences in the polyacrylamide-gel electrophoresis patterns of the two peaks (Smuckler & Tata, 1971). That this is not due to column overloading is suggested by the application of identical protein loads to columns of identical geometry. Thus administration of α-amanitin *in vivo* decreases the activity of RNA polymerase B in a manner which is qualitatively similar to that observed with the exotoxin of *B. thuringiensis*. On the other hand, administration of cordycepin to mice *in vivo*, in doses and at times known to inhibit RNA synthesis markedly (Table 4), does not cause any change in the relative amount of the two main RNA polymerase fractions A and B (Fig. 6). The actions *in vivo* of the exotoxin of *B. thuringiensis* and of cordycepin on the activity of liver RNA polymerases are not alike in this respect, but the exotoxin and α-amanitin produce similar responses.

The experiments presented above were carried out with the addition of DNA isolated from calf thymus or liver of mice. To test whether a further alteration of chromatin and/or DNA template activity might also cause the decreased RNA polymerase activity of nuclei from exotoxin-treated mice, both chromatin and DNA were isolated from nuclei of control and exotoxin-treated mice. Analysis of chemical content showed that the chromatin prepared from exotoxin-treated mice has slightly less protein and much less RNA than that from control animals (Table 5). The absorbance spectra and the 'melting' curves for DNA and chromatin isolated from control and exotoxin-treated mice were identical. DNA prepared from the chromatin of both control and exotoxin-treated mice had less than 1% of protein and no detectable amounts of RNA. Further, with both DNA preparations, CsCl-density-gradient centrifugation revealed patterns previously described for mouse liver DNA and its satellite (Kit, 1961) (Fig. 7). The density in g/cm^3 of the major DNA fraction is 1.702 and that of its satellite is 1.692, and the values are in good agreement with published values (Kit, 1961; Webb, 1963). This DNA showed identical template activity when isolated from treated or control animals.

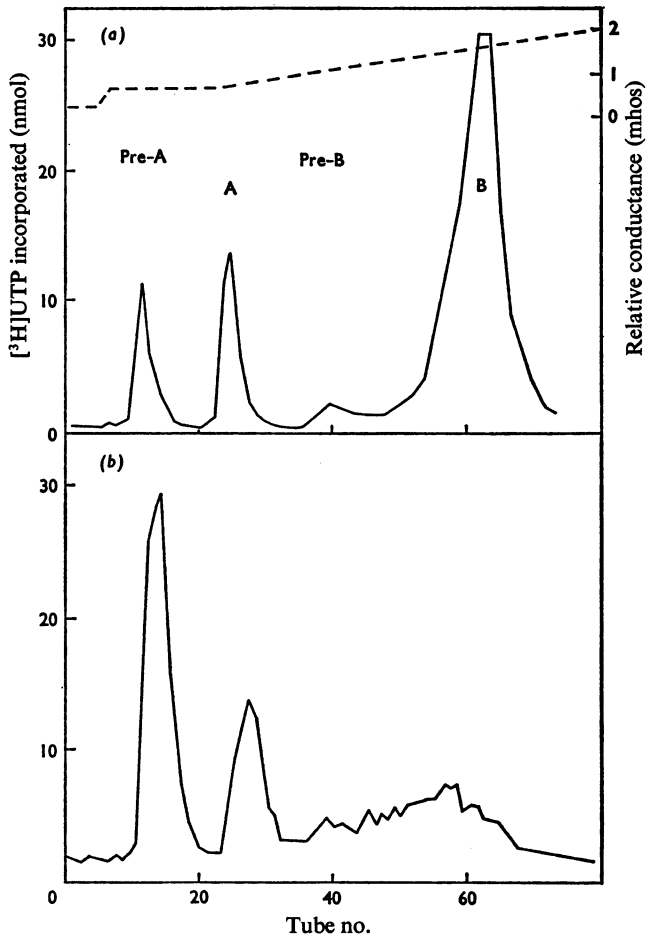

Fig. 5. *Elution profile on DEAE-Sephadex of rat liver RNA polymerase from control and α-amanitin-treated rat liver*

α-Amanitin (1 μg/g body wt.) was injected and the rats were killed at 20 min. Isolation and characterization were carried out as described in the text. In the control pattern (*a*), four peaks of activity are apparent. Peaks Pre-A, A and Pre-B are α-amanitin-insensitive, peak B is inhibited to 95% by 2 μg of α-amanitin/ml. In the elution profile of material isolated from the α-amanitin-treated animals (*b*), there is a significant decrease in activity of peak B. The salt gradient is shown by ----.

The template activity of chromatin from control and exotoxin-treated mice was studied by using RNA polymerases A and B isolated from control and exotoxin-treated mice (Table 6). Similar experiments were carried out with RNA polymerases A and B isolated from normal rat liver (Table 6). The results of these experiments clearly show that both RNA polymerase A and B can utilize chromatin as template; however, a pronounced selectivity of the two enzymes for the template is apparent. RNA polymerase A (α-amanitin-insensitive) uses chromatin less well as template compared with RNA polymerase B (α-amanitin-sensitive), which is significantly more active. [During preparation of this manuscript similar results were reported by Butterworth *et al.* (1971). Preliminary experiments indicated maximal incorporation at about 100 μg of chromatin DNA/ml under the condition of assay, i.e. 0.1 M-$(NH_4)_2SO_4$. In these experiments endogenous activity was subtracted. These results are in accord with the findings of Butterworth *et al.* (1971).] With specific regard to our studies, no differences in chromatin template activity were noted in the exotoxin-treated and control preparations.

These results indicate that treatment of mice *in vivo* with the exotoxin does not alter the template activity

Table 4. *Effect of α-amanitin and cordycepin in vivo on RNA formation by nuclei measured in vitro*

Male Sprague–Dawley rats were given 1 μg of α-amanitin /g body wt. intraperitoneally and were killed at 20 min. Male CBA mice were given 250 μg of cordycepin/g body wt. intraperitoneally and were killed at 8 h. Nuclei were prepared and incubated as described in the text. Incorporation of [^{14}C]ATP was carried out for 10 min, that of [^{3}H]UTP for 20 min and the trichloroacetic acid-precipitable material was obtained by precipitation and washing with 10% (w/v) trichloroacetic acid. After ethanol and ether washing, this material was collected on Millipore filters and assayed by liquid-scintillation spectrophotometry. These experiments with α-amanitin were carried out by Dr. Jamshed Tata, who has kindly permitted us to use these results.

Conditions of incubation	Incorporation of [^{14}C]ATP (c.p.m./mg of DNA)			Incorporation of [^{3}H]UTP (c.p.m./mg of DNA)		
	Control mice	α-Amanitin-treated mice	% of control	Control mice	Cordycepin-treated mice	% of control
$MnCl_2$ (3 mM)	4850	3480	72	2160	1060	49
$MgCl_2$ (6 mM)	3020	3020	100	1110	745	67
$MnCl_2$ (3 mM) + $(NH_4)_2SO_4$ (114 mM)	9000	4070	45	8960	2440	27
$MgCl_2$ (6 mM) + $(NH_4)_2SO_4$ (114 mM)	3800	3400	84	5800	2050	36

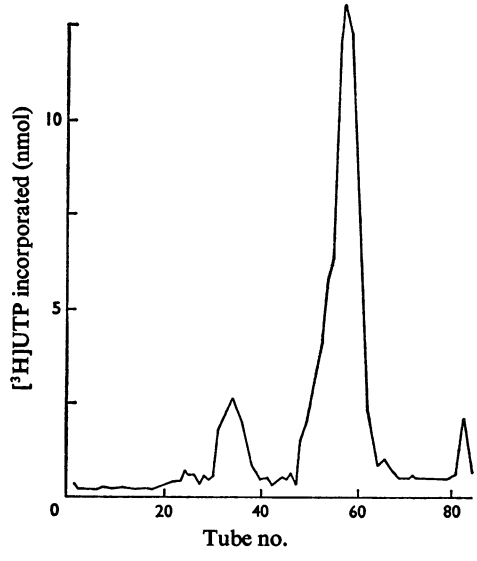

Fig. 6. *Elution profile on DEAE-Sephadex of mouse liver after cordycepin treatment in vivo*

Mice received 250 μg of cordycepin/g body wt. intraperitoneally and were killed after 8 h. The nuclear enzymes were isolated and analysed as described in the text.

of either chromatin or DNA. The site of action of the exotoxin administered *in vivo* is primarily on the molecule of the nuclear RNA polymerases. The α-amanitin-sensitive RNA polymerase B is much more strongly affected in exotoxin-treated mice as compared with RNA polymerase A, which does not seem to be altered. The fact that administration of exotoxin *in vivo* alters RNA polymerase B, shows that the effect of exotoxin on nuclear RNA polymerases is more complex than it just being a simple competitive inhibitor of ATP incorporation into RNA chains. Alteration of the enzyme molecule of RNA polymerase B is further supported by the fact that the elution pattern after DEAE-Sephadex chromatography resembles that obtained for liver RNA polymerases isolated from animals treated *in vivo* with α-amanitin. Also, treatment of mice *in vivo* with cordycepin, which leads to the formation of the ATP analogue cordycepin 5′-triphosphate, does not alter the elution profile of isolated liver RNA polymerases.

Inhibition of isolated liver RNA polymerases by exotoxin

To test the mechanism of the inhibition of RNA polymerases by exotoxin, experiments were carried out with the pooled enzyme fractions constituting peaks A and B comparing the effect of α-amanitin and testing the kinetics of exotoxin inhibition. In agreement with previous observations (Fiume & Wieland, 1970), it was found that RNA polymerase A was insensitive to α-amanitin at concentrations up to 10 μg/ml, whereas RNA polymerase B is 95% inhibited by 2 μg/ml of α-amanitin. The exotoxin inhibition of RNA polymerases A and B at various concentrations of inhibitor and/or ATP was investigated. At a final concentration of ATP of 0.4 mM, 50% inhibition of RNA polymerase B from control mice was caused by 0.18 μM-exotoxin. A similar figure was

Table 5. *Chemical composition of chromatin from liver of control and exotoxin-treated mice*

The amount of protein and RNA is given in mg/mg of DNA. Each value represents the mean of triplicate assays on chromatin preparations from six mice. The separate values represent analyses of two sets of independent preparations of chromatin.

	Protein/DNA ratio	RNA/DNA ratio
Control mice	0.975; 0.990	0.102; 0.116
Exotoxin-treated mice	0.815; 0.850	0.072; 0.074

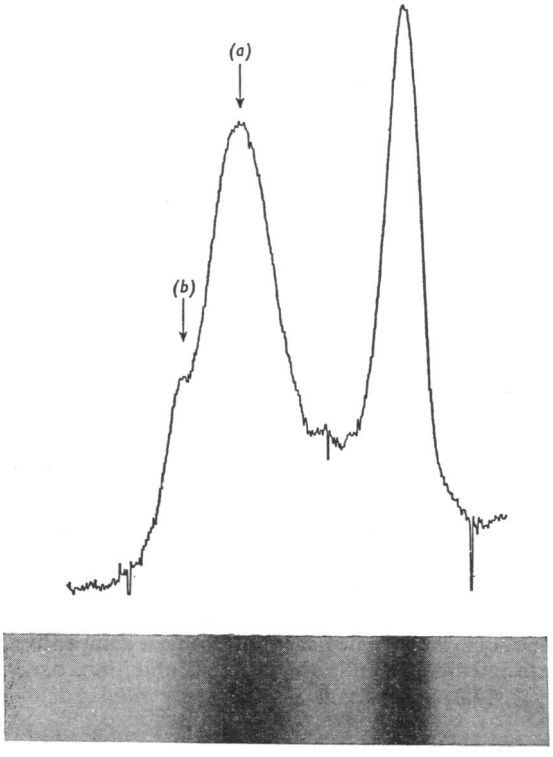

Fig. 7. *Buoyant-density analysis of the DNA isolated from exotoxin-treated mouse liver*

Measurement revealed a major band (*a*) with a density of 1.701 g/cm^3 and a satellite band (*b*) at 1.692. Identical results were obtained with control preparations. The major single peak to the right of the figure is *Micrococcus* DNA used as a density marker.

found when RNA polymerase B from normal rat liver was tested. In the case of RNA polymerase B from exotoxin-treated mice, the same extent of inhibition required a higher exotoxin concentration of 0.34μM. The exotoxin/ATP ratio for 50% inhibition of RNA polymerase B is in the range of $4.5–8.5 \times 10^{-4}$, a much lower figure than the reported ratio of 1.25 (for 70% inhibition) for the *E. coli* RNA polymerase (Sebesta & Horska, 1968, 1970).

In the case of RNA polymerase A, markedly higher concentrations of exotoxin were needed to attain the same degree of inhibition. Thus 50% inhibition of RNA polymerase A from control mice was observed at a final concentration of 0.117mM-exotoxin. In this case, too, 50% inhibition of RNA polymerase A from exotoxin-treated mice required a higher concentration of exotoxin, i.e. 0.36mM. The exotoxin/ATP ratio at 50% inhibition of RNA polymerase A is thus in the range of $4.25–8.5 \times 10^{-2}$, which approaches the values reported for the *E. coli* RNA polymerase (Sebesta & Horska, 1970). The above results clearly indicate that liver RNA polymerase A is about 100-fold more resistant to the inhibitory action of the *B. thuringiensis* exotoxin than is RNA polymerase B.

The kinetics of the RNA polymerase reaction in the presence of the exotoxin was studied and Lineweaver–Burk plots at different concentrations of exotoxin for RNA polymerase A and B are shown in Fig. 8. Considering the limitations imposed by the complexity of the RNA polymerase system, it appears that in the case of RNA polymerase A, exotoxin acts as a competitive inhibitor of ATP. The inhibition of RNA polymerase B shows more complex kinetics. Here, the intersection of the linear curves for the inhibited and non-inhibited reactions does not fall on either axis, which may be interpreted as showing a mixed type of inhibition of RNA polymerase B by exotoxin (Webb, 1963). The results from the above experiments were analysed by regression analysis by using the least-mean-square procedure to determine the line, its slope and intercept. In the case of rat liver RNA polymerases with ATP as substrate the K_m for RNA polymerase A was 58μM and that for RNA polymerase B was 26μM. The K_i in the presence of 1.78μM-exotoxin was 2.78 for RNA polymerase A and that for RNA polymerase B was 0.204.

Summarizing the results on the kinetics of RNA polymerase inhibition by exotoxin, it appears that RNA polymerase B is much more strongly inhibited than is RNA polymerase A, and it is likely that the action of the exotoxin on the molecule of RNA polymerase B involves a more complex mechanism than mere competition with the substrate.

Table 6. *Template activity of chromatin isolated from control and exotoxin-treated mouse liver nuclei*

Chromatin was prepared as described in the text from control and exotoxin-treated mice. To determine the capacity of the chromatin to act as template for rat liver polymerases A and B, experiments were carried out at a concentration equivalent to 100μg of DNA/ml and with $(NH_4)_2SO_4$ added to 0.1M for both enzymes (see the Experimental section). Incorporation was carried out for 30min and the radioactivity was assayed as indicated. The values represent the mean of triplicate analyses, with the endogenous activity of the chromatin subtracted.

Chromatin source	RNA polymerase activity (nmol of [^{3}H]UTP incorporated/30min per mg of enzyme protein)	
	Peak A	Peak B
Control mice	1.43	31.5
Exotoxin-treated mice	1.47	33.5

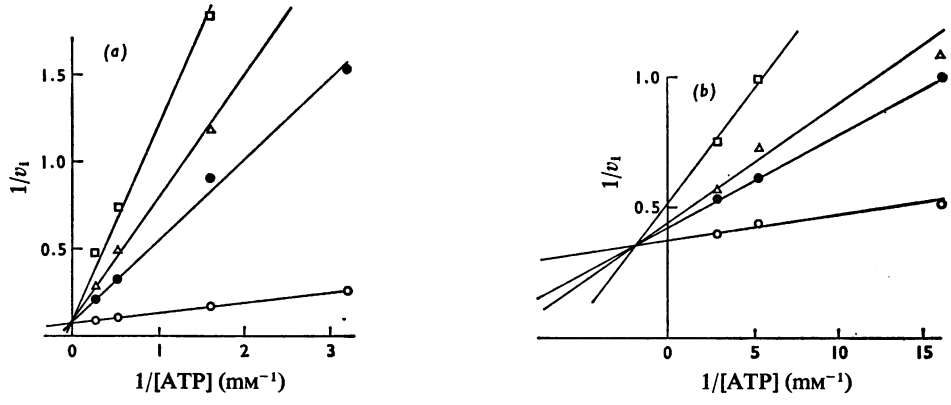

Fig. 8. *Lineweaver–Burk reciprocal plot of the effect of different exotoxin concentrations at various ATP concentrations on enzymes A and B*

For details see the text. The intersection of the lines for enzyme A (*a*) is at the ordinate, indicating a competitive inhibition, whereas with enzyme B (*b*) it is between the ordinate and abscissa indicating a mixed inhibition. o, no added exotoxin; ●, 0.178μM-exotoxin; △, 1.78μM-exotoxin; □, 17.8μM-exotoxin.

Discussion

The structural similarity of ATP and *B. thuringiensis* exotoxin suggested that the latter acts as an ATP analogue in inhibiting RNA formation. Investigation of the mechanism of decreased RNA production by using bacterial DNA-dependent RNA polymerase did indeed reveal that there was a competitive inhibition with ATP in cell-free systems (Sebesta & Horska, 1970). There was some indication for greater complexity in eukaryotic cells, since a selective inhibition of ribosomal RNA formation was demonstrated after administration of exotoxin to mice (Mackedonski *et al.*, 1970). With this in mind, an analysis of the effect of the exotoxin on mammalian liver polymerase was carried out *in vivo* and *in vitro*. A comparison was made with the effect of α-amanitin, a cyclic peptide that does not act as an ATP analogue, and cordycepin, which is believed to be triphosphated and to result in premature RNA chain termination (Schildkraut *et al.*, 1962; Shigeura & Boxer, 1964; Klenow & Frederiksen, 1964).

α-Amanitin inhibits one species of RNA polymerase by interacting with the enzyme protein, but not with the other components of the enzyme complex nor with the other polymerase molecules (Lindell *et al.*, 1970; Jacob *et al.*, 1970*a*,*b*). The inhibition by *B. thuringiensis* exotoxin also seems to be caused by interaction with the enzyme protein, at least in the case of RNA polymerase B. The following observations are relevant here: (*a*) the amount of active RNA polymerase B is decreased in exotoxin-

treated mice in a similar way to the decrease caused by α-amanitin administration *in vivo*; this effect is not shared by all ATP analogues, since cordycepin (probably cordycepin 5'-triphosphate in the cell) does not alter the normal elution pattern of liver RNA polymerases from DEAE-Sephadex; (*b*) treatment *in vivo* with exotoxin does not alter the template activity of either chromatin or DNA, whereas the specific activity of RNA polymerase B is markedly decreased and the specificity toward chromatin as a template is altered; (*c*) a rough estimate shows that about 70 molecules of exotoxin/molecule of RNA polymerase are needed to attain 50% inhibition, a figure which compares with similar estimates about α-amanitin (Jacob *et al.*, 1970*a*,*b*). The above considerations point to the possibility of a strong and selective binding of exotoxin to the molecule of RNA polymerase B. Since by its chemical structure (Farkas *et al.*, 1969; Bond *et al.*, 1969), exotoxin is an analogue of ATP, it seems reasonable to assume that this latter nucleotide (or its derivatives) participates directly in the blocking of the active centre of RNA polymerase B, rather than being just a substrate for the polymerization reaction. The mixed-type inhibition observed by the action of exotoxin *in vitro* on RNA polymerase B and the altered selectivity toward the chromatin template are consistent with this interpretation.

The higher sensitivity of RNA polymerase B to exotoxin inhibition contrasts with the behaviour of RNA polymerase A. In addition, exotoxin seems to act as a competitive inhibitor of ATP in the polymerization reaction with this enzyme species. In this respect, liver RNA polymerase A resembles the *E. coli* enzyme, inhibited in a competitive manner by *B. thuringiensis* exotoxin (Sebesta & Horska, 1968, 1970). Significant differences in intracellular distribution also contrast enzyme A with enzyme B; the former is suggested to be nucleolar and the latter nucleoplasmic in location (Roeder & Rutter, 1969). The former is also believed to be active in formation of ribosomal RNA, whereas the latter participated in the formation of more DNA-like RNA. Enzyme A is eluted in a larger quantity of protein than enzyme B, enzyme A has been shown to have at least two components by chromatography (Chesterton & Butterworth, 1971) and to have at least 17 distinct migratory bands in polyacrylamide–sodium dodecyl sulphate electrophoresis (in comparison with three major and two minor bands for enzyme B). Kinetic studies also reveal a twofold difference in apparent K_m values for ATP: that of enzyme A is twice that of enzyme B. The K_i for the two enzymes is also remarkably different under identical inhibitor conditions: the K_i of enzyme B is tenfold less than the K_i for enzyme A. Since not all the protein eluted with enzyme B may be enzymic and functional, some reservation must be made in interpreting the K_m and K_i values from these determinations. It is also possible that one or more of the protein components that appear on gel electrophoresis protect the enzyme from exotoxin, further complicating kinetic analysis.

The preferential inhibition of isolated RNA polymerase B by the addition of exotoxin *in vitro* is in sharp contrast to the effects *in vivo* of the exotoxin in RNA synthesis. As shown previously (Mackedonski *et al.*, 1970), exotoxin treatment of mice inhibits preferentially the labelling *in vivo* of ribosomal RNA, namely the 45S rRNA precursor, whereas DNA-like labelling is much less affected. In the present studies treatment *in vivo* with exotoxin showed a decreased RNA polymerase activity of isolated nuclei at both Mg^{2+}, low ionic strength (corresponding to the nucleolar enzyme) and Mn^{2+}, high ionic strength (corresponding to the nucleoplasmic enzyme) (Widnell & Tata, 1966). Further, addition of exotoxin to nuclei was inhibitory to the RNA polymerase activity at all concentrations of Mg^{2+} and Mn^{2+} both at low and high ionic strength. Thus it seems that a discrepancy exists between the action of the exotoxin *in vitro* (on isolated RNA polymerases) and *in vivo* (on whole liver or intact nuclei). A similar observation was reported by Jacob *et al.* (1970*a*,*b*) when the action *in vivo* and *in vitro* of α-amanitin on RNA synthesis and RNA polymerase activity was compared. Our results with *B. thuringiensis* exotoxin support the suggestion that nucleolar ribosomal RNA synthesis is controlled by the activity of nucleoplasmic RNA polymerase by a mechanism which is sensitive to α-amanitin and exotoxin. However, alternative explanations are also possible and further investigations are required to clarify the different responses *in vivo* and *in vitro* to inhibitors affecting the molecule of nuclear RNA-dependent RNA polymerases.

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