INHIBITION OF RNA POLYMERASE AND RNA SYNTHESIS IN RAT LIVER NUCLEI BY *Bacillus thuringiensis* EXOTOXIN (THURINGIENSIN)

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Thuringiensin inhibits *in vitro* the activity of Mg^{2+}- and Mn^{2+}-activated RNA polymerase in rat liver nuclei. In analogy to actinomycin D thuringiensin inhibits the high-ionic strength activated enzyme to a considerably lower degree. Furthermore a pronounced inhibition *in vivo* of nuclear RNA polymerases (50% inhibition following the administration of 0·85 mg of thuringiensin per kg) has been observed after the intraperitoneal injection of the compound; in this case both RNA polymerases have been affected practically equally. The effect of thuringiensin sets in immediately after the administration and the maximum inhibition is reached after a period of 60 to 90 min. However, at 16 h following the administration of thuringiensin at a dose level of 25% of LD_{50} the activity of both polymerases returns to a normal level. Simultaneously with the inhibition of the enzyme activity the utilization of orotic acid for the synthesis of nuclear RNAs is impaired; this occurs not only *in vitro* during the incubation of isolated liver nuclei with thuringiensin but also *in vivo* following its intraperitoneal administration. The synthesis of the enzymes responsible for the formation of pyrimidines *de novo* is not affected. The inhibitory effect of thuringiensin in mammalian cells is discussed.

B. thuringiensis produces thuringiensin which together with protein like endotoxin contributes considerably to the toxic effects of this microorganism directed against insects[1,2]. Following the purification and characterization of this substance[3–5], its structure has been elucidated[1,6] and the main causes of its toxicity have been determined. Thuringiensin is an unusual derivative of adenosine and is inhibitory to the synthesis of RNA even in mammals[7–9]. The administration of thuringiensin leads to the decreased utilization of orotic acid and to the impaired incorporation of adenosine and cytidine into liver RNAs; the synthesis of RNA is inhibited at dose levels that do not interfere[7] with the protein synthesis and with the formation of DNA. These observations led us to study the effect of thuringiensin on the isolated enzyme systems taking part in the synthesis of RNA. In *Escherichia coli* we have found previously that the compound inhibits DNA-dependent RNA polymerase acting as a structural analogue of adenosine 5′-triphosphate which displaces natural 5′-triphosphate from the specific binding site in RNA polymerase-DNA complex[10–12].

In distinction to the mechanism of the inhibition of RNA polymerase which is

well established, the effects *in vivo* of thuringiensin are less clear. We have investigated[8,9] these problems more in detail in collaboration with the Institute of Biochemistry in Sofia. The results indicated that in analogy to bacterial systems also in mammals the inhibition of DNA-dependent RNA polymerase was a prerequisite underlying the action of thuringiensin. For this reason the present report deals with the following questions: *1*) Is the inhibition of RNA polymerase the only site in RNA biosynthesis that is affected? and, *2*) Does the effect of thuringiensin *in vivo* and *in vitro* on individual nuclear DNA-dependent RNA polymerases correlate with the inhibition of liver RNA synthesis? Since numerous inhibitors (rifamycins[13,14], α-amanitin[15,16], aflatoxin B_1 (ref.[17])) affect bacterial and mammalian RNA polymerase – in the latter case also the nucleolar and nucleoplasmic one – to a different degree these questions are apparently of fundamental importance.

EXPERIMENTAL

Chemicals. Thuringiensin was isolated from the culture of *B. thuringiensis* var. *gelechiae* as described[3]. Actinomycin D was supplied by Merck, Sharp and Dohme, West Point. GTP and ATP were delivered by Calbiochem and CTP by Reanal. Uridine-[2-^{14}C] (44mCi/mmol) and orotic[-6^{14}C] acid (47 mCi/mmol) were prepared by the Institute for Research, Production and Uses of Radioisotopes, Prague.

Animals. For the experiments male rats (160–180 g) kept under standard conditions were used. Each group of animals included 4–6 individuals starved 14–18 h prior to the experiment. The compounds were dissolved shortly before application carried out by the intraperitoneal route. The details of the experimental conditions are given in legends to Figures.

Isolation of rat liver nuclei. The nuclei were isolated using a modified technique of Widnell and Tata[18] from rat livers. The livers were excised immediately after killing and transferred to 2 volumes of cold 2·4M sucrose with 1·5 mM Mg^{2+}-ions. They were cut to pieces and homogenized under cooling in a Potter-Elvehjem glass homogenizer with a Teflon pestle; the homogenate was filtered through a double layer of gauze and through cheese cloth. After dilution (1 : 5) with 2·2M sucrose the suspension[19] was thoroughly mixed and centrifuged (Spinco, rotor 30, 20000 r.p.m., 60 min, 2°C). The nuclear sediment was washed and suspended in 0·25M sucrose in 0·01M-Tris-HCl buffer (pH 8·1) with 1·5 mM Mg^{2+}-ions to obtain 80–100 . 10^6 nuclei per ml. All operations were carried out under cooling.

DNA-*dependent* RNA *polymerase assay.* Incubation was carried out[20] at 37°C in a Dubnoff shaking incubator in a total volume of 0·5 ml containing 0·1M-Tris-HCl buffer (pH 8·1) with 0·08M mercaptoethanol and 0·025M-KCl. To study the low- or high-ionic strength activated enzyme[21] the reaction mixture contained 6 mM Mg^{2+}-ions or 6 mM Mn^{2+}-ions with 0·5M ammonium sulfate and each of 0·75 mM nucleoside 5'-triphosphates, with the exception of 0·03 mM uridine-[2-^{14}C] 5'-triphosphate. As a source of the enzyme the suspension of nuclei (10^7) was added and the incubation lasted for 10 min. To terminate the reaction 2 ml of ice-cold 10% trichloroacetic acid or in case of high-ionic mixture 1 ml of distilled water and 3 ml of 10% trichloroacetic acid was added; after cooling and centrigufation the sediment was washed 3 times in 4 ml of ice-cold 5% trichloroacetic acid[19]. The resulting sediment was suspended in 0·5 ml of formic acid and an aliquot was used to measure the radioactivity in a liquid scintillation

spectrometer (Packard). The enzyme activity is expressed in pmol of incorporated uridine 5'-triphosphate in the presence of 10^7 rat liver nuclei during a 10-min incubation period.

Metabolic transformation of orotic acid in vitro. The incubation of orotic-[6-^{14}C] acid, 0·1 mM, with the liver cell-free extract was carried out as described[22] in 0·04M-Tris-HCl buffer (pH 7·4) at 37°C for 10 min in a total volume of 0·5 ml containing 0·4 mM 5-phosphoribosyl-1-pyrophosphate. The analysis of aliquots of the reaction mixture was carried out by chromatography using Whatman No 1 paper in a solvent system composed of isobutyric acid–ammonium hydroxide–water (66 : 1·5 : 33) and subsequent rechromatography in a solvent system composed of 2-propanol–ammonium hydroxide–water (7 : 1 : 2). The zones corresponding to individual substances determined according to standards were cut out and their radioactivity measured in a Packard liquid scintillation counter.

RNA *synthesis in rat livers.* For the determination of the rate of synthesis of total liver ribonucleic acids the specific radioactivity of uridine 2'(3')-phosphate isolated from an alkaline hydrolysate of liver ribonucleic acids following the administration of orotic-[6-^{14}C] acid (3 μCi/μmol) was measured. To groups of 4 male rats fasted for 16 h thuringiensin or saline and after 2 horotic [6-^{14}C] acid were administered *i.p.* The animals were killed 2 h following the label and their livers were homogenized under cooling in 3 volumes of ice-cold 0·2M-$HClO_4$; subsequent homogenate was centrifuged and the extraction was repeated twice. The sediment was subjected to alkaline hydrolysis (1M-KOH, 18 h, 20°C). The hydrolysate was neutralized with 10% $HClO_4$, centrifuged, and after cooling the clear supernatant fraction was chromatographed[23] in a solvent system composed of isobutyric acid–ammonium hydroxide–water. After the elution of the zone corresponding to uridine 2'(3')-phosphate the sample was rechromatographed in a similar way in a solvent system composed of 2-propanol–hydrochloric acid–water (170 : 39 : 41). Following the elution the specific radioactivity of isolated nucleotide was determided.

Labelling of nuclear ribonucleic acids. To groups of 4—6 male rats fasted for 16 h thuringiensin and after 2 h orotic-[6-^{14}C] acid (4 μCi/0·1 μmol) were administered. The animals were killed at different time intervals, and the nuclei isolated from the livers[18,19] were suspended under

TABLE I

In vitro Inhibitory Effect of Thuringiensin, Actinomycin D and of their Combination on RNA Polymerases in Rat Liver Nuclei

Nuclei isolated from the livers of 16 h starved male rats were suspended in 0·01M Tris-HCl, pH 8·1, with 0·25M sucrose and 1·5 mM Mg^{2+}-ions. High and low ionic strength activated RNA-polymerase activities were measured during a 10-min incubation period of 37°C, using 10^7 liver nuclei. The activity of enzymes is expressed as nmol of uridine-[2-^{14}C]5'-triphosphate incorporated per 10^7 liver nuclei ± standard error of the mean.

Inhibitor μmol/ml	RNA polymerase activity (nmol per 10^7 nuclei ± S.E.)			
	Mg^{2+}-activated	%	Mn^{2+}-activated	%
Control	0·44 ± 0·06	100	1·27 ± 0·12	100
Thuringiensin (0·1)	0·11 ± 0·03	25·0	0·64 ± 0·04	50·4
Actinomycin D (0·01)	0·09 ± 0·02	20·4	0·68 ± 0·07	53·4
Combination	0·06 ± 0·01	13·6	0·40 ± 0·05	31·5

cooling in 0·25M sucrose containing 0·01M-Tris-HCl buffer (pH 8·1) with 1·5 mM Mg^{2+}-ions. An aliquot of the suspension corresponding to 10^7 nuclei was repeatedly extracted with ice-cold 5% trichloroacetic acid and the sediment was dissolved in formic acid for the radioactivity assay (Packard). The incorporation of orotic acid into the nuclei is expressed in nmol of orotic-[6-^{14}C] acid incorporated per 10^7 nuclei.

Analysis of acid-soluble liver pool. The supernatant fraction obtained after the extraction (cold 0·2M-$HClO_4$) of rat livers labelled with orotic-[6-^{14}C] acid in the presence and absence of thuringiensin was adsorbed to activated charcoal under shaking. The extent of adsorption was determined by measuring the radioactivity. The charcoal was washed with water and the desorption at 45°C was carried out with 50% ethanol containing 4% ammonium hydroxide. The material thus obtained was subjected to chromatography using Whatman paper No 3 in a solvent system composed of isobutyric acid–ammonium hydroxide–water. The corresponding substances were eluted using appropriate standards.

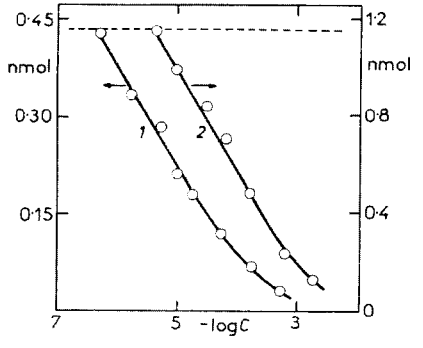

FIG. 1

In vitro Inhibition of Nuclear RNA Polymerases from Rat Liver by Thuringiensin

Liver nuclei (10^7) suspended in 0·01M Tris-HCl (pH 8·1) with 0·25M sucrose and 1·5 mM-$MgCl_2$ were incubated with the drug at 37°C for 10 min in 0·1M Tris-HCl (pH 8·1) with 0·08M 2-mercaptoethanol, 0·025M-KCl and 6 mM-Mg^{2+}-ions (*1*) or 6 mM-Mn^{2+}-ions with 0·5M ammonium sulfate (*2*) in a total volume 0·5 ml. 0·75 mM each of the common nucleoside 5′-triphosphates except of uridine-[2-^{14}C] 5′-triphosphate (0·03 mM) were present. RNA polymerase activity is expressed as nmol of uridine 5′-triphosphate incorporated per 10^7 of liver nucleic. *c* Concentration of thuringiensin in nmol/ml.

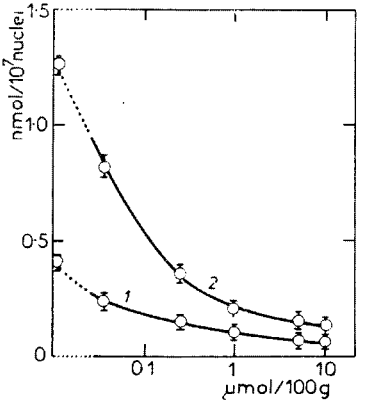

FIG. 2

Effect of Thuringiensin Administered *in vivo* on the Activity of RNA Polymerases in Isolated Rat Liver Nuclei

Thuringiensin (μmol/100 g) was administered intraperitoneally 3 h before killing to groups of 16 h starved male rats (4—6 animals; 160—170 g). The activity of Mg^{2+} — (*1*) and Mn^{2+}-activated (2) RNA polymerases in isolated liver nuclei was assayed as in Fig. 1 and is expressed as nmol of uridine 5′-triphosphate incorporated per 10^7 nuclei ± standard error of the mean.

RESULTS

Effect of Thuringiensin on RNA *Polymerase Activity in Isolated Rat Liver Nuclei* in vitro

The decreased activity of both DNA-dependent RNA polymerases during the incubation of isolated rat liver nuclei in the presence of increasing concentrations of thuringiensin is indicated in Fig. 1. The nucleolar enzyme was inhibited by thuringiensin considerably more than the extranucleolar one activated by high-ionic strength. The comparison of inhibitory effects of thuringiensin and actinomycin D is indicated in Table I. The total inhibition of the enzyme activity has not been observed even at high concentrations of these compounds. Since the substances affect RNA polymerase by different mechanisms (competition of thuringiensin with adenosine 5'-triphosphate[10,11] and binding of actinomycin D to deoxyguanosine[25,26] of the template DNA) it could be expected that their simultaneous addition into the reaction mixture might enhance their inhibitory effect. However, not even an additive inhibitory effect was observed as indicated in Table I.

RNA *Polymerase in Liver Nuclei after the Administration of Thuringiensin to Rats*

In analogy to the situation *in vitro* the administration of thuringiensin *in vivo* leads to the depression of the activity of both RNA polymerases in isolated rat liver nuclei. The depression of enzyme activities is dose-dependent (Fig. 2). 50% inhibition occurs in low- as well as in high-ionic strength activated enzyme following the administration of thuringiensin at the dose level of 0·85 mg per kg that is practically non-toxic[7] (LD_{50} 18 mg per kg). The comparison of the effect of thuringiensin and of actinomycin D following their administration *in vivo* is given in Table II. The decreased activity of Mg^{2+}-activated RNA polymerase is in distinction to the conditions *in vitro* nearly equal to the effects of thruringiensin on Mn^{2+}-activated enzyme. The combination *in vivo* of thuringiensin and actinomycin D does not lead to further enhancement of the inhibition. Nevertheless, it is necessary to take into account the rate of clearance of thuringiensin from the organism[27]; moreover *in vivo* it is also subjected to metabolic transformations[27].

The time course of the activity of both RNA polymerases following a single dose of thuringiensin to rats is shown in Fig. 3. The administration of the drug is associated with an immediate depression of the enzyme activity and the course of the maximal inhibition is identical in both cases during the time period of 60–90 min. The activity of both RNA polymerases begins to increase only after 8 h, while the functioning of both enzymes is within normal limits at 16 h after the administration. In this connection is should be noted that dephosphorylated thuringiensin (25 mg per kg) did not affect either *in vivo* or *in vitro* the activity of RNA polymerases.

Synthesis of Ribonucleic Acids in the Liver of Thuringiensin Treated Rate

In one of our first papers dealing with the inhibitory effect of thuringiensin in mammals we reported the decreased utilization of orotic acid for the synthesis of total liver ribonucleic acids[7]. DNA synthesis and the formation of proteins under these conditions remained unaffected. This finding is extended by the data concerning the incorporation of orotic acid into nuclear RNA of rat livers following thuringiensin as given in Fig. 4. 50% inhibition of incorporation occurs after the administration of 0·1 μmol per 100 g of the compound which corresponds to the dose level that causes *in vivo* the inhibition of the activity also of RNA polymerase to the same extent (Fig. 2). For this reason we have followed the activity of RNA polymerases and the rate of synthesis of total liver ribonucleic acids in relation to different dose levels of thuringiensin. Table III indicates that the impaired activity of both enzymes is associated with the depressed synthesis of liver ribonucleic acids.

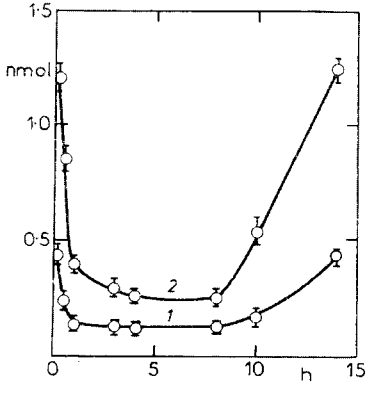

FIG. 3

Time Course of Inhibition of Nuclear Mg^{2+} (*1*) and Mn^{2+}-Activated (*2*) RNA Polymerases after a Single Dose of Thuringiensin Administered *in vivo*

Groups of 4—6 male rats (170 g) starved for 16 h were injected intraperitoneally with the compound (0·5 μmol/100 g) at different time intervals before killing. RNA polymerases of isolated liver nuclei was assayed as in Fig. 1.

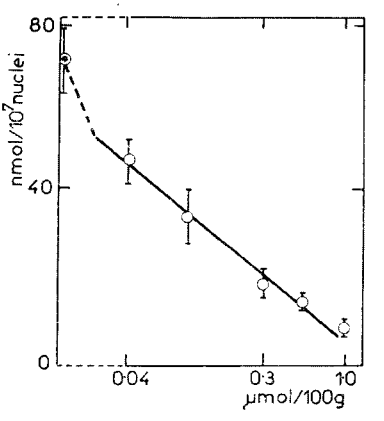

FIG. 4

Inhibition of RNA Synthesis in Liver Nuclei *in vivo* after the Administration of Thuringiensin to Rats

Thuringiensin (μmol/100 g) was injected intraperitoneally to groups of 4 male rats (160 g) after 16 h of starvation 2 h before orotic-[6-^{14}C] acid (4 μCi/0·1 μmol per rat). The animals were killed 3 h after the label. Radioactivity of nuclear ribonucleic acids is expressed as pmol of orotic-[6^{14}C] acid incorporated per 10^7 of isolated rat liver nuclei ± standard error of the mean.

The time course of the utilization of orotic-[6-^{14}C] acid for the formation of nuclear ribonucleic acids in rat livers is given in Fig. 5. The administration of thuringiensin simultaneously with orotic-[6-^{14}C] acid leads to the decreased uptake of the precursor into liver nuclei that remains constant over the time interval studied (70% inhibition at the dose level of thuringiensin of 0·3 μmol per 100 g). Comparing the effect of this substance with actinomycin D (2 h exposition, 0·1 μmol per 100 g) on the synthesis of liver ribonucleic acids it was found that the effect of thuringiensin was less considerable than that of actinomycin D (49 – 46% and 23 – 30% of the control value, respectively).

TABLE II

Activity of Nuclear RNA Polymerases from the Liver of Rats Treated with Thuringiensin and Actinomycin D

Groups of 6 male rats (180 g) starved 16 h were injected intraperitoneally with thuringiensin, actinomycin D or their combination 2 h before killing.

Administered μmol/100 g	RNA polymerase activity (nmol per 10^7 nuclei ± S.E.)			
	Mg^{2+}-activated	%	Mn^{2+}-activated	%
Control	0·39 ± 0·03	100	1·23 ± 0·09	100
Thuringiensin (1)	0·09 ± 0·01	23	0·27 ± 0·02	22
Actinomycin D (0·1)	0·12 ± 0·02	31	0·42 ± 0·05	34
Combination	0·11 ± 0·01	28	0·24 ± 0·03	20

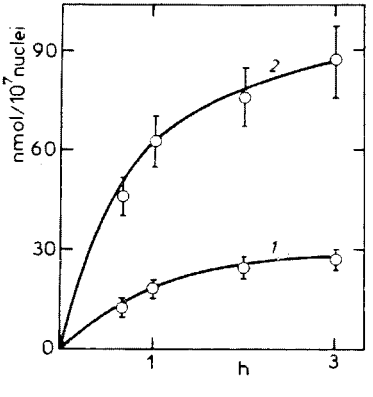

FIG. 5

Labelling of Nuclear RNA and Its Inhibition by Thuringiensin Administered *in vivo*

Thuringiensin (0·3 μmol/100 g) (*1*) or saline (*2*) were injected intraperitoneally to 16 h starved male rats 2 h before orotic-[6-^{14}C] acid (4 μCi/0·1 μmol per rat). Groups of 4 animals were killed at different time intervals thereafter (h) and the labelling of nuclear RNA's was measured as in Fig. 4.

While studying the effect of thuringiensin on the incorporation of orotic acid, its metabolites in the liver have been investigated. We have observed that the administration of thuringiensin at the dose level that inhibits the utilization of orotic acid by 70%, the level of orotidine 5'-phosphate and uridine 5'-phosphate is not changed. Equally the activity of enzymes which take part in the metabolic transformations of orotic acid (orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase) was not affected by thuringiensin administered *in vivo*. Apparently the inhibitory effect of thuringiensin on the utilization of orotic acid for the synthesis of liver ribonucleic acids is due to the specific interference of this compound with RNA polymerase system.

DISCUSSION

Liver nuclei have often been used for the studies of the properties and the role of RNA polymerases[28-32]. Using new techniques for the isolation of different RNA polymerases in rat liver nuclei it has been unequivocally established that more than one DNA-dependent RNA polymerase are present in rat liver[21,34,35]. In the present report we have employed intact nuclei isolated from rat livers and the effect of thuringiensin on individual RNA polymerases has been assayed in the presence of different ions. The addition of Mg^{2+}-ions and of a low ionic strength leads preferentially[21,35] to the synthesis of G—C-rich (ribosomal-like) RNAs, and the polymerase is localized in the nucleolus[34,35]. On the contrary, the addition of Mn^{2+}-ions and of of high-ionic strength is a requisite for the formation of DNA-like ribonucleic acid and the activity of the respective enzyme is located in the nucleoplasma[34,35].

TABLE III

Decrease of Nuclear RNA Polymerase Activities and Total RNA Synthesis in the Liver of Rats after the Administration of Thuringiensin

Groups of 4 male rats (170 g) starved 16 h were injected intraperitoneally with thuringiensin 2 h before killing or orotic-[6^{14}-C] acid administration (4 μCi/0·1 μmol per rat). The animals were killed 1 h after the label. Uridine 2'(3')-phosphate was isolated from the liver RNA and its specific radioactivity (dpm/μmol) measured.

Thuringiensin μmol/100 g	RNA polymerase activity, nmol per 10^7 nuclei ± S.E.				Incorporation of orotic acid	
	Mg^{2+}-activated	%	Mn^{2+}-activated	%	dpm/μmol ± S.E.	%
0	0·46 ± 0·04	100	1·25 ± 0·09	100	14 457 ± 810	100
0·04	0·28 ± 0·02	16	0·80 ± 0·04	64	9 785 ± 1·630	69
0·20	0·14 ± 0·01	30	0·34 ± 0·03	28	4 220 ± 975	29

The results of our experiments indicate that thuringiensin inhibits not only bacterial[10,11] but also mammalian DNA-dependent RNA polymerases. This finding is in agreement with the proposed mechanism of action of thuringiensin, *i.e.*, its competition with adenosine 5′-triphosphate for the specific binding site on the enzyme–template complex[11,12]. It is probable that the spatial structure of the binding site for the substrate is identical for DNA-dependent RNA polymerases of different origin[36]. This assumption is probably not valid for the adjacent loci of the binding site since α-amanitin which interacts with them inhibits RNA polymerases of different origin to a varying degree[15,28].

Our findings apparently indicate that thuringiensin interferes with cellular metabolism essentially during the final stages of RNA synthesis. The dose level of thuringiensin that inhibits the uptake of orotic acid into liver ribonucleic acids by 70% does not affect the quantity of orotic acid metabolites in the liver acid-soluble pool. Furthermore thuringiensin does nor influence the activity of enzymes taking part in the metabolic transformation of orotic acid (orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase).

As far as the effect of thuringiensin on nuclear RNA polymerases is concerned, the nucleolar enzyme is inhibited *in vitro* more extensively than the nucleoplasmic one. The effect is analogous to that observed with actinomycin D in HeLa cells[34] and Ehrlich ascitic cells[37,38]. Consequently the sensitivity toward thuringiensin of enzyme reactions leading to the formation of ribosomal and DNA-like ribonucleic acids is different[39]. In analogy following the administration of the compound *in vivo* prior to isolation of liver nuclei both enzymes are influenced to a different degree[40].

The described observations stand somewhat in a contradiction to the studies concerning the fate of thuringiensin in mammalian organism[27]. It has been shown in mice that the substance is excreted from the organism at a high rate and rapidly dephosphorylated to an inactive product[27]. For this reason a possibility cannot be excluded at present that the prolonged depression of the activity of nuclear RNA polymerases (Fig. 3) is due to the interference of thuringiensin with the polymerase system or that it is accounted for by nonspecific changes such as the interference with cellular regulatory systems (*e.g.* adenyl cyclase[24]) or changes in nucleolar ultrastructure[41] as seen for example in the case of aflatoxin B_1 (ref.[17]).

The administration of low doses of thuringiensin results in a preferential inhibition of the synthesis of liver 28 S, 18 S and 5 S ribosomal RNA including the 45 S precursor ribosomal RNA while the synthesis of nuclear "DNA-like" RNA is less affected[8,9]. However, in thuringiensin-treated mice the labelling of ribosomal RNA and of messenger RNA in cytoplasmic ribonucleoprotein fraction was inhibited to the same extent[9]. The *in vivo* administration of thuringiensin decreased the activity of nucleoplasmic RNA polymerase while the nucleolar enzyme was affected to a lower degree[40]. The results suggest that the discrepancy between the effect of thuringiensin on liver RNA polymerases and its interference with the synthesis of individual fractions

of RNA in the liver of rats treated with drug does not necessarily reflect the impairment of DNA-dependent RNA polymerase reaction only. Another compound affecting maturation of ribosomal RNA in the liver, 5-azacytidine[42,43], is without inhibitory effect on the activity of liver RNA polymerase. It is thus possible that the differences between the action of thuringiensin on the labelling of nuclear "DNA-like" RNA and messenger RNA in cytoplasmic fractions are due to the post-transcriptional modifications by the drug of messenger RNA processing leading to the formation of cytoplasmic messenger RNA.

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