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Studies on the Pathogenesis of Liver Necrosis by α-Amanitin

EFFECT OF α-AMANITIN ON RIBONUCLEIC ACID SYNTHESIS AND ON RIBONUCLEIC ACID POLYMERASE IN MOUSE LIVER NUCLEI

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1. Injection of α-amanitin to mice causes a decreased incorporation of [6-^{14}C]-orotic acid into liver RNA *in vivo*. 2. The activity of RNA polymerase activated by Mn^{2+} and ammonium sulphate is greatly impaired in liver nuclei isolated from mice poisoned with α-amanitin, and is inhibited by the addition of the same toxin *in vitro*. 3. The activity of the Mg^{2+}-activated RNA polymerase is only slightly affected by α-amanitin either administered to mice or added *in vitro*.

α-Amanitin is the most powerful toxin of the toadstool *Amanita phalloides*. It has been isolated by Wieland, who identified it as a bicyclic polypeptide and recently elucidated its structural formula (for reviews see Wieland & Wieland, 1959; Wieland, 1964). Studies on the biochemical alterations induced by this toxin are reported in Wieland & Wieland (1959).

Mice injected with the purified toxin die within 2–5 days with severe fatty degeneration and necrosis of liver and kidney, the first morphological lesions being observable in the nuclei (Fiume & Laschi, 1965), where fragmentation of nucleoli is visible as early as 1hr. after the poisoning. Cytoplasmic structures appear undamaged in the electron microscope even 15hr. after the administration of α-amanitin (Fiume & Laschi, 1965).

An analysis of the DNA, RNA and protein content of liver nuclei revealed a progressive decrease of the RNA content that was significant as early as 1hr. after the poisoning, the content of DNA and of protein being unchanged even after 24hr. (Fiume & Stirpe, 1966). These results prompted us to study the effect of α-amanitin on the synthesis of RNA in mouse liver. The incorporation of injected orotic acid into RNA was greatly impaired in poisoned animals, and consequently we investigated the effect of the toxin on the two RNA polymerase reactions (EC 2.7.7.6) studied by Tata & Widnell (1966). It was observed that the activity of the Mn^{2+}–ammonium sulphate-activated RNA polymerase reaction was decreased in the nuclei isolated from poisoned animals, and that the same reaction was inhibited by the addition *in vitro* of α-amanitin. This toxin had only a slight inhibitory effect either *in vivo* or *in vitro* on the Mg^{2+}-activated RNA polymerase reaction. A preliminary account of this work has been published (Stirpe & Fiume, 1967).

EXPERIMENTAL

Animals. Male Swiss mice weighing 25–30g. were used. They were maintained on a complete laboratory diet; food was withdrawn when poisoned and control mice were injected. α-Amanitin was injected intraperitoneally at the dosage of 5μg./10g. body wt. as a 0·005% solution in 0·9% NaCl. Control animals received an equal amount of saline and were killed at the same time as poisoned animals, the experiments being run at the same time.

Chemicals. ATP, CTP, UTP (all as sodium salts) and yeast RNA were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; DNA (salmon sperm) was from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; GTP (sodium salt) and [8-^{14}C]ATP (21mc/m-mole) were purchased from Schwarz BioResearch Inc., Orangeberg, N.Y., U.S.A.; [6-^{14}C]orotic acid (44·5mc/m-mole) was from The Radiochemical Centre, Amersham, Bucks. α-Amanitin was supplied by Professor T. Wieland, Frankfurt am Main, West Germany. All other chemicals were of analytical grade. Solutions were prepared in glass-redistilled water.

Isolation of nuclei. Nuclei were isolated as described by Widnell & Tata (1964*a*), the livers from three or four animals being pooled in each experiment. The high-speed centrifugation was performed in an MSE 40 centrifuge (8×25ml. rotor). Nuclei were used within 15min. from isolation.

Incorporation of orotic acid into RNA. Mice received an injection of [6-^{14}C]orotic acid (10μc/ml. in 0·9% NaCl), given intraperitoneally at the dosage of 1μc/10g. body wt. After 15min. the animals were killed, the livers were homogenized and nuclei isolated. All sucrose solutions contained 2μg. of polyvinyl sulphate (potassium salt)/ml. to inhibit ribonuclease (Scherrer & Darnell, 1962). RNA was extracted with $HClO_4$ as described by Munro & Fleck (1966) from samples of unfractionated homogenates and of nuclear suspensions. Portions of the RNA extracts and of the acid-soluble fractions were taken for the determination of the RNA and of the radioactivity. For this, small amounts of extract were mixed in counting vials with 5ml. of ethylene glycol monomethyl ether and 10ml. of scintillation fluid [0·05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0·4% 2,5-diphenyloxazole in toluene], and were counted in a

Nuclear–Chicago mark I scintillation counter (counting efficiency about 55%, background 22 counts/min.). The counts were corrected to 100% efficiency.

Assay of RNA polymerase. The activities of the Mg^{2+}-activated and of the Mn^{2+}–$(NH_4)_2SO_4$-activated polymerases of isolated nuclei were determined as described by Tata & Widnell (1966). The RNA was extracted by the method of Widnell & Tata (1964*a*), and hydrolysed by boiling in 1 ml. of N-HCl for 60 min. The samples were dried under vacuum, the residue was redissolved in 0·5 ml. of water, and 0·4 ml. was taken for the determination of the radioactivity as described above.

Hydrolysis of nuclear RNA. [6-^{14}C]Orotic acid was injected intraperitoneally into mice at the dosage described above, and the animals were killed after 30 min. Liver nuclei were isolated and were incubated in the same medium used for the assay of the Mn^{2+}–$(NH_4)_2SO_4$-activated polymerase, except that the ATP was entirely unlabelled. At zero time and after 1 hr. of incubation 5 ml. of 0·5 N-$HClO_4$ was added, and after centrifugation the radioactivity of the supernatant and of the precipitated nuclear RNA was determined.

Determination of RNA and DNA. RNA was determined as described by Munro & Fleck (1966) or by the method of Schneider (1957), with yeast RNA as a standard in the latter case. DNA was determined by the method of Burton (1956), with salmon sperm DNA as a standard.

RESULTS

The experiments reported in Table 1 showed that the incorporation of [6-^{14}C]orotic acid into the RNA of liver homogenates and of isolated nuclei was already decreased by about 50% when orotic acid was injected 30 min. after α-amanitin poisoning. The inhibition was somewhat greater in the nuclei 1 hr. after the poisoning. The radioactivity in the acid-soluble fraction of homogenates and nuclei was the same in control and poisoned mice, thus indicating that α-amanitin has no effect on the precursor pools.

To identify the site of action of α-amanitin in the RNA-forming systems, the activity of RNA polymerase was studied in nuclei isolated from poisoned mice. According to Tata & Widnell (1966), two RNA polymerase reactions occur in isolated liver nuclei, one activated by Mg^{2+} and the other one activated by Mn^{2+} and ammonium sulphate. These two reactions were assayed. The activities obtained with normal nuclei were somewhat lower than those generally reported in the literature for rat liver nuclei. We have observed that the activities of both polymerase reactions were higher in rat liver than mouse liver nuclei and this was confirmed by Dr J. R. Tata (personal communication). The results (Table 2) showed that the activity of the Mn^{2+}–ammonium sulphate-activated reaction was much lower in the nuclei isolated from animals treated with α-amanitin, the impairment being already well marked 1 hr. after the poisoning, whereas the Mg^{2+}-activated reaction was slightly and not constantly decreased after the administration of α-amanitin.

The effect of α-amanitin *in vitro* on the same systems was tested, and it was observed (Fig. 1) that the Mn^{2+}–ammonium sulphate-activated polymerase reaction was inhibited up to a maximum of 80% by the toxin at a concentration of 0·015 μg/ml. The activity of the Mg^{2+}-activated polymerase reaction was not significantly affected by α-amanitin added *in vitro* even at a much higher concentration (μμmoles of ATP incorporated/10 min./mg. of DNA: 118 ± 16 without α-amanitin; 85·6 ± 14 with 10 μg. of α-amanitin/ml., mean values of four experiments, $P > 0{\cdot}05$). Control experiments have shown that the different effect of α-amanitin on the two RNA polymerase reactions is conditioned by the high concentration of ammonium sulphate, and is not due to the other differences between the two experimental systems (pH, Mn^{2+} instead of Mg^{2+}, length of incubation).

Cunningham & Steiner (1966) have reported that ammonium sulphate inhibits nuclear ribonuclease. Therefore the hydrolysis of nuclear labelled RNA was measured in the reaction mixtures containing

Table 1. *Effect of α-amanitin poisoning on the incorporation of* [6-^{14}C]*orotic acid into RNA of mouse liver homogenate and nuclei*

Experimental conditions were as described in the Experimental section.

		Sp. activity of RNA (counts/min./mg.)						Radioactivity in acid-soluble fraction (counts/min./g. of wet liver)			
		Homogenate			Nuclei			Homogenate		Nuclei	
Expt. no.	Time of orotic acid injection* (min.)	Controls	Poisoned	Inhibition (%)	Controls	Poisoned	Inhibition (%)	Controls	Poisoned	Controls	Poisoned
1	30	1338	608	54	15892	10779	32	211276	270106	363	312
2	30	1121	507	55	18229	7765	57	213197	228019	297	321
3	60	1546	563	64	21720	5801	73				
4	60	808	390	52	20760	8030	61	237004	332174	472	469

* Time after injection of α-amanitin. Mice were killed 15 min. after the injection of orotic acid.

Table 2. *Effect of α-amanitin poisoning on the* Mn^{2+}*–ammonium sulphate-activated and* Mg^{2+}*-activated RNA polymerase reactions in mouse liver nuclei*

The reaction mixtures and the conditions of incubation were identical with those described by Tata & Widnell (1966). Activities are expressed as $\mu\mu$moles of ATP incorporated into RNA/45min./mg. of DNA for the Mn^{2+}–$(NH_4)_2SO_4$-activated reaction, and as $\mu\mu$moles of ATP incorporated/10min./mg. of DNA for the Mg^{2+}-activated reaction. Values given are after correction for the dilution of radioactive ATP and subtraction of zero-time blanks. The results are mean values ± S.E.M., and the numbers of experiments are given in parentheses.

Time after poisoning (hr.)	Mn^{2+}–$(NH_4)_2SO_4$-activated enzyme		Mg^{2+}-activated enzyme	
	Controls	Poisoned	Controls	Poisoned
1	679 ± 77 (4)	199 ± 57 (4)	119 ± 16 (4)	97 ± 18 (4)
3	561 ± 77 (5)	140 ± 17 (4)	118 ± 16 (4)	108 ± 15 (4)

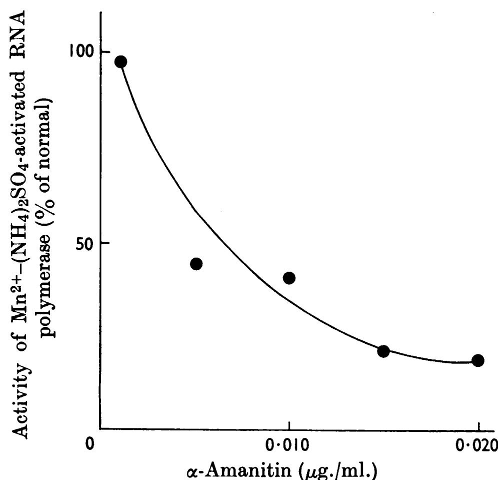

Fig. 1. Effect of α-amanitin added *in vitro* on the Mn^{2+}–$(NH_4)_2SO_4$-activated RNA polymerase reaction. Experimental conditions are as described in Table 2. Activity without α-amanitin is made equal to 100.

ammonium sulphate. No radioactivity was released from RNA into the acid-soluble fraction, regardless of the presence of α-amanitin up to 1 µg./ml., thus excluding the possibility that the effect of α-amanitin could be due to reactivation of the ribonuclease.

DISCUSSION

The experiments reported above demonstrate that: (i) poisoning with α-amanitin causes a rapid and marked impairment of RNA synthesis in the mouse liver; (ii) the activity of the Mn^{2+}–ammonium sulphate-activated RNA polymerase reaction is lowered in the nuclei isolated from the liver of poisoned mice; (iii) very low concentrations of α-amanitin inhibit the same reaction *in vitro*.

A peculiar aspect of the action of α-amanitin is that this toxin inhibits markedly the Mn^{2+}–ammonium sulphate-activated reaction, with little effect on the Mg^{2+}-dependent one, whereas other inhibitors of RNA polymerase tested on similar systems interfere strongly with both reactions, and actually are more inhibitory for the Mg^{2+}-activated polymerase, as has been shown for actinomycin D (Widnell & Tata, 1966) and for aflatoxin B_1 (Gelboin, Wortham, Wilson, Friedman & Wogan, 1966).

Several hypotheses have been formulated to explain the effect of the ammonium sulphate on RNA polymerase. It has been suggested that this salt acts by activating the DNA–RNA polymerase complex (Goldberg, 1961; Pegg & Korner, 1965), or by unmasking a second DNA-dependent RNA polymerase activity (Widnell & Tata, 1964*b*, 1966), or by removing inhibitory histones from DNA (Chambon, Ramuz & Doly, 1965), whereas Steiner & King (1966) suggested that its enhancing effect on the incorporation of precursors into RNA could be due to the inhibition of the hydrolysis of newly formed RNA by ribonuclease. The difference between the RNA produced by the two reactions (Tata & Widnell, 1966; Widnell & Tata, 1966; Blackburn & Klemperer, 1967) is a strong argument against the last hypothesis, and further support is given by the fact that α-amanitin inhibits the reaction activated by Mn^{2+} and ammonium sulphate without affecting the hydrolysis of RNA.

The coincidence between the effect of this toxin on the Mn^{2+}–ammonium sulphate-activated reaction and on the incorporation of orotic acid into RNA *in vivo* suggests that the reaction activated *in vitro* by Mn^{2+} and ammonium sulphate operates *in vivo* as well. At the same time, these findings indicate that the impairment of orotic acid incorporation *in vivo* is probably due to the inhibition of the above-mentioned reaction. The inhibition of RNA synthesis *in vivo* may well account for the decreased concentration of RNA observed in the liver nuclei of poisoned mice (Fiume & Stirpe, 1966). Further experiments are required to assess the role of this inhibition in the pathogenesis of liver necrosis induced by α-amanitin. From the analysis of the base composition (Tata & Widnell, 1966; Widnell & Tata, 1966; Blackburn & Klemperer, 1967) it has been shown that the RNA formed *in vitro* by the Mn^{2+}–ammonium sulphate-activated polymerase is more of the DNA type. Should the same type of RNA be produced *in vivo* by the reaction that is activated *in vitro* by Mn^{2+} and ammonium sulphate, one would expect a lack

of DNA-like RNA in the liver of α-amanitin-poisoned mice.

The fact that α-amanitin inhibits markedly only one of the two RNA polymerase reactions tested can throw some light on how this toxin, contrary to all other known inhibitors of RNA synthesis, does not prevent bacterial growth and the replication of both DNA and RNA viruses (Fiume, La Placa & Portolani, 1966). The mechanism whereby α-amanitin inhibits the Mn^{2+}–ammonium sulphate-activated polymerase remains to be elucidated, but the different effect of this toxin on the Mg^{2+}-activated polymerase suggests that it does not act by binding to DNA, or at least not in the same way as do actinomycin D (Reich & Goldberg, 1964) and aflatoxin B_1 (Sporn, Dingman, Phelps & Wogan, 1966).

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