REVIEW LETTER

AMANITINS. CHEMISTRY AND ACTION

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1. Introduction

Amanitins are the slow acting toxic components of the poisonous mushroom *Amanita phalloides* [1], which most probably cause the fatalities observed several days after ingestion of this toadstool. The structural formula (Ia) of α-amanitin has been elucidated [2, 3] as a cyclic octapeptide whose ring is divided by a sulfoxide bridge from an original cysteine sulfur to the 2-position of the indole nucleus of a tryptophan unit. Besides α-amanitin, which is a carboxamide, the corresponding carboxylic acid, β-amanitin (Ib) has also been isolated. In the white mouse the toxicity of Ib is a little less than that of Ia, the LD_{50} being 0.3 mg/kg body weight and 0.4 mg/kg respectively. The carboxylic group can be used as a handle for binding the toxin to various proteins, e.g. via thiophenylester [4] or carbodiimides [5].

2. Structure activity relationship

Nature offers several examples for relationship of structure and toxicity. The δ-OH group in a side chain is not essential for the poisonous action for γ-amanitin (Ic) is even more toxic (LD_{50} = 0.15 mg/kg) than Ia. The phenolic hydroxyl in 6-position also has no effect on toxicity: amanin (Id), which is devoid of it, has an amanitin-like action, as do the 6-methoxy derivatives of Ia, Ib and Ic obtained by reaction with diazomethane. Neither does the sulfoxide part of the molecule influence toxicity, since the thioether prepared from 6-*O*-methyl-α-amanitin by treatment with Raney-nickel [6] was still toxic. Nontoxic variants of the molecule occur also in nature. Amanullin (Ie) is an ineffectual variant lacking only the γ-OH group in a side chain of γ-amanitin (Ic) [7], and the same is true for amanullic acid, the corresponding carboxylic compound [8]. The decisive γ-OH can also be removed by oxidation (of 6-*O*-methyl-α-amanitin) with periodate. The resulting aldehydic compound ("aldo-amanitin") with a $-CH(CH_3)-CHO$ side chain, instead of $-CH(CH_3)-CH(OH)-CH_2OH$, shows no toxicity. Upon reduction with $NaBH_4$ the side chain is transformed to the γ-OH-containing $-CH(CH_3)-CH_2OH$ with recovery of toxicity [9]. Destruction of the molecular shape by elimination of the SO-bridge or by opening one of the peptide bonds also results in disappearance of activity [1].

3. Symptoms of intoxication

All laboratory animals except the rat are very sensitive to α-amanitin. The LD_{50} on intraperitoneal injection is 0.2–0.3 mg/kg body weight in mice [1]. The lethal doses for the guinea pig and the dog are 0.05 mg/kg and 0.15 mg/kg respectively [10], the rabbit is also very sensitive but the lethal dose has not been determined. On the other hand the rat survives doses 10 times higher than those which kill the above mentioned animals [10]. In amphibians like frogs or toads sensitivity is much reduced, LD_{50} being about 15 mg/kg [11].

In the mouse d-amanitin causes steatosis and necrosis of the liver and kidneys. In the adult male mouse one minimal lethal dose (MLD) of α-amanitin always pro-

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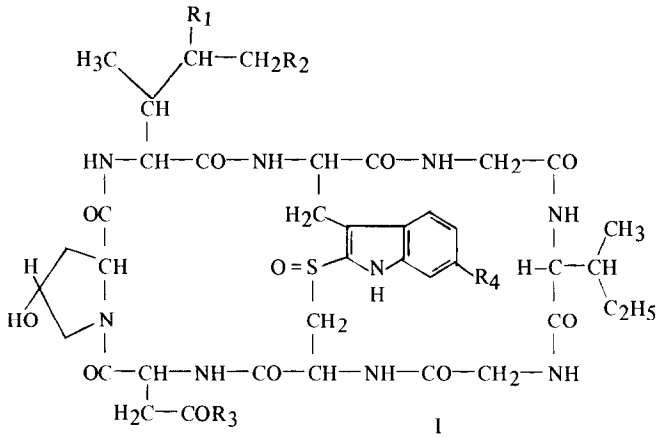

I

	R_1	R_2	R_3	R_4
a α-Amanitin	OH	OH	NH_2	OH
b β-Amanitin	OH	OH	OH	OH
c γ-Amanitin	OH	H	NH_2	OH
d Amanin	OH	OH	OH	H
e Amanullin	H	H	NH_2	OH

duces necrosis of the kidneys but never of the liver; liver necrosis only appears with doses above MLD [12]. Even after injection of 3 MLD, necrosis of the kidneys never appears in less than 3 days; where there is necrosis of the liver it can result in death, before necrosis of the kidney has had time to develop. So in all mice given more than one MLD and dying within 2 days there is necrosis of the liver, but not of the kidneys [12].

Amongst the decreases of concentrations of other biomolecules (glutathione, NAD, etc.) during α-amanitin poisoning in mice [13] a striking drop in serum protein was noticed, mainly in the albumin fraction, but also in other components, whose synthesis in liver seemed to be impaired by the poison [11].

4. Mechanism of toxic action

As an approach to the study of the mechanism of α-amanitin action, Fiume and Laschi [14] investigated the morphological lesions occurring in mouse liver and kidneys a few hours after toxin administration. They found that the targets of the toxic action of α-amanitin are the nuclei, which within 1 hr after injection of the toxin looked vesicle-like. Electron microscopic studies of mouse hepatocytes have confirmed that the first cellular organelle to show lesions after injection of α-amanitin is the nucleus [14]. Changes in cytoplasmic organelles appear much later. The most striking and precocious lesions in the nucleic are observed in the nucleoli. A few minutes after the injection of α-amanitin, they break up and their fibrillar and granular components segregate [12]. Another striking and early ultrastructural lesion is the condensation of chromatin, which causes the vesicle-like aspect of the nuclei of the liver and of the kidneys in histological sections. The finding of chromatin condensation in liver and kidney nuclei led to a study of the action of α-amanitin on the loops of lampbrush chromosomes. α-Amanitin was found to produce a retraction of normal loops in the oocytes of *Triturus cristatus carnifex* [15]. α-Amanitin also causes a shrinkage of puffs of giant chromosomes in salivary glands of *Chironomus* larvae [16].

In the mouse kidneys α-amanitin affects only the cells of the proximal convoluted tubules [12]. This finding led to the hypothesis that kidney damage is due to reabsorption in these tubules of α-amanitin from the glomerular filtrate. Confirmation of this

hypothesis was provided by experiments with a conjugate of β-amanitin (Ib) with rabbit serum albumin [5] which is filtered through the glomeruli only in minute quantities. After conjugation β-amanitin toxicity was increased more than ten times for the liver [5] but was completely lost for the kidneys [12].

In rat hepatocytes, which are very resistant to α-amanitin [10], the toxin produces lesions similar in appearance, in rapidity of onset and in severity to those in mouse liver cells, but which in contrast regress and disappear within 24 hr [12]. Unlike hepatocytes rat renal cells, even after injection of very high doses of α-amanitin, never show any lesions. This finding has been explained by suggesting that the cells of rat renal proximal tubules, in contrast to those of the mouse, do not have the capacity to reabsorb amanitin from the glomerular filtrate. Therefore these cells are not damaged and there is a rapid elimination of amanitin with the urine and a consequent rapid fall of the blood level. This explains the quick recovery of the nuclear lesions in liver cells and the resistance of the rat to amanitin.

The action of α-amanitin has also been studied in cultures of carcinomatous cells of the KB-Eagle line and in primary cultures of human amniotic cells [17]. α-Amanitin kills these cells when its concentration in the medium reaches 2 μg/ml. Here also the first morphological lesion is at the level of the nuclei where fragmentation of nucleoli was observed.

α-Amanitin was found not to inhibit the multiplication of three species of bacteria (*Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*) nor the replication of two RNA viruses (Poliovirus type 2 and parainfluenza virus type 3) and two DNA viruses (vaccinia virus and the virus causing bovine infective rhinotracheitis) [17].

After having identified one site of cytopathic attack of α-amanitin in the nucleus, the first step in an attempt to identify the underlying biochemical lesion was to determine the protein, DNA and RNA contents of the nuclei isolated from mouse hepatocytes after injection of α-amanitin [18]. It was found that while the DNA and protein contents were unchanged even 24 hr after α-amanitin injection the nuclear RNA was decreased significantly after only 1 hr. A study of the action of α-amanitin on RNA synthesis in mouse liver showed a 50% inhibition of the incorporation of ^{14}C-orotic acid into RNA 30 min after the injection of 1.5 MLD [19]. Consequently the effect of α-amanitin on the two RNA-polymerase reactions described in isolated liver nuclei by Widnell and Tata [20] was studied. One of them is activated by Mg^{2+} and produces an RNA of ribosomal type, the other one is activated by Mn^{2+} and a high concentration of ammonium sulphate and produces DNA-like RNA [21, 22]. The first reaction is localized chiefly in the nucleoli, the second in the nucleoplasm [22, 23]. The Mn^{2+}-ammonium sulphate-activated RNA-polymerase is strongly impaired in liver nuclei isolated from mice poisoned with α-amanitin and the same reaction is inhibited by 80% when α-amanitin at a concentration of 1.5×10^{-8} M is added *in vitro* to nuclei from normal mouse hepatocytes. The Mg^{2+}-activated RNA-polymerase is only slightly affected by α-amanitin either administered to mice or added *in vitro*. The slight inhibition of this reaction by 10^{-8} M α-amanitin does not increase even when the concentration is increased 1000-fold. The finding that α-amanitin only inhibits the Mn^{2+}-ammonium sulphate-activated RNA-polymerase [19] suggested that it does not act by binding to DNA and anticipated a specific lack of DNA-like RNA in the liver of α-amanitin-poisoned mice (provided this enzyme makes the same type of RNA *in vivo* as *in vitro*). Subsequently the action of α-amanitin on RNA polymerase solubilized from rat liver nuclei was studied [24–26] and it was found that the solubilized RNA-polymerase is inhibited to the same degree as the Mn^{2+}-ammonium sulphate-activated RNA-polymerase in isolated nuclei. Since the soluble enzyme, deprived of its deoxyribonucleo-protein template, requires for its activity the addition of DNA, it was possible to demonstrate by increasing the concentration of DNA or of the enzyme that α-amanitin exerts its inhibitory effect by binding to the enzyme and not to DNA. This mode of action resembles that of rifamycins on bacterial RNA-polymerase [27, 28]. In contrast to α-amanitin rifamycins do not inhibit mammalian RNA-polymerase [29]. On the other hand α-amanitin was found completely ineffective on bacterial RNA-polymerase [24, 26, 30] and on the RNA-polymerase of vaccinia-virus [31]. These results are consistent with the findings that α-amanitin has no effect on bacterial growth nor on vaccinia-virus replication [17] and indicate that the toxin is a specific inhibitor of RNA-polymerase of eukaryotic organisms. Interestingly the nontoxic

"aldo-amanitin" [9] has no effect on RNA-polymerase solubilized from rat liver nuclei [32].

Meanwhile Roeder and Rutter isolated several distinct RNA-polymerase activities from nuclei of eukaryotic cells [33]. From rat liver nuclei these authors solubilized two RNA polymerase activities [I and II). Polymerase II requires for its action a Mn^{2+}/ Mg^{2+} ratio and an ionic strength higher than does polymerase I. Polymerase I resides in the nucleolus and polymerase II in the nucleoplasm [34]. Probably these solubilized enzymes correspond to the two RNA-polymerase reactions of isolated nuclei [33], polymerase II being more easily solubilized than is polymerase I. In the experiments reported above, where the inhibitory effect of α-amanitin on solubilized RNA-polymerase was detected [24–26], the methods for solubilization [35, 36] used very probably only solubilized polymerase II [33].

Recently it was found [37, 38] that α-amanitin strongly inhibits the RNA polymerase of nucleoplasm (polymerase II) and does not affect the enzyme of nucleoli (polymerase I). These results fit in with the previous finding of Stirpe and Fiume [19] on the two RNA-polymerase reactions in isolated nuclei and substantiate the difference between polymerase activity in nucleoli and in the extranucleolar region of the liver cell nucleus. A selective inhibition by α-amanitin was observed also by Kedinger et al. [30], who found that this cyclopeptide inhibits only one of the two RNA-polymerases which they succeeded in isolating from calf thymus nuclei.

Also RNA-polymerase from yeast, which is resistant to rifamycin, is completely inhibited by α-amanitin, although the amount of toxic peptide required is about 100 times higher (ca. 500 molecules per molecule of polymerase) than for the mammalian enzymes [39].

In the light of the new observations the decrease in protein content of the blood of α-amanitin poisoned animals [11] can be explained as a consequence of the inhibition of RNA synthesis.

As far as the mechanism of inhibitory action of α-amanitin on the extranucleolar RNA-polymerase is concerned the only thing known up to now is that α-amanitin inhibits the elongation step of transcription since its addition after start of polymerization does not modify the extent of inhibition [24–26, 30]. The effect of the toxin on the initiation step is not yet known.

As a specific inhibitor of only one of the enzymes α-amanitin will make it possible to differentiate the functions of the two RNA-polymerases present in mammalian nuclei. Therefore it will be an extremely useful tool in biological research.

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