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Inhibition by α-Amanitin of Ribonucleic Acid Polymerase Solubilized from Rat Liver Nuclei

By F. NOVELLO, L. FIUME and F. STIRPE
Istituto di Patologia generale dell'Università di Bologna, 40126 *Bologna, Italy*

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1. α-Amanitin inhibits *in vitro* the RNA polymerase solubilized from isolated rat liver nuclei. 2. In contrast with previous observations with whole nuclei, the inhibition occurs approximately to the same extent in the presence and in the absence of ammonium sulphate. 3. Evidence is presented that the toxin acts by interacting with the enzyme itself and not with DNA or other components.

α-Amanitin, a toxic principle from *Amanita phalloides* (Wieland, 1968), has been shown to cause hepatic necrosis in mice, with early nuclear damage and fragmentation of nucleoli (Fiume & Laschi, 1965). Administration of the toxin to mice was followed by a progressive decrease of the nuclear RNA content in the liver (Fiume & Stirpe, 1966), by an impaired incorporation of orotic acid into RNA *in vivo* and by a marked decrease of the RNA polymerase activity of nuclei isolated from such livers; an inhibition of RNA polymerase was also observed on addition of α-amanitin directly to isolated nuclei from liver of normal mice (Stirpe & Fiume, 1967). These effects of α-amanitin on RNA polymerase were visible only if the enzyme activity of isolated nuclei was assayed in the Mn^{2+}–ammonium sulphate system described by Widnell & Tata (1966), whereas the toxin had scarcely any effect on the enzyme activity of nuclei assayed at low ionic strength in the presence of Mg^{2+}. α-Amanitin also seems to abolish the stimulatory effect of ammonium sulphate and of heparin on the enzyme activity (Novello & Stirpe, 1969).

The experiments reported in this paper describe the effect of α-amanitin on RNA polymerase (EC 2.7.7.6) solubilized from rat liver nuclei. It was observed that the toxin inhibits the solubilized enzyme both at high and at low ionic strength, and that the inhibition seems to be due to strong binding of α-amanitin to the enzyme. A preliminary account of this work has been given (Novello, Fiume & Stirpe, 1969).

EXPERIMENTAL

Chemicals. ATP, CTP, UTP and GTP (all as sodium salts), spermine and DNA (calf thymus) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; [8-^{14}C]-ATP (31.2 μCi/μmol) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; α-amanitin was a generous gift from Professor T. Wieland, Heidelberg, Germany. Calf thymus DNA was denatured by immersing the solution (1 mg/ml of the buffer used for the preparation of RNA polymerase) in boiling water for 3 min, followed by rapid cooling in a salt–ice bath (Jacob, Sajdel & Munro, 1968).

Preparation and assay of RNA polymerase. Nuclei were isolated and RNA polymerase was solubilized from them as described by Jacob *et al.* (1968). The enzyme preparation was kept at −80°C until use. The enzyme activity was also assayed as described by these investigators, but with the following modifications: (1) DNA was added at a final concentration of 100 μg/ml; (2) the concentrations of nucleotide substrates were threefold higher; (3) the incubation time was decreased to 10 min. The reaction was stopped by addition of 1 mg of bovine serum albumin (in 0.1 ml) and 5 ml of cold 0.5 M-$HClO_4$. The resulting suspension was filtered through glass-fibre discs (Whatman GF/C) and the material on the filters was washed twice with 3 ml of cold 0.2 M-$HClO_4$. The filters were transferred to counting vials with 10 ml of scintillation fluid [0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene], and their radioactivity was counted in a Nuclear-Chicago mark I scintillation counter with an efficiency of about 80%.

RESULTS

Inhibition of solubilized RNA polymerase by α-amanitin. The inhibition of the enzyme solubilized from rat liver was observed on addition of α-amanitin, and the dose–response curve was very similar to that obtained with mouse liver nuclei (Stirpe & Fiume, 1967). The effect of α-amanitin was the same with either native or denatured DNA as template (Fig. 1).

A peculiar feature of the effect of α-amanitin on RNA polymerase of isolated nuclei was that marked inhibition occurred only in the presence of ammonium sulphate (Stirpe & Fiume, 1967). With the solubilized enzyme the effect of the toxin was seen regardless of the presence of ammonium sulphate, although it was more marked when the salt was present. The inhibition was not modified if spermine was omitted from the reaction medium (Table 1). The effect of

α-amanitin in the presence of spermine alone could not be tested, since under these conditions RNA polymerase activity was very low owing to precipitation of DNA (F. Novello & F. Stirpe, unpublished work).

Characteristics of the inhibition of RNA polymerase by α-amanitin. The effect of α-amanitin on RNA polymerase could be due to interaction of the toxin with DNA or with the enzyme. The inhibition was not relieved by increasing amounts of DNA in the reaction medium, nor was the apparent K_m for DNA modified in the presence of the toxin (Fig. 2).

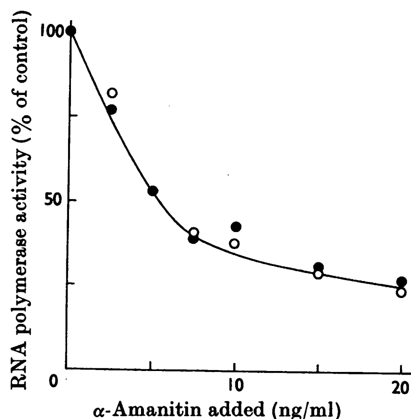

Fig. 1. Inhibition *in vitro* by α-amanitin of RNA polymerase solubilized from rat liver nuclei. The reaction mixture contained, in a final vol. of 0.75ml: 40μmol of tris–HCl buffer, pH 8.0, 2μmol of $MgCl_2$, 1.5μmol of $MnCl_2$, 2μmol of NaF, 7μmol of cysteine, 2μmol of spermine, 0.9μmol each of CTP, GTP and UTP, 0.20μmol of ATP including [^{14}C]ATP (0.21μCi), 100μg of DNA, 0.015ml of saturated $(NH_4)_2SO_4$ soln., brought to pH 8.0, the indicated amounts of α-amanitin, and 0.2ml of solubilized enzyme (added last). The reaction was run at 37°C for 10min. ●, Native DNA; ○, heat-denatured DNA.

When the effect of α-amanitin was tested with different concentrations of enzyme, the degree of inhibition produced by the toxin decreased as the amount of enzyme increased (Fig. 3). This result suggested that α-amanitin could act directly on the enzyme, and this was corroborated by the experiments reported in Table 2. The enzyme, or the other components of the reaction system, or both, were mixed with α-amanitin in 0.25ml, i.e. in one-third of the volume used for the assay of RNA polymerase. The samples were preincubated at 37°C for 5min and then the volume was brought to 0.75ml and the reaction was started by adding the missing component(s). An inhibition of AMP incorporation was observed that was equal to or even more marked than the inhibition obtained when three times the quantity of α-amanitin is added to the normal volume of assay mixture. The presence of DNA, nucleotides or both in the preincubation mixture did not influence this inhibition of the enzyme and no enhancement of the inhibition was observed if these components were preincubated in the presence of α-amanitin without the enzyme. It should be noted (1) that α-amanitin inhibited RNA polymerase activity even when it was added to the enzyme after DNA and nucleotides, i.e. when the RNA synthesis was already in progress, and (2) that a lower activity was observed, even without α-amanitin, when the enzyme was preincubated with nucleotides. With separate experiments (not shown) it was observed that the presence of ammonium sulphate in the preincubation mixtures did not influence the inhibition by α-amanitin, and as a control the inhibition of RNA polymerase was

Table 1. *Effect of α-amanitin on RNA polymerase activity assayed in the presence or absence of ammonium sulphate and spermine*

Experimental conditions were as described in Fig. 1.

Assay conditions				RNA polymerase activity (pmoles of AMP incorporated/10min)	Inhibition by α-amanitin (%)
$(NH_4)_2SO_4$	Spermine	DNA	α-Amanitin		
Absent	Absent	Native	None	194	
			10ng/ml	70	64
Present	Absent	Native	None	182	
			10ng/ml	52	71
Present	Present	Native	None	280	
			10ng/ml	68	76
Absent	Absent	Denatured	None	317	
			10ng/ml	129	59
Present	Absent	Denatured	None	532	
			10ng/ml	164	69
Present	Present	Denatured	None	670	
			10ng/ml	212	68

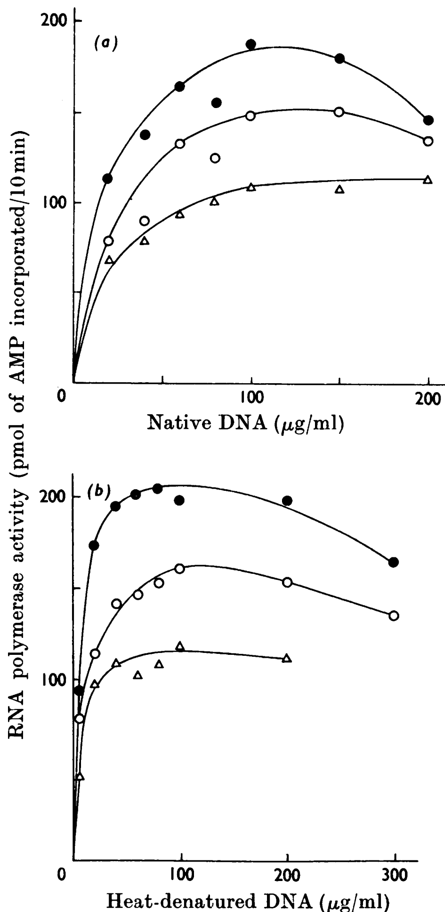

Fig. 2. Inhibition of RNA polymerase by α-amanitin in the presence of different concentrations of DNA. Experimental conditions were as indicated in Fig. 1, except for the concentrations of native (*a*) or heat-denatured (*b*) DNA. The experiments with native and denatured DNA were performed with two different enzyme preparations. ●, No α-amanitin; ○, 2.5ng of α-amanitin/ml; △, 5ng of α-amanitin/ml.

not enhanced by preincubation of the enzyme with α-amanitin in the final assay mixture with all the components except [^{14}C]ATP.

Finally, the effect of α-amanitin was studied in the presence of different concentrations of nucleotides and it was observed (Fig. 4) that the inhibition was not relieved by increasing amounts of substrates.

DISCUSSION

The results presented in this paper demonstrate that α-amanitin inhibits solubilized RNA polymerase to the same extent as was observed with isolated

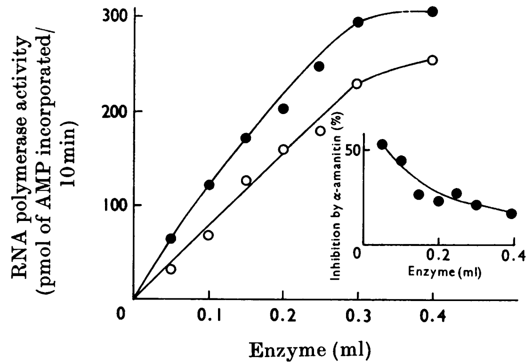

Fig. 3. Effect of enzyme concentration on the inhibition of RNA polymerase by α-amanitin. Experimental conditions were as indicated in Fig. 1, with 50μg of DNA and with the indicated quantities of enzyme. ●, No α-amanitin; ○, 3.2ng of α-amanitin/ml.

nuclei (Stirpe & Fiume, 1967). However, the inhibition of the solubilized enzyme occurs at approximately the same extent at low and at high ionic strength, and high ionic strength seems unnecessary for the interaction of α-amanitin with the enzyme (see below). Under the conditions of our experiments it seems that, in the absence of spermine, ammonium sulphate stimulates RNA polymerase only when denatured DNA is used as a template (Table 1). This stimulation is abolished by α-amanitin, and this is in agreement with the observations on isolated nuclei (Novello & Stirpe, 1969).

The preincubation experiments reported in Table 2 demonstrated that the inhibition of RNA polymerase was proportional to the concentration of α-amanitin to which the enzyme was exposed initially. These experiments and those with different amounts of enzyme (Fig. 3) indicate that α-amanitin acts on the enzyme itself and not on DNA or other components. The fact that dilution of the enzyme–amanitin mixture does not decrease the extent of inhibition suggests that α-amanitin acts by binding to RNA polymerase, probably with a rather strong and not easily reversible binding. Thus the mode of action of this toxin differs from those inhibitors of RNA polymerase that act by binding to DNA, such as actinomycin D (Reich & Goldberg, 1964), aflatoxin (Sporn, Dingman, Phelps & Wogan, 1966) or chromomycin (Kersten, Kersten & Szybalski, 1966), but resembles that of rifamycins on bacterial RNA polymerase (Wehrli, Knüsel, Schmid & Staehelin, 1968). However, in contrast with rifamycin (Sippel & Hartman, 1968), α-amanitin inhibited RNA polymerase even after the reaction had begun, i.e. after the initiation complex had formed.

Table 2. *Influence of preincubation of components of RNA polymerase assay on the inhibition of AMP incorporation by α-amanitin*

Experimental conditions were as described in Fig. 1. The preincubation was at 37°C in 0.25ml (one-third of the final volume) with 0.8ng of α-amanitin (3.2ng/ml) for 5min, after which missing components were added, to a final volume of 0.75ml, thus lowering the concentration of α-amanitin to 1.06ng/ml. The reaction was stopped 10min later. All components of the medium not mentioned were divided proportionally between the preincubated mixture and the addition, thus ensuring constant composition of the medium. Heat-denatured DNA was used in Expts. 1 and 2 and native DNA was used in Expt. 3.

		RNA polymerase activity (pmol of AMP incorporated/10min)								
Components preincubated in 0.25ml (⅓ of final volume)		Expt. 1			Expt. 2			Expt. 3		
α-Amanitin present during preincubation	Quantity ..	None		0.8ng	None		0.8ng	None		0.8ng
	Concn. ..	—		3.2ng/ml	—		3.2ng/ml	—		3.2ng/ml
Enzyme		145		50	156		36	64		22
Enzyme, DNA		134		48	149		54	79		29
Enzyme, ATP, CTP, GTP, UTP		79		43	89		31	46		23
Enzyme, DNA, ATP,* CTP, GTP, UTP		126		55	118		49	73		24
DNA		140		122						54
ATP, CTP, GTP, UTP		163		105						55
DNA, ATP, CTP, GTP, UTP		152		104	139		82	70		53
α-Amanitin omitted during preincubation, but present in the assay	Quantity ..				None	0.8ng	2.4ng			
	Concn. ..				—	1.06ng/ml	3.2ng/ml			
Enzyme, DNA, ATP,* CTP, GTP, UTP					118	85	62			
Non-incubated samples										
α-Amanitin present in the assay	Quantity ..	None	0.8ng	2.4ng	None	0.8ng	2.4ng	None	0.8ng	2.4ng
	Concn. ..	—	1.06ng/ml	3.2ng/ml	—	1.06ng/ml	3.2ng/ml	—	1.06ng/ml	3.2ng/ml
None		140	100	62	154	97	65	86	65	51

* Except [^{14}C]ATP, which was added at the end of preincubation.

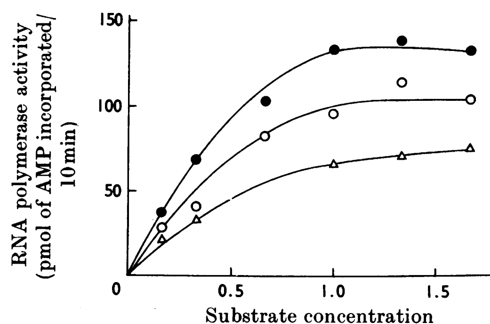

Fig. 4. Inhibition of RNA polymerase by α-amanitin at different substrate concentrations. Experimental conditions were as described in Fig. 1, except for the concentrations of substrates. Substrate concentration 1.0 corresponded to that of Fig. 1. ●, No α-amanitin; ○, 2.5ng of α-amanitin/ml; △, 5ng of α-amanitin/ml.

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