AMATOXINS, PHALLOTOXINS, PHALLOLYSIN, AND ANTAMANIDE: THE BIOLOGICALLY ACTIVE COMPONENTS OF POISONOUS *AMANITA* MUSHROOMS

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I. INTRODUCTION AND SCOPE

Extracts of the deadly poisonous *Amanita* mushroom have been objects of investigation for more than a century. These extracts were mainly from the green species *Amanita phalloides*. They were called "amanita toxin" and contained a heat-stable toxin fraction. As we know today, this fraction is composed of two groups of toxic peptides, the amatoxins and the phallotoxins, each consisting of several analogous cyclic peptides.

Over the three decades stretching from the isolation of the first crystalline toxins at the end of the 1930's,[1,2] to the elucidation of their structures,[3,4] to today, many of the contours of this landscape, originally so blurred, have become distinct. Reviews on the history, chemistry, and pharmacology of the toxic peptides have been published between 1958 and 1972.[5-10] One of the most recent essays on poisonous mushrooms appeared in 1975,[11] concentrating on the species of northern America.

At the center of interest lie the amatoxins, which are the sole cause of fatal human mushroom intoxications. These toxic peptides inhibit RNA polymerase B and hence protein synthesis of mammalian cells. The main component, α-amanitin, has been widely used in the past years as a potent inhibitor and is a valuable tool in molecular biology.

As for the second group, the phallotoxins, we will summarize our knowledge of its interaction with actin and try to relate this interaction to the many lesions observed in hepatocytes on treating with phalloidin, the toxin most widely used in this group. The virotoxins, found exclusively in *Amanita virosa*, will not be considered here, and are currently under investigation in our laboratory. Though differing in structure from the phallotoxins, their toxic action appears to be closely related to the latter. Another biologically active peptide to be discussed here is antamanide. To date, only a single peptide of this type has been found in *Amanita phalloides*, which, nevertheless, has been widely modified in its structure due to its availability via synthesis. The outstanding biological activity of this compound is its protecting effect from phalloidin poisoning.

Phallolysin, a biologically active protein component found in some *Amanita* species, will also be discussed. Due to its hemolytic activity, this heat-labile compound "phallin" was

the first toxin to be detected in *Amanita phalloides* by Kobert[12,13] and Abel and Ford.[14] Although it does not contribute to human mushroom intoxication, it is by far the most potent mushroom toxin when parenterally applied.

It is the aim of the present review to characterize the various biologically active substances on the basis of our present understanding and to explain, as well as possible, the molecular events by which the processes initiated by the toxins take their fatal turn. Therefore, we will consider only those publications which contribute to the understanding of the molecular mechanisms of action. Hence, although valuable in other respects, some papers reporting experiments with toxin mixtures in crude extracts of mushrooms or dealing solely with survival rates of laboratory animals under the influence of a host of drugs will be disregarded. Likewise, a series of clinical and clinicochemical investigations will not be considered here. Despite being of interest with respect to human mushroom intoxication, these papers are beyond the scope of molecular toxicology or biochemistry. For the same reason, the chemistry of the toxins will be treated in this review only in as far as it is concerned with questions of analysis or structure-activity relationships. However, in order to be comprehensive, all papers dealing exclusively with synthetic work will be cited in the list of references.

II. OCCURRENCE, CHARACTERIZATION, AND ANALYSIS OF THE PEPTIDIC TOXINS

A. Occurrence

Amatoxins occur in minute amounts in several mushrooms, including edible ones,[15] and are probably present in all mushroom species. The low concentration (1 to 10 ng/g fresh tissue) can only be detected in bioassays.

Carpovores which contain high concentrations of amatoxins (10 to 200 μg/g fresh tissue) and which can cause lethal intoxications are rather scarce. The green death cap *Amanita phalloides,* found growing all over central Europe, was the main object of the authors' investigations. The white "albino" variety, *Amanita phalloides* var. *verna,* is rarely seen. Nevertheless, this mushroom, known as the "deadly agaric," could well be more dangerous than the green one because it may be mistaken more easily for the edible white *Tricholoma* and *Agaricus* species. *Amanita virosa* Fr., also white, is found in some parts of Europe but appears to be widely spread in some states of North America. Tanghe and Simons[16] have reported the occurrence of *Amanita phalloides* in the eastern U.S. *Amanita tenuifolia* MURR and *Amanita bisporigera* ATK., which have not been observed in Europe to our knowledge, are reported to be rich in toxins.[17,18] Recently, amatoxins have also been found in *Amanita ocreata*[19] and, most interestingly, also in some species of *Galerina* by Tyler et al.;[20] *Galerina* mycelium has also been reported to produce amatoxins under conditions of submerged fermentation.[21] Furthermore, amatoxins, in amounts sufficient for their detection on thin layer plates, have been found in small *Lepiota* species by Gerault and Girre.[391]

Using combined chromatographic procedures,[22,23] the toxins of various *Amanita* species could be quantitated, also allowing a comparison of analogous species in Europe and northern America. Toxin contents are listed in Table 1. The table shows that *A. phalloides* is the most toxic species which, apart from rare exceptions, contains 2 to 3 mg of amatoxins per gram of dry tissue. *A. virosa* contains 1.2 to 2.6 mg amatoxins per gram of dry tissue; however, some American samples were devoid of amatoxins. One sample of a mushroom with a high content of amanitin, not belonging to the species *Amanita (Galerina marginata),* contained 0.4 mg α-amanitin per gram of dry tissue.[23] Most recently, the amount of amatoxins in *Galerina autumnalis* has been quantitated by Johnson et al.[45] This mushroom contains 0.8 to 1.5 mg/g dry weight of α- and β-amanitin.

Besides the well-characterized amatoxins (eight compounds), phallotoxins (seven compounds), and the recently detected virotoxins (at least two components), no further notable peptidic toxins have been discovered in our laboratory. The observation of Courtillot and Staron,[24] cited in detail in the review by Litten,[11] that amatoxins or phallotoxins can exist in additional forms of high molecular weight (>10.000 dalton) called "viroisine" or "phalloisine," could not be confirmed in our laboratory. An attempt to isolate phalloisine from

TABLE 1

Amounts of Various Toxins Present in Four Species of Deadly Poisonous *Amanita* Mushrooms[39,48]

Toxin	*A. phalloides* I (E)	II (E)	III (E)	IV (E)	V (NA)	VI (NA)	*A. verna* I[a] (E)	II–V (NA)	*A. virosa* I (E)	II (E)	III (NA)	IV (NA)	V (NA)	VI (NA)	*A. bisporigera* I (NA)
Acidic phallotoxins	4.2	3.2	2.6	2.9	4.3	2.8	1.9	—	1.6	0.7	—	—	—	—	—
Phalloidin (with phallisin)	0.9	1.1	1.2	1.3	1.7	1.2	0.5	—	2.1	2.1	0.4	0.6	0.9	—	0.9
Phalloin	<0.1	—	0.3	0.3	0.1	0.4	—	—	—	—	0.1	0.2	0.1	0.4	0.4
Amanin	<0.1	—	0.3	—	X	X	—	—	—	—	X	0.6	0.5	X	X
β-Amanitin	1.4	0.6	<0.1	0.7	1.7	2.3	0.2	(<0.1)[b]	—	—	—	<0.1	—	—	—
α-Amanitin	1.0	0.9	<0.1	1.1	1.2	1.0	0.3	—	1.2	1.4	2.6	—	—	1.9	1.8
γ-Amanitin	0.1	0.1	—	0.2	0.1	0.3	<0.1	—	—	—	<0.1	—	—	—	0.6
Amanullin	—	<0.1	—	0.1	X	X	—	—	—	—	—	—	—	—	—
Virotoxins	—	—	—	—	—	—	—	—	1.3[c]	1.3[c]	X	X	X	X	X

Note: E = European, NA = North America, and X = not determined.

[a] Possibly not *A. verna*, but *A. phalloides* var. *verna*.
[b] Traces in one case.
[c] Expressed in μmoles per gram of dry tissue since the molecular weight is unknown; calculated by using ε_M of phalloidinsulfone.

A. phalloides following the method described resulted in a preparation consisting of a mixture of the well-known low molecular weight toxins.[25] Seeger[26] also did not detect any derivatives of peptidic toxins in the high molecular weight fraction of aqueous extracts of *A. phalloides*. The material isolated by the French authors possibly represents the salts of some acidic toxins with an unknown basic compound of high molecular weight.

The analysis of the chromatographic patterns of toxins obtained from single specimens of various *Amanita* species are useful for chemotaxonomic evaluation: only in *A. phalloides* did we find the total set of toxins known thus far; *A. verna* generally contains fewer and sometimes no toxins; and *A. virosa* is impressive in its relatively high content of the neutral toxins α-amanitin and phalloidin, but lacks most of those toxins with a lower number of hydroxyl groups in the side chains. Finally, so far, virotoxins have only been isolated from *A. virosa* and may be useful in characterizing this *Amanita* species.

B. Characterization and Toxicity

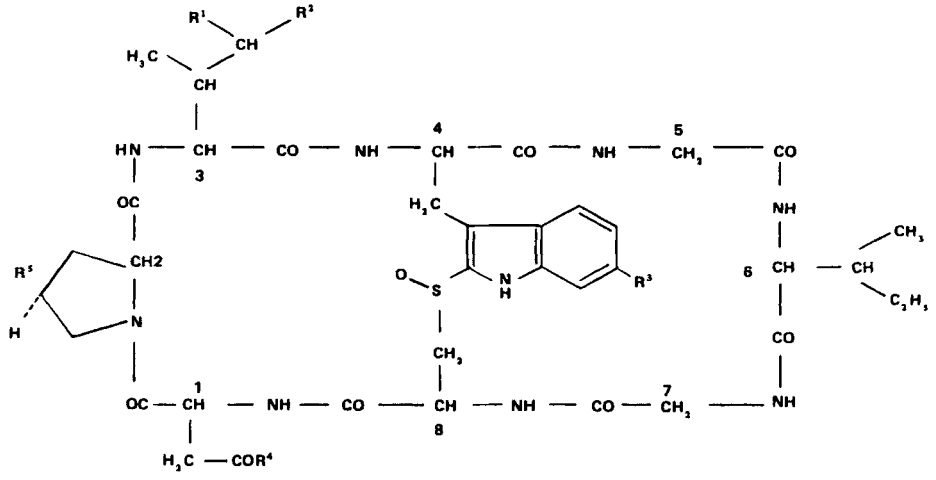
The toxic peptides are generally isolated from methanolic extracts of the mushrooms; their purification was last described in 1974.[27] Both amatoxins and phallotoxins are colorless, mainly crystalline compounds, and soluble in water, methanol, and other polar organic solvents. They are best characterized by chromatographic procedures followed by specific color reactions. They may also be characterized by amino acid analysis, UV spectra, ORD and CD spectra, and by their toxicity in the white mouse.

Amatoxins as well as phallotoxins are rather stable cyclic peptides. There is no enzyme known which is capable of degrading them. Heating to 100°C for several minutes will not destroy them either. On the other hand, both kinds of toxins, especially the amatoxins, decompose slowly in aqueous solutions if stored in open vessels. Rapid decomposition of amatoxins was observed on thin silica layers exposed for some hours during daylight in the open air.

The naturally occurring amatoxins cause death in relatively small doses; only amanullin and proamanullin are nontoxic (Table 2). Susceptibility to the poison differs among the various animal species. In the white mouse, the LD_{50} is ~0.3 mg/kg body weight and death occurs in 2 to 5 days. Compared with the white mouse, the rat is more resistant (LD_{50} about 4 mg/kg body weight), whereas dogs are much more sensitive, with an LD_{50} of only 0.1 mg/kg if administered intravenously. The various animal species also differ in their ability to resorb the toxin from the gastrointestinal tract. Whereas in mice and rats, the poison is resorbed extremely slowly or not at all, it causes

TABLE 2

Naturally Occurring Amatoxins, Their Toxicity (LD_{50}, mg/kg for the White Mouse, i.p.), and Concentration (*M*)[a] Causing 50% Inhibition of RNA Polymerase B (or II)(= approximate K_i)



	Name	R^1	R^2	R^3	R^4	R^5	LD_{50}	K_i (*M*)
A1	α-Amanitin[b]	CH_2OH	OH	OH	NH_2	OH	0.3	$0.5 \cdot 10^{-8}$
A2	β-Amanitin	CH_2OH	OH	OH	OH	OH	0.5	$0.5 \cdot 10^{-8}$
A3	γ-Amanitin	CH_3	OH	OH	NH_2	OH	0.2	$0.5 \cdot 10^{-8}$
A4	ε-Amanitin	CH_3	OH	OH	OH	OH	0.3	$0.5 \cdot 10^{-8}$
A5	Amanullin	CH_3	H	OH	NH_2	OH	>20	10^{-5}
A6	Amanullinic acid	CH_3	H	OH	OH	OH	>20	—
A7	Proamanullin	CH_3	H	OH	NH_2	H	>20	$5 \cdot 10^{-5}$
A8	Amanin	CH_2OH	OH	H	OH	OH	0.5	$0.5 \cdot 10^{-8}$

[a] The concentrations of amatoxins expressed in moles per liter (*M*) times 10^3 correspond roughly to milligrams amatoxin per milliliter.

[b] Systemic name: cyclic (L-Asparaginyl-4-hydroxy-L-prolyl-(*R*)-4,5-dihydroxy-L-isoleucyl-6-hydroxy-2-mercapto-L-tryptophylglycyl-L-isoleucylglygyl- L-cysteinyl) cyclic (4 → 8)-sulfide-(*R*)-S-oxide.

death in guinea pigs, cats, and dogs after peroral application of doses of a few milligrams per kilogram. Humans are at least as sensitive as dogs and resorb the toxin rather quickly, since the consumption of a single mushroom (5 to 6 mg amatoxin per 40 g fresh weight) seems to be sufficient to kill an adult.

Recently, the complex symptoms of amanitin intoxication have been analyzed in beagle dogs.[28-34] Early symptoms are hyperglycemia, followed by hypoglycemia, which then caused death in most dogs after 1 to 2 days. If compensated with glucose, the dogs developed an acute liver dystrophy, causing death after 2 to 3 days. Both of these symptoms were overlayed with severe hemorrhages in various organs, which was the main cause of death in some cases. A late symptom of the intoxication was kidney failure from which a few dogs died, though not before 7 days after intoxication.

The naturally occurring phallotoxins are less toxic than the amatoxins. In the white mouse, the LD_{50} is about 2.5 mg/kg body weight. In contrast to the amatoxins, rats are more susceptible to phallotoxins than mice. Peroral application leading to death of either animal species did not occur. Therefore, the phallotoxins do not seem to play a role in human mushroom intoxication. Death following i.p., s.c., or i.v. administration occurs within 2 to 5 hr after gross swelling of the liver. By the time of death, the liver will have reached threefold its original weight by having withdrawn a great deal of peripheral blood. Therefore, the main cause of death appears to be hemodynamic shock. It is noteworthy that swelling of the liver is less severe if the blood cells are absent. Erythrocytes or other kinds of blood cells may well be an additional factor in lethal phalloidin intoxication. Rats with a portocaval shunt need higher doses of phallotoxins but still develop a swelling of the liver.[35]

In retrospect, antamanide has received a name which does not cover its biological activity. Antamanide neither protects animals from amanitin intoxication nor from treatment with total extracts of *Amanita* mushrooms. On the contrary, there is a slight synergistic activity of antamanide in amanitin intoxication. However, antamanide does protect animals very effectively against phallotoxins. Protection is effected with only 0.15 mol of antamanide for 1 mol of toxin, provided that antamanide is applied prior to or together with the toxin. Following the toxin, much higher doses are needed and after 10 min has elapsed, no protecting effect can be seen at all. Antamanide is excreted rapidly but displays its antiphalloidin effect for at least 2 hr after administration.

C. Analysis of Toxins of *Amanita* Species

1. Qualitative Analysis

Paper chromatography has been of great value for the detection of all *Amanita* toxins. The best separation was achieved by a mixture of butanone per acetone per water (30:3:5). Figure 1 is a diagram of descending paper chromatography showing all those components of *A. phalloides* identified to date.

The same solvent was useful in developing

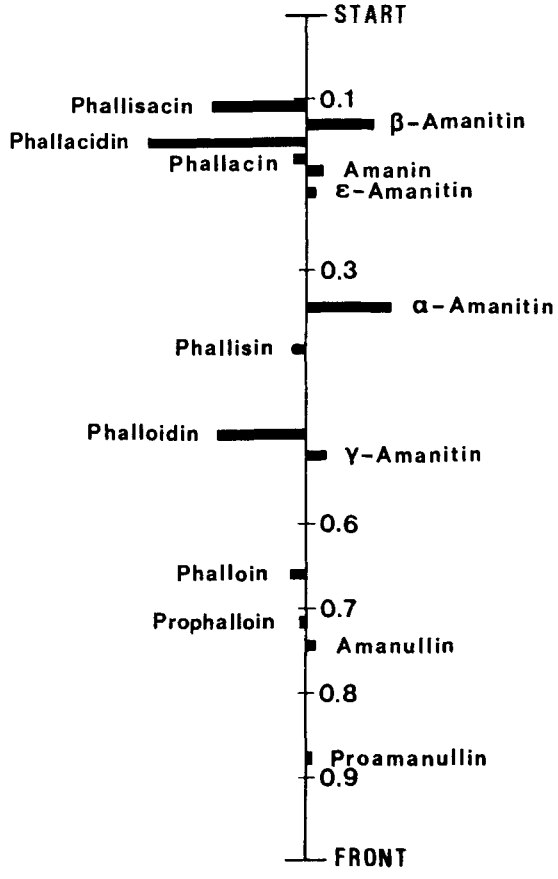


FIGURE 1. Diagrammatic representation of a descending paper chromatogram of *Amanita phalloides* toxins (R_f values) in butanone-acetone-water (30:3:5 vol). For clarity, the amatoxins (right) are arranged separately from the phallotoxins (left). The length of the bars indicates the relative amounts.

the toxins on cellulose thin-layer plates and also permitted a two-dimensional analysis with a second solvent.[36] Some hundreds of solvent mixtures have been tested on silica plates by Palyza in order to find a reliable and rapid method of detecting and estimating the toxins in biological fluids. He recommends a mixture of ethylenglycolmonobutylether per 25% ammonia (7:3)[37] and in one of his papers [38] gives an evaluation of all chromatographic methods used up to 1970. In the author's laboratory, neutral toxins on silica thin layers are usually developed with secondary butanol per ethylacetate per water (14:12:5) or chloroform per methanol per water (65:25:4). Optimum separation of acidic toxins was achieved with secondary butanol per 3% ammonia (100:44).

Several color reagents have been proposed for visualization of the toxin spots.[38] The reaction with cinnamic aldehyde in HCl vapor which yields violet and light-blue colors with amatoxins and phallotoxins, respectively,[39] is most commonly used for the detection of the toxins. In the same communication a spot test for amatoxins has also been described using old newspaper. A drop of the solution is brought onto the paper and contacted either with the vapor of fuming hydrochloric acid or, after drying, is spotted with approximately 8 *N* hydrochloric acid. A blue color will develop within several minutes indicating amatoxins in a concentration as low as 20 $\mu g/m\ell$. Since the concentration of the amatoxins in the mushrooms is ten to one hundred times that value, a discrimination of amanitin-containing mushrooms is feasible by this test. The reaction is most probably due to the lignin components of the paper. The color reaction with cinnamic aldehyde per HCl is, of course, also useful for the same purpose. This reagent allows the detection of amatoxins in amounts as low as 0.025 μg.[40] The color reaction of phallotoxins is about ten times less sensitive. Recently, croconic acid (1,2-dihydroxycyclopentene-trione) in HCl vapor was found to give a wine-red color with amatoxins and a yellow color with phallotoxins.[41]

2. *Quantitative Analysis*

a. Chromatography and Spectrophotometry

Isolation and quantitative analysis of up to 11 toxins in a methanolic extract from single mushrooms have been achieved by a combination of chromatographic procedures: after chromatography on a Sephadex® LH-20 column, the toxins were further purified on thin layer plates, eluted, and characterized spectrophotometrically.[22] Using this method, the toxin pattern of *A. phalloides* could be compared with those of *A. phalloides* var. *verna*, *A. virosa*, *A. bisporigera*, and *Galerina marginata*.[23] The method also proved useful in the detection of two new acidic phallotoxins in *A. phalloides* and a group of novel toxins in *A. virosa* (virotoxins). Recently, the toxins of the *Amanita* species of the northeastern U.S. have been analyzed using a similar method by Yocum and Simons.[42]

The chromatographic procedures described yielded pure toxins in microgram amounts. They could easily be quantitated by spectrophotometry using the molar extinction coefficients $\varepsilon_{310} = 13.500$ for all amatoxins and $\varepsilon_{300} = 10.100$ for phallotoxins (all compounds in aqueous solution). The amatoxins contain 3 or 4 mol of water of crystallization: α-amanitin, $C_{39}H_{54}N_{10}O_{14}S \cdot 4\ H_2O$, 990 mol wt; β-amanitin, $C_{39}H_{53}N_9O_{15}S \cdot 3\ H_2O$, 973 mol wt; and γ-amanitin, $C_{39}H_{54}N_{10}O_{13}S \cdot 4\ H_2O$, 974 mol wt. Neutral phallotoxins crystallize with 5 mol of water of crystallization:[1] phalloidin, $C_{35}H_{49}N_8O_{11}S \cdot 5\ H_2O$, 879 mol wt; phalloin, $C_{35}H_{49}N_8O_{10}S \cdot 5\ H_2O$, 863 mol wt; and phallisin, $C_{35}H_{49}N_8O_{12}S \cdot 5\ H_2O$, 895 mol wt. Acidic phallotoxins such as phallacidin, phallacin, and phallisacin have been obtained in lyophilized form only.

b. Bioassays

The most sensitive assays for amatoxins were established by making use of either their inhibition of RNA polymerase or by binding to immunoglobulins in a radioimmunoassay. With a lower sensitivity, phallotoxins could be determined using muscle actin as a binding protein. None of the bioassays permitted discrimination between the different members of a toxin family. On the other hand, the sum of amatoxins or phallotoxins could be determined with high specificity for there is no cross reaction of amatoxins with actin or of phallotoxins with RNA polymerase or amanitin-binding immunoglobulins. Inhibition of RNA polymerase activity by phalloidin with doses 100 times that of amatox-

ins reported earlier had probably been occasioned by small amounts of amatoxins present as impurities in phalloidin.[43]

Inhibition of RNA polymerase B has been used for a quantitative estimation of amatoxins in *A. verna* by Preston et al.[44] and in *Galerina autumnalis* by Johnson et al.,[45] the limit of detection being around 1 ng/mℓ. Operating with a similar sensitivity, the assay of inhibition of RNA polymerase was useful in our studies[15] in detecting amatoxins in several species of carpovores generally considered as nontoxic or even edible.

Antibodies against amatoxins have been raised by Fiume et al.[46] and in our laboratory.[47] Fiume made use of the high resistance of the rat to amatoxins, including the macromolecular derivatives of β-amanitin (β-Ama-BSA). Our antigen was β-Ama-BSA cross-linked and partially precipitated with glutaraldehyde, which was tolerated to some extent by rabbits. The concentration of amanitin-binding globulins produced in both animal species remained low, about 20 μg/mℓ. Both assays had a limit of detection of 0.5 ng amatoxins per milliliter. It made no difference whether the free amatoxins were separated by adsorption onto charcoal,[47] or by precipitation of the globulin-bound toxin by ammoniumsulfate.[46] The assay using charcoal is disturbed by components present in biological fluids like serum, urine, or mushroom sap, which lower the sensitivity of the assay by a factor of 50. Biological samples containing amatoxins should preferably be purified before the assay.

For the assay of phallotoxins, the charcoal technique was successfully applied to a system containing 3×10^{-7} *M* rabbit-muscle actin (in 0.1 *M* Kcl) as the binding protein. The limit of detection was 160 ng of phallotoxin per milliliter of aqueous solution.[48] In this assay, the sensitivity is limited by the dissociation of F-actin polymers into monomers in highly diluted solutions.

III. CHEMISTRY OF THE TOXINS

Chemical work on the *Amanita* toxins has consisted of the elucidation of the structures of the amatoxins as well as of the phallotoxins; chemical modifications of the molecules, including labeling with radioactive isotopes; conjugation of the toxins to molecules of high molecular weight; and attempts to synthesize the natural products and artificial analogs. Exploration of the structures of all the toxic peptides in *Amanita* phalloides and also of the antitoxic peptide antamanide can be regarded as almost finished; several compilations have been given.[5-10] For separate reviews of amatoxins, also see References 49 and 50; for phallotoxins, Reference 51; and for antamanide, Reference 52. The structures of the phallotoxin-like acting components of *Amanita* virosa (virotoxins) are still under investigation.

A. Chemistry of the Amatoxins

*1. Naturally Occurring Amatoxins**

The family of amatoxins now consists of eight defined members, and in addition, two esters of β-amanitin have been isolated.[27] The formulas of the amatoxins identified are represented in Table 2 with their respective toxicities and inhibitory capacities towards RNA polymerase B (or II).

With one exception, all the amatoxins inhibit RNA polymerase B (or II) in minimal concentrations. Only proamanullin (A7)[27] shows a 500 times lower inhibitory capacity. The lack of affinity for RNA polymerase B (or II) is accompanied by loss of in vivo toxicity in A7. Since the CD-spectrum of A7 (Figure 2) suggests a conformation identical to that of the toxic amatoxins, the lack of the hydroxyl group of hydroxyproline, not a different molecular shape, must be responsible for the weak affinity of proamanullin to RNA polymerase B.

As for amanullin (A5), its slightly decreased affinity for the enzyme cannot sufficiently explain its loss of in vivo toxicity. The rapid dissociation of the amanullin-enzyme complex is able to account for the nontoxicity.[53]

2. Chemical Derivatives and Radioactive Labeling: Synthetic Approaches

Affinity for the polymerase and the consequential toxicity can be chemically modified as follows:

1. Monocyclic derivatives of amatoxins have

* Numbering of the amino acid residues in the amatoxins will from now on start from the alphabetically first residue (Asn = No. 1). This corresponds to the numbering in the phallotoxins, but may differ from numbering in previous papers.

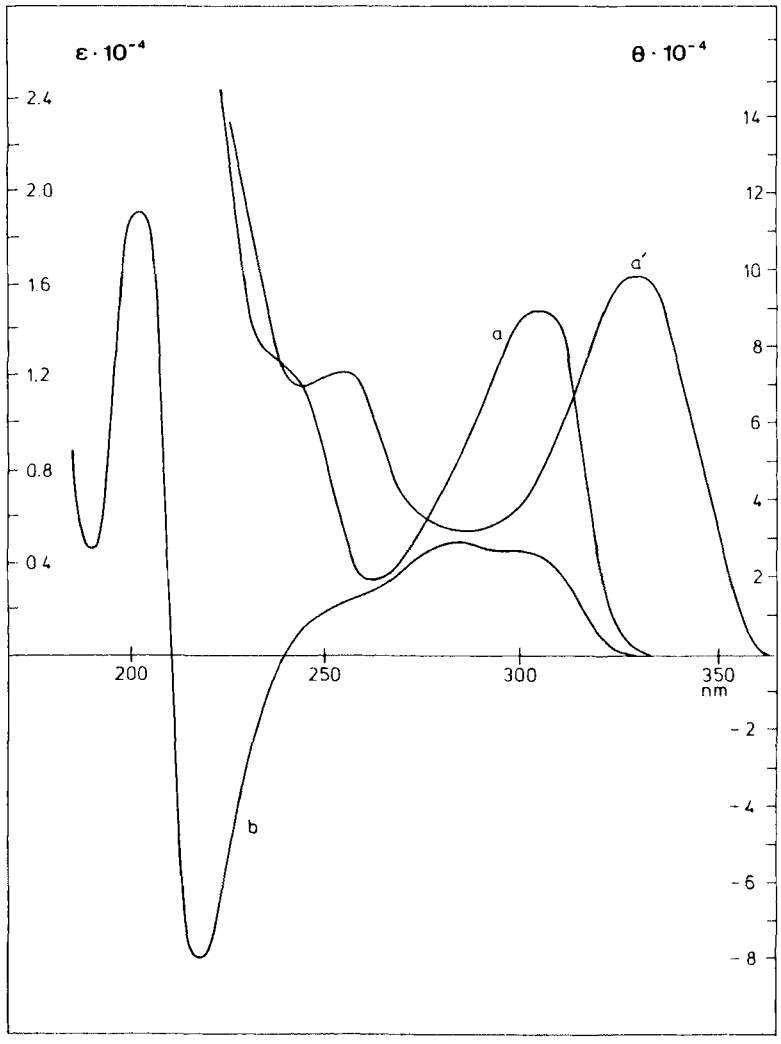


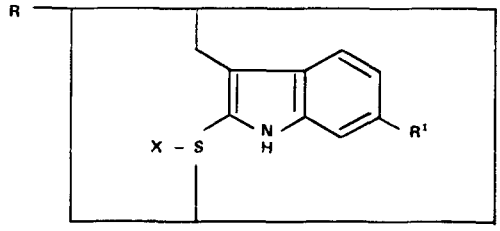
FIGURE 2. Ultraviolet absorption spectra of α− (and β−, γ−) amanitin in water at pH 7 (a) and pH 11 (a') and the circular dichroism spectrum at pH 7 (b).

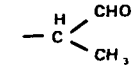
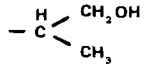
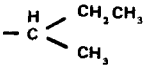
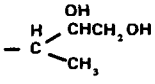
no toxicity. The peptide bond between residues 3 and 4 can be specifically cleaved by the action of trifluoroacetic acid at room temperature due to the proximity effect of the hydroxyl group in γ-position (lactone formation). The resulting seco-compounds are nontoxic.

2. Shortening of the side chain of residue 3 alters the affinity for the enzyme. Although replacement of the δ-positioned hydroxyl group of α-amanitin (A1) by hydrogen (γ-amanitin, A3) has little influence, oxidation of 6′-methoxy-α-amanitin (toxic) by periodate leading to an aldehyde group (A9) dramatically reduces the affinity for the enzyme and abolishes toxicity. The formation of the aldehyde group is associated with a change in the conformation of the molecule (CD spectrum),[54] which causes a 100 times lower affinity for the enzyme. Reduction of O-methylaldoamanitin to O-methyldesmethyl-λ-amanitin (A10) restores the conformation together with a good deal of affinity and toxicity.[55] As with O-methylaldoamanitin, a synthetic analog with a side chain of only three carbon atoms, (A11), has a 500 times lower affinity and is completely nontoxic (Table 3). The constants of inhibition and dissociation and dissociation rates of some amatoxins varying in the side chain at position 3 are discussed under Section VI.A.4.a.

TABLE 3

Chemically Modified Amatoxins, Their Inhibition Constants (M) for RNA Polymerase B (or II)(Approximative K_i Values), and Their LD_{50} Values (mg/kg for the White Mouse)



No.	R	X	R′	Compound	K_i (M)	LD_{50}
A9		(R) O	OCH_3	O-Methylaldoamanitine	10^{-6}	—
A10		(R) O	OCH_3	**O-Methyl-dehydroxymethyl-α-amanitin**	$2 \cdot 10^{-8}$	3.0
A11	$-CH_2-CH_2OH$	—	H	Dinor-S-deoxo-amanin amide	$5 \cdot 10^{-5}$	—
A12		—	H	6′-Dehydroxy-S-deoxoamanullin	10^{-7}	—
A13 (*R*)	Same	(*R*) O	H	(*R*)-6′-Dehydroxyamanullin	10^{-7}	—
A13 (*S*)	Same	(*S*) O	H	(*S*)-6′-Dehydroxyamanullin	10^{-5}—10^{-4}	—
A14			OCH_3	**O-Methyl-S-deoxo-α-amanitin**	10^{-6}	0.5—1
A15 (*R*)	Same	(*R*) O	OCH_3	**O-Methyl-α-amanitin (*R*)-sulfoxide**	10^{-6}	0.5
A15 (*S*)	Same	(*S*) O	OCH_3	**O-Methyl-α-amanitin (*S*)-sulfoxide**	10^{-7}	**III>10**
A16 to 21	Same	(*R*) O	**OX**	**Various 6′-ethers of α-amanitin with X = ethyl, propyl, + hexyl, ω-aminohexyl, 2.4-dinitrophenyl, 1-phenyl-5-tetrazolyl**	10^{-8}	0.5—1.0 A21 nontoxic
A22	**Same**	**(*R*)O**		**6′-Ether of β-amanitin with X = 1-phenyl-S-tetrazolyl**		

3. Oxygen at the sulfur bridge affects the biological activity of the amatoxins in a way similar to that in phallotoxins.[56] 6′-Methoxy-α-amanitin (A15 [*R*], Table 3) was deoxygenated by treating with Raney nickel.[57] The equally toxic 6′-methoxy-S-deoxo-α-amanitin (A14) could be reoxidized by peracetic acid to yield the two expected diastereomeric sulfoxides A15 (*R*) and A15 (*S*). The (*R*)-isomer of A15 was identical to the starting material. The (*S*)-isomer showed no toxicity up to a dose of 10 mg/kg and had a K_i ten times smaller than that of the (*R*)-form.[58] The (*R*) and (*S*) forms also differed widely in their CD spectra.[58] On further oxidation, both diastereomers yielded the same sulfone ($-SO_2-$), which had the same toxicity as the starting material. In summary, for maintaining toxicity of amatoxins, the bridging sulfur atom can either be free or oxidized to the (*R*)-sulfoxide or the sulfone. Oxygen in (*S*)-configuration yields a sulfoxide which is nontoxic due to a changed conformation.
4. The carboxylic group of amatoxins may be free (A2, A4) or amidated (A1, A3). Esterification with diazomethane forming a methylester does not influence toxicity.
5. The phenolic hydroxy group in the 6′-position is not essential for toxicity as shown by the naturally occurring amanin (A8). Replacement of OH by hydrogen could be achieved by catalytic hydrogenation of the toxic 1-phenyl-5-tetrazolylether of α-amanitin.[59] The formation of various ethers at the phenolic hydroxyl group in 6′-position generally has little influence on toxicity and binding to RNA polymerase B (or II).[60] Table 3 also includes some 6′-alkoxy and 6′-aryloxy compounds synthesized in our laboratory (A16 to A19).

Radioactive labels have been incorporated into the amatoxins to date in the form of four different compounds:

1. 6′-O-[^{14}C] (or ^{3}H) methyl-α-amanitin[61] was obtained by reacting the sodium salt of α-amanitin, obtained from α-amanitin and sodium methylate in dimethylformamide, with labeled methyliodide.
2. (1)-γ-[^{3}H] dehydroxymethyl-6′-O-methyl-α-amanitin.* This is obtained by reduction of 6′-O-methylaldoamanitin (A9, Table 3) in methanolic solution with [^{3}H] $NaBH_4$.
3. 6′-[^{3}H]-amanin (A8-^{3}H). Catalytic [^{3}H]-hydrogenation of 6′-(1-phenyl-5-tetrazolyloxy)-β-amanitin (A22) gave the 6′-tritiated amanin.[62] The compound was used for covalently labeling the amatoxin-binding site of calf thymus RNA polymerase B (or II) as described under Section VI.A.4.6.
4. Most recently, Morris et al.[392] reported the [^{125}I]-iodination of α-amanitin. The compound was characterized as 7′-iodo-α-amanitin by proton magnetic resonance.

a. Synthetic Approaches

Also included in Table 3 are four totally synthetic amatoxin-like compounds (A11, A12, A13 [*S*], and A13 [*R*].[63] The bicyclic skeleton of the natural amatoxins has been synthesized in all of them. One of the compounds, dinor-S-deoxoamanin-amide (A11), lacks the 6′OH-group as well as the sulfoxide oxygen, both being nonessential for biological activity. Thus, the lack of the two carbon atoms in the side chain of residue 3 is responsible for the low affinity of A11 to RNA polymerase B (or II)($K_i = 5 \times 10^{-5}$ *M*); again, this demonstrates the importance of a minimal number of carbon atoms in the side chain of position 3. The other products of synthesis were 6′-dehydroxy-S-deoxoamanullin (A12) and its two sulfoxides A13 (*R*), corresponding to natural A5 and the nonnatural isomer A13 (*S*). The only difference between A13 (*R*) and A5 is the lack of the OH group in the 6′ position. Indeed, A13 (*R*) has a K_i comparable with that of A5. It is also comparable with the mother compound A12.

3. Chiroptical Data

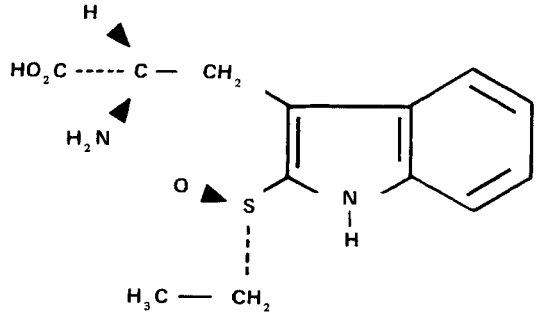
Measurement of optical rotatory dispersion (ORD) and of circular dichroism (CD) has been very useful in structural and synthetic work in the amatoxin field.[54] The CD spectrum of α-amanitin together with its UV spectrum is shown in Figure 2.

In water, all biologically active amatoxins such as α-, β-, and γ-amanitine, as well as the nontoxic amanullin, exhibit almost identical CD spectra. At least three positive Cotton ef-

* New nomenclature; former name: {^{3}H}-O-methyl-demethyl-γ-amanitin.

fects, at 305, 285, and 255 nm, and one negative Cotton effect at about 232 nm originate from the aromatic part of the molecule, the 2-sulfoxido-6-hydroxy-indole moiety. A strongly negative Cotton effect at 220 nm and another positive one at 205 nm are attributed to amide chromophores. However, the nontoxic O-methyl-aldo-amanitin (A9) lacks the negative Cotton effect at 220 nm and shows higher ellipticity at 205 nm. This points to a conformational difference possibly caused by the hydrogen bond accepting property of the aldehyde group. The CD spectrum of the nontoxic (*S*)-sulfoxide A15 (*S*) differs fundamentally from that of the toxic one by showing a negative Cotton effect centered around 305 nm and a second, strongly negative one at about 250 nm, clearly indicating a basic change in the molecular structure. According to the results from CD, the nontoxicity of amanullin is not due to a changed conformation. Though less strongly, the compound is able to bind to RNA polymerase B (or II), as do the toxic amapeptides (Table 2). Attempts at understanding its lack of toxicity have considered the dissociation rate constants of the toxin · enzyme complex.

The ORD curves of amanin, a natural sulfoxide of the amatoxin series (A8, Table 2), and of an acetylderivative of γ-amanitin exhibit positive Cotton effects around 300 nm (Figure 3, Curves b and c). They closely resemble the ORD curve of the toxic diastereomeric sulfoxide (Curve a) obtained by oxidation of phalloidin (P21, Table 5). All these curves are very similar to the ORD curve of a synthetically prepared sulfoxide of 2-ethylthio-L-tryptophan, whose structure was proven by X-ray analysis to be the (*R*)-diastereomer (Structure 1).[64] This allowed the assignment of (*R*)-configuration to



STRUCTURE 1

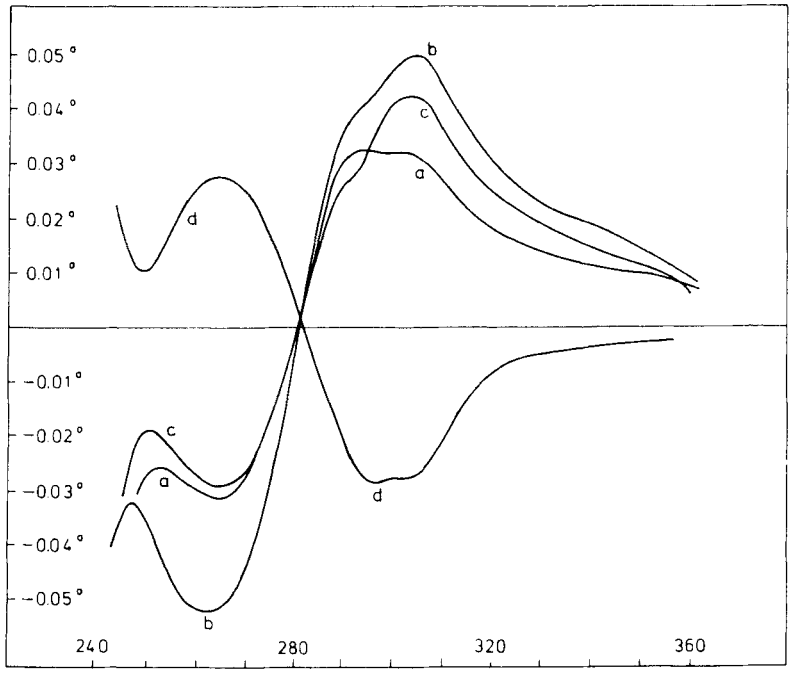


FIGURE 3. Optical rotatory dispersion curves of (a) the toxic phalloidin sulfoxide P 21 (*R*), (b) amanin A 8, (c) 6′-O-acetyl-λ-amanitin, and (d) of the nontoxic phalloidin sulfoxide P 21 (*S*).

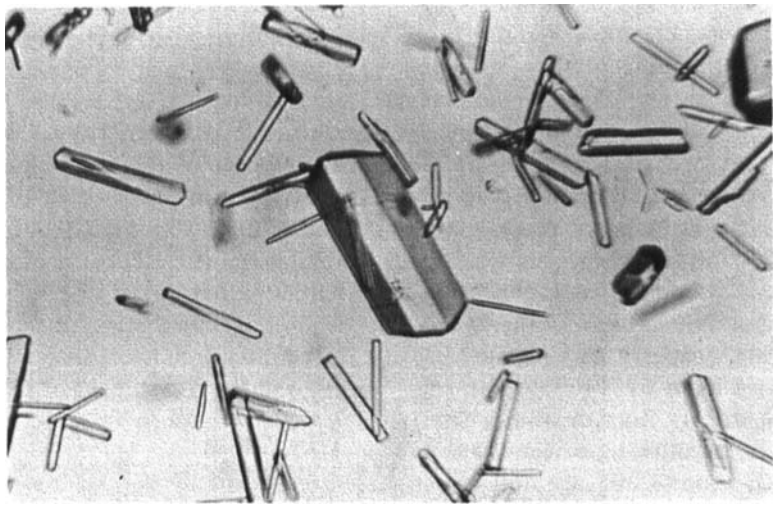
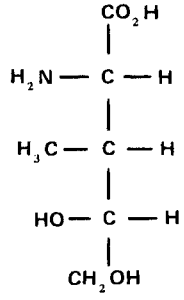


FIGURE 4. Crystals of β-amanitin obtained from ethanol. (Courtesy of R. R. Yocum.)

the amatoxins and to the toxic sulfoxide of phalloidin. X-Ray analysis was also used for the structural analysis of the lactone-hydrobromide of the hydroxylated isoleucine[65] of position 3 of α-amanitin, which is (2*S*, 3*R*, 4*R*)-2-amino-3-methyl-4,5-dihydroxyvaleric acid (Structure 2).



STRUCTURE 2

Both of these stereochemical properties have been confirmed together with all other molecular features elucidated, by a recent X-ray structural analysis of β-amanitin (Figure 4) from the laboratory of Lipscomb et al.[66] (Figure 5). The recent structure analysis of β-amanitin in crystals was used to decide a few ambiguities in the nuclear magnetic resonance studies of α-amanitin dissolved in dimethylsulfoxide. According to this work by Tonelli et al.,[393] the conformations of amatoxins are very similar in the solid state and in solution.

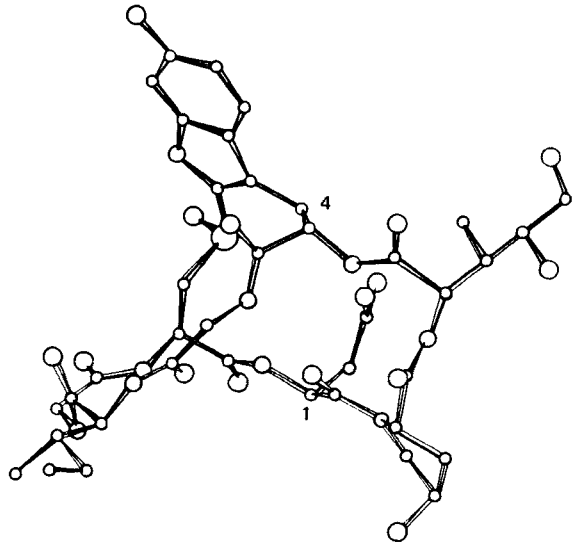


FIGURE 5. Structure of β-amanitin as determined by X-ray analysis.[66]

4. Conjugation of Amatoxins to High Molecular Weight Compounds

As early as 1959, β-amanitin was conjugated to bovine serum albumin after activation of the acidic toxin as a thiophenylester.[67] In 1969, Cessi and Fiume[68, 69] used the carbodiimide method for coupling the toxin to the protein. Recently in our laboratory, amatoxins have been conjugated to macromolecular compounds, either by an azolinkage[70] or as amides via *N*-hydroxysuccinimidesters of β-amanitin.

The carrier molecules included albumin, γ-globulins, polylysine, and dextranes.[71] On the average, one to three toxin molecules could be attached per protein molecule.

Unexpectedly, the protein-conjugated amatoxins were more toxic than β-amanitin itself.[68,72] Toxicity was increased six to ten times, depending on the coupling method. Additional coupling of a β-amanitin-albumin conjugate with fluorescein-isothiocyanate increased the toxicity three to four times further.[73] Generally, the conjugates obtained with carbodiimide were the most toxic ones, because by this reaction, 6′-amatoxinesters were also formed with the protein, which can be cleaved inside the cell to release the free toxin. Furthermore, carbodiimides can effect cross-linking of proteins, therefore increasing their molecular weight. In contrast to this, activation of the carboxyl group of β-amanitin forms exclusively amide bonds with the protein. Amatoxins conjugated to albumin still inhibit RNA polymerase B (or II) although in many-fold higher concentrations than in the unbound state.[73]

B. Chemistry of the Phallotoxins

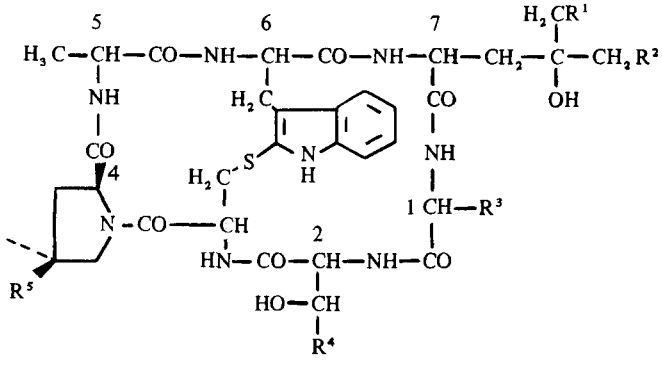
1. Naturally Occurring Phallotoxins

The family of phallotoxins is now comprised of seven defined members. Since the last review,[10] in addition to the four known phallotoxins, three further components have been discovered in the mushrooms: phallacin (P5), phallisacin (P7),[41] and prophalloin (P3).[74] The formulas of the identified phallotoxins are quoted in Table 4 together with their LD_{50} values for the white mouse. All the natural phallotoxins are toxic with LD_{50} values ranging from 1.5 to 4.5 mg/kg, except prophalloin (P3), which is nonlethal even in doses up to 100 mg/kg body weight. The nontoxicity of P3 is consistent with a previous observation on a synthetically prepared Pro^5-analog of norphalloin (P16), which also showed no toxicity due to the absence of the *allo*-positioned OH group at the pyrrolidine ring. All natural toxins exhibit typical ORD[75] and CD spectra in water, as reproduced in Figure 6. This suggests a similar conformation for all toxins, including the nontoxic compounds (P3).

A molecular model of phalloin obtained by

TABLE 4

Naturally Occurring Phallotoxins, Their Toxicity (LD_{50}, mg/kg for the White Mouse, i.p.) and Their Relative Affinity (A) to Rabbit Muscle Actin (phalloidin = 1)



No.	Name	R^1	R^2	R^3	R^4	R^5	LD_{50}	A_{actin}
P1	Phalloidin[a]	OH	H	CH_3	CH_3	a-OH	2.0	1.00
P2	Phalloin	H	H	CH_3	CH_3	a-OH	1.5	0.74
P3	Prophalloin	H	H	CH_3	CH_3	H	>100	0.01
P4	Phallisin	OH	OH	CH_3	CH_3	a-OH	2.5	0.55
P5	Phallacin	H	H	$CH(CH_3)_2$	CO_2H	a-OH	1.5	0.44
P6	Phallacidin	OH	H	$CH(CH_3)_2$	CO_2H	a-OH	1.5	0.91
P7	Phallisacin	OH	OH	$CH(CH_3)_2$	CO_2H	a-OH	4.5	0.58

[a] Systemic name: cyclic (L-Alanyl-D-threonyl-L-cysteinyl-*cis*-4-hydroxy-L-prolyl-L-alanyl-2-mercapto-L-tryptophyl-4,5-dihydroxyleucyl) cyclic (3 → 6)-sulfide.

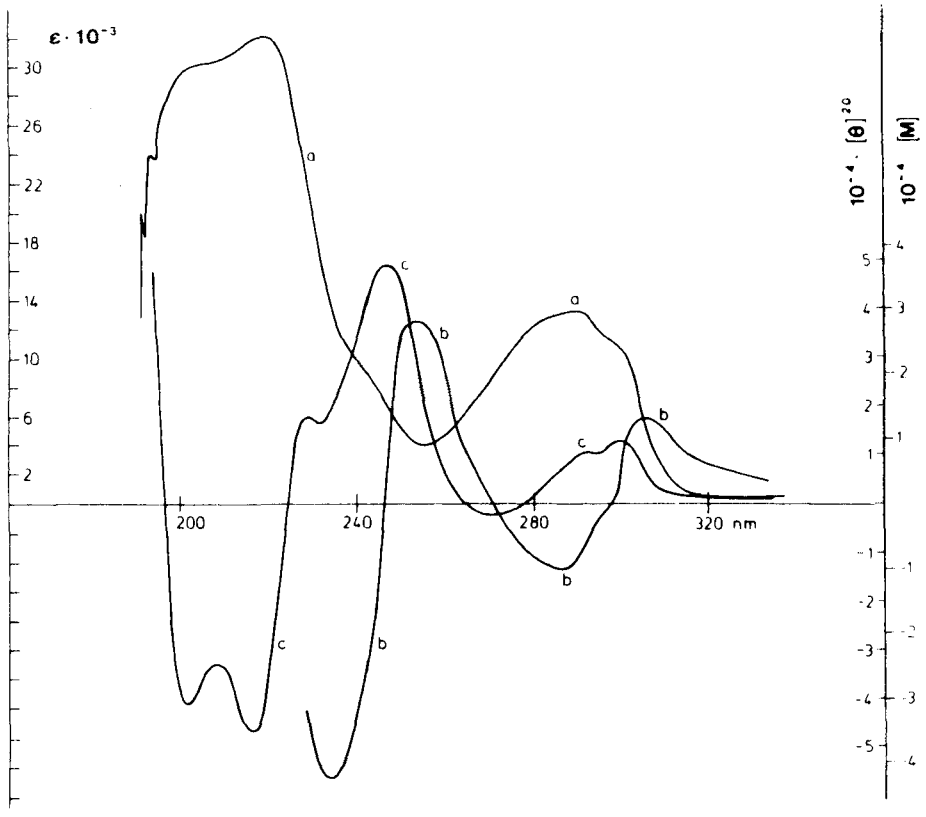


FIGURE 6. Ultraviolet absorption spectrum (ε) × 10^{-3} (a), optical rotatory dispersion spectrum (M) × 10^{-4} (b), and circular dichroism spectrum (θ)^{20} × 10^{-4} (c) of phalloidin in water.

^{1}H nuclear magnetic resonance analysis and from approximate potential energy calculations by Patel et al.[76] is shown in Figure 7. This model agrees rather well with the suggestion that R^5, CH_3 and the whole indole part form the binding sites of phallotoxins to actin.

Table 4 also informs us that the two δ-OH groups in residue number 1 are not essential for the toxicity, that the alkyl group R^3 in number 1 can be methyl or isopropyl, and that methyl in residue 2 is replaceable by carboxyl without affecting the toxicity of the molecule (R^4). Further information on the structure-activity relationship has been obtained from derivatives obtained both via chemical reactions and total synthesis.

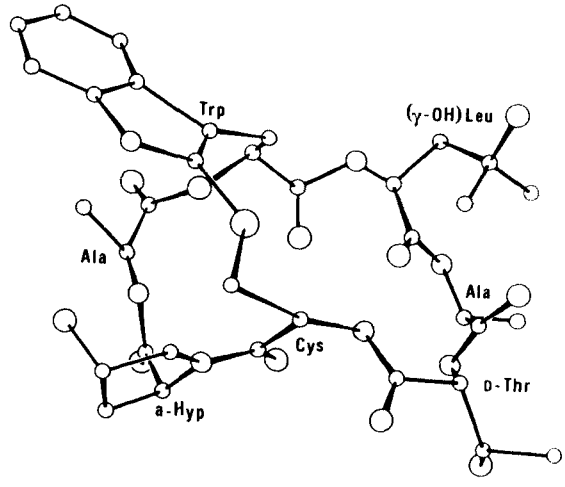


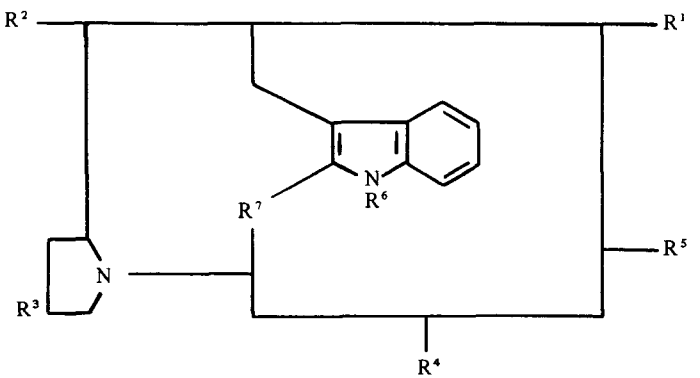
FIGURE 7. Molecular model of phalloidin as derived from nuclear magnetic resonance data and minimum energy calculations.[76] Helicity of indolylthioether moiety modified according to NMR results of J. Dabrowski (unpublished).

2. Derivatives of Phallotoxins

The derivatives in Table 5 have been obtained by transformation of natural phallotoxins or by synthesis.[10] In phallotoxins, as with amatoxins, opening one ring of the bicyclic system removes the toxicity. Cleavage of the weakest peptide bond between residue 7 and 1 (lactone formation) or elimination of the sulfur bridge by hydrogenation with Raney nickel leads to monocyclic secophallotoxins or dethiophallotoxins,

TABLE 5

Chemically Modified Phallotoxins: Their Structure, Toxicity (LD_{50}, mg/kg for the White Mouse), and Relative Affinities (A) for Rabbit Muscle Actin (phalloidin = 1)



No.	Compound	R¹	R²	R³	R⁴	R⁵	R⁶	R⁷	LD_{50}	A_{actin}
P8	Ketophalloidin	CH_2-CO-CH_3	CH_3	a-OH	CH(OH)CH_3	CH_3	H	R⁷	LD_{50}	A_{actin}
P9	Demethylphalloin	CH_2-CH(OH)CH_3	CH_3	a-OH	CH(OH)CH_3	CH_3	H			
P10	Norphalloin	CH_2-CH_2-CH_3	CH_3	a-OH	CH(OH)CH_3	CH_3	H	S	2.0	0.34
P11	Leu⁷-phalloidin	CH_2-$CH(CH_3)_2$	CH_3	a-OH	CH(OH)CH_3	CH_3	H	S	2.0	0.64
P12	Gly⁴-norphalloin	C_2H_5	H	a-OH	CH(OH)CH_3	CH_3	H	S	1.5	0.60
								S	2.0	0.40
P13	Leu¹-phalloidin	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	CH(OH)CH_3	CH_2-$CH(CH_3)_2$	H	S	>26.0	<0.01
P14	Phe¹-phalloidin	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	CH(OH)CH_3	$CH_2C_6H_5$	H	S	2.0	0.28
P15	Gly¹-phalloidin	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	CH(OH)CH_3	H	H	S	>20.0	<0.01
P16	Pro⁴-norphalloin	C_2H_5	CH_3	H	CH(OH)CH_3	CH_3	H	S	7.5	0.02
P17	D-Abu²-norphalloin	C_2H_5	CH_3	a-OH	CH_2CH_3	CH_3	H			
								S	>20.0	—
P18	Phallacidinamide	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CONH_2$	$CH(CH_3)_2$	H	S	3—4	—
P19	Phallacidinmethylamide	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	CONHCH_3	$CH(CH_3)_2$	H	S	3—4	—
P20	Phallacidindimethylamide	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CON(CH_3)_2$	$CH(CH_3)_2$	H	S	6.5	0.64
P21	Phalloidinsulfoxide (*S*)	$CH_2C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	CH(OH)CH_3	CH_3	H	S	9.0	0.21
(*S*)								(*S*) SO	>20	<0.01

TABLE 5 (continued)

Chemically Modified Phallotoxins: Their Structure, Toxicity (LD_{50}, mg/kg for the White Mouse), and Relative Affinities (A) for Rabbit Muscle Actin (phalloidin = 1)

No.	Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7	LD_{50}	A Actin
P21 (*R*)	Phalloidinsulfoxide (*R*)	$CH_3C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CH(OH)CH_3$	CH_3	H	(*R*) SO	2.5	0.10
P22	*Phalloidinsulfone*	$CH_3C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CH(OH)CH_3$	CH_3	H	SO_2	2.5	0.33
P23	N^{im}-methyl phalloidin	$CH_3C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CH(OH)CH_3$	CH_3	CH_3	S	2.5	—
P24	N^{im}-ethyl phalloidin	$CH_3C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CH(OH)CH_3$	CH_3	CH_2CH_3	S	10.0	0.22
P25	N^{im}-carbamoylmethylphalloidin	$CH_3C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CH(OH)CH_3$	CH_3	CH_2-CO NH_2	S	7.0	0.06
P26	N^{im}-propyl phalloidin	$CH_3C(OH)(CH_3)$-CH_2OH	CH_3	a-OH	$CH(OH)CH_3$	CH_3	CH_2-CH_2-CH_3	S	>20.0	<0.01
P27	Triacetylphalloidin	$CH_3C(OH)(CH_3)$-CH_2-OAc	CH_3	a-OAc	$CH(OAc)CH_3$	CH_3	H	S	>20.0	<0.01

respectively. Both derivatives are nontoxic by conformational changes as indicated by their CD spectra (Figure 8).

The contributions to the toxicity of the different side chains in the intact molecule and of other features introduced by synthesis are summarized in Table 5. The important residues have been numbered R^1 to R^7 in the schematic formula.

R^1 — Shortening of the side chain of residue 7 does not alter the affinity. Oxidation of phalloidin (P1) by KIO_4 leads to a ketone (ketophalloidin, P8) of equal toxicity; reduction of P8 by sodium borohydride yields demethylphalloin (P9), which also has the same toxicity. P8 was the starting material for several transformations, among others, norphalloin (P10), a phallotoxin with a norvaline side chain.[77] Without any oxygen at the side chain of 7, this compound turned out to be even a little more toxic than P1. Similarly, Leu^7-phalloidin (P11) has full toxicity.[78]

R^2 — The methyl group in position 5 is essential for toxicity; Gly^5-norphalloin (P12) is not toxic.[79]

R^3 — The *allo*-positioned hydroxy group (*cis* to carboxyl) of residue number 4 is also essential for toxicity: Pro^4-norphalloin (P16) and Hyp^4-norphalloin with translocated OH are not toxic.[79] This is in line with the lack of toxicity in naturally occurring prophalloin (P3) and in the synthetic triacetyl-phalloidin (P27).[75]

R^4 — The hydroxy group of the D-threonine residue makes only a small contribution to biological activity; the analog with D-α-aminobutyric acid instead of D-Thr (P17) is toxic.[80] The methyl group in R^4 may also be replaced, e.g., by carboxyl (P5 to 7), without loss of toxicity of the molecule. However, voluminous residues at the carboxylic group lower toxicity: phallacidin- amide (P18), - methylamide (P19), and - dimethylamide (P20) exhibit decreasing toxicities.[41]

R^5 — From the various natural compounds, we learn that R^5 can consist of a methyl group or an isopropyl group (P5 to 7). Synthetically obtained Leu^1-phalloidin (P13) was equally toxic;[81] however, Phe^1-phalloidin (P14), with a more bulky side chain, had very little toxicity if any. Gly^1-phalloidin (P15) had a reduced toxicity.

R^6 — The hydrogen atom at the indole nitrogen may be substituted only by a chain of two carbon atoms; otherwise toxicity will be lost. N^{ind}-Methylphalloidin (P23) is as toxic as the parent compound, whereas the N^{ind}-ethyl (P24) and N^{ind}-carbamoylmethyl compounds (P25) exhibit a decreased toxicity. N^{ind}-Propylphalloidin (P26) is nontoxic as a result of a changed conformation.[75]

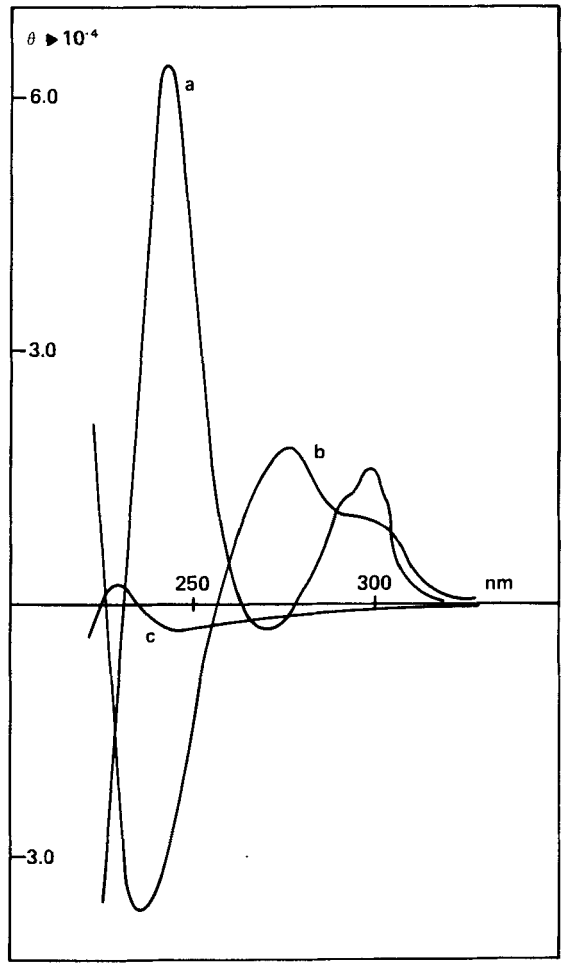


FIGURE 8. Circular dichroism spectra of phalloidin (a), seco-phalloidin (b), and dethiophalloidin (c) in water.

R^7 — Two sulfoxides of phalloidin have been obtained by oxidation of the thioether by peracetic acid.[56] The diastereomers separated by chromatography differed strikingly. The (*S*)-diastereomer of the sulfoxides (P21 [*S*]) is non toxic, whereas the (*R*)-diastereomer (P21 [*R*]) is as toxic as phalloidin. The ORD spectra of the two diastereomers show inverse curves (Figure 3, Curves a and d), thus indicating the SO group as an inherently dissymmetric chromop-

hore. The absolute configuration was found by comparing their chiroptical behavior with a model sulfoxide of tryphophan, whose structure has been analyzed by X-ray diffraction.[64] On further oxidation, both sulfoxides yielded the identical sulfone (P22), which has full toxicity. As with the amatoxins, in a toxic phallopeptide, the sulfur atom is either a sulfide, a sulfoxide with (*R*) configuration, or a sulfone. The sulfoxide with (*S*)-configuration is nontoxic.

3. Synthesis of Phallotoxins, Radioactive Labeling

Proof of the correct formula of the phallotoxins was first obtained by the synthesis of norphalloin (P10).[82] Several analogs have since been prepared using corresponding synthetic pathways.[79,80] Most recently, the total synthesis of phalloin (P2) was achieved by Munekata et al.;[78] by a similar synthetic technique, Leu[7] phalloidin (P11) has also been obtained.

Besides the laborious *de novo* synthesis of toxin derivatives, a second route to analogs substituted in position 1 has been worked out. Secophalloidin, the product of the acidic cleavage of phalloidin (P1) at the peptide bond between γ, δ-dihydroxy-leucine and 1-alanine, can be recyclized yielding the toxin by the mixed anhydride method,[83] The seco-compound was subjected to an Edman degradation procedure, which shortened the peptide chain by the 1-alanine. Glycine, L-leucine, or L-phenylalanine was introduced at the terminal amino group of the new secocompound. On cyclization, the modified seco-compounds yielded the phallotoxins P13, P14, and P15.[81]

a. Introduction of Radioactive Atoms

For radioactive labeling, one of the methods described below is used starting either from natural toxins or derivatives of natural toxins.

1. A ^{35}S-containing dithiolane was formed from ketophalloidin (P8)(Structure 3).[84]
2. Hydrogenation of ketophalloidin (P8) by ^{3}H-containing $NaBH_4$ yielded [^{3}H] demethylphalloin (P9).[85]
3. Methylation of the indole-*N* of phalloidin by ^{14}C-methyliodide resulted in N^{ind}-^{14}C-methylphalloidin.[75]

STRUCTURE 3

All labeled toxins had a toxicity similar to that of phalloidin.

4. Conjugation of Phallotoxins to Bovine Serum Albumin (and to Sepharose)

Phallacidin (P6) was covalently bound to bovine serum albumin by its carboxylic group to give a conjugate in which up to five molecules of the toxin were bound to one molecule of protein, mainly by amide bonds between carboxyl and ε-amino groups of lysine residues.[86] Another procedure, coupling of phalloidin (P1) to bovine serum albumin by means of a water-soluble carbodiimide, gave a conjugate with a ratio of 2.3 equivalents of toxin per protein in which the toxin, lacking a free carboxylic group, was bound by esterification of its hydroxyl groups with carboxyls of the protein.[87] The latter kind of covalent bond is more prone to hydrolysis leading to the occurrence of free phalloidin inside cells after phagocytosis.

Biological activity of phallotoxins bound to albumin can only be expected after release of the toxin inside the cell since phallotoxins attached to albumin bind to muscle actin only slightly, if at all.

IV. ANTAMANIDE

Antamanide[88] is a cyclic decapeptide isolated in our laboratory from *Amanita phalloides* mushrooms by tracing back the antagonistic effect against phalloidin of one lipophilic fraction of the mushroom extracts.[89] Use of 0.5 to 1.0 mg of the crystalline substance prevents death in white mice from the lethal doses of 5 mg phalloidin per kilogram of body weight.

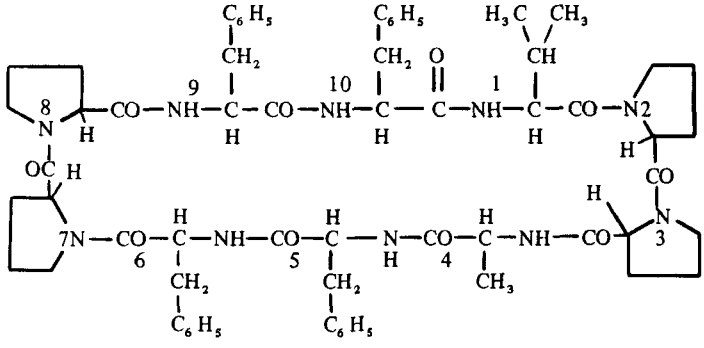
Administration must be made intravenously, intraperitoneally, or subcutaneously; there is no effect following a peroral application. The rapid excretion of antamanide with the bile[90] requires that antamanide not be administered earlier than 2 hr before application of phalloidin. Highest activity is achieved by administration immediately before or together with the toxin. A small protective effect was observed by application up to 10 min after poisoning, which disappeared, however, when antamanide was administered any later than 10 min after the toxin.

A. Biological Action

Concerning the mechanism of the antitoxic activity, it is clear that antamanide does not compete with the phallotoxins for one and the same receptor site. This was ruled out by the following experiments. High doses of phalloidin applied prior to a [^{14}C]-labeled derivative of antamanide did not reduce the amount of antamanide incorporated into a mouse liver.[90] However, in reverse, the uptake of phalloidin in rat livers was distinctly reduced, in vivo as well as during perfusion, if antamanide was present. Therefore, we must assume that antamanide strengthens some unknown membrane structure against the attack by phalloidin. A similar retarding effect of antamanide has been observed on the uptake of dyestuffs by the perfused liver, as described by Jahn.[91] For example, mice were injected i.p. with 5 mg of antamanide per kilogram of body weight 10 to 20 min prior to i.v. application of 20 mg/kg indocyanine green (a) or 50 mg/kg bromsulphalein (b). In the antamanide-treated animals, the uptake and thereafter the elimination of the dyes was heavily retarded as followed by their concentration in the serum: (a) 55 ± 9 ng/mℓ (control 20 ± 2) and (b) 113 ± 30 ng/mℓ (control < 10).

B. Structure and Chemical Synthesis of Antamanide and of Several Analogs

The cyclic decapeptide antamanide contains the four L-amino acids alanine, valine, proline, and phenylalanine in a ratio of 1:1:4:4. They are arranged as in Structure 4.



STRUCTURE 4

The first synthesis of antamanide was achieved in 1969 by cyclization of linear peptides, e.g., H-Phe^6-(Pro^{7,8})$_2$-(Phe^{9,10})$_2$-Val^1-(Pro^{2,3})$_2$-Ala^4-Phe^5-OH obtained by conventional methods of peptide synthesis.[92] Shortly thereafter, peptide synthesis on a solid phase was applied, resulting in a similar linear decapeptide, which was likewise cyclized to antamanide.[93] Later, many analogs were synthesized, reviewed in Reference 52 or described in References 94 to 96. Much of the chemical effort was put in to investigate the structure-activity relations of antamanides against the background of the ion-complexing capacities of these compounds. In general, peptide synthesis on solid supports has proved extremely helpful in the authors' laboratory for the synthesis of this kind of cyclopeptides. Another great convenience was the ready crystallization of antamanide and most analogs. This also allowed cyclization of the appropriate peptides from not entirely purified linear precursor peptides.

1. Molecular Features Essential for Antitoxic Activity

Our knowledge of the amino acid residues re-

garded as crucial for antitoxic activity includes information from a wealth of antamanide analogs. Two dozen of these compounds have been selected for Figure 9.

Among the analogs of antitoxic activity reduced to zero are those in which one of the prolines has been omitted or replaced by glycine (Numbers 8, 18). Supposedly, the four prolines function as a framework which enables the molecule to accept a conformation suitable for complexing ions and/or fits a possible acceptor site on hepatocytes. Other features essential for

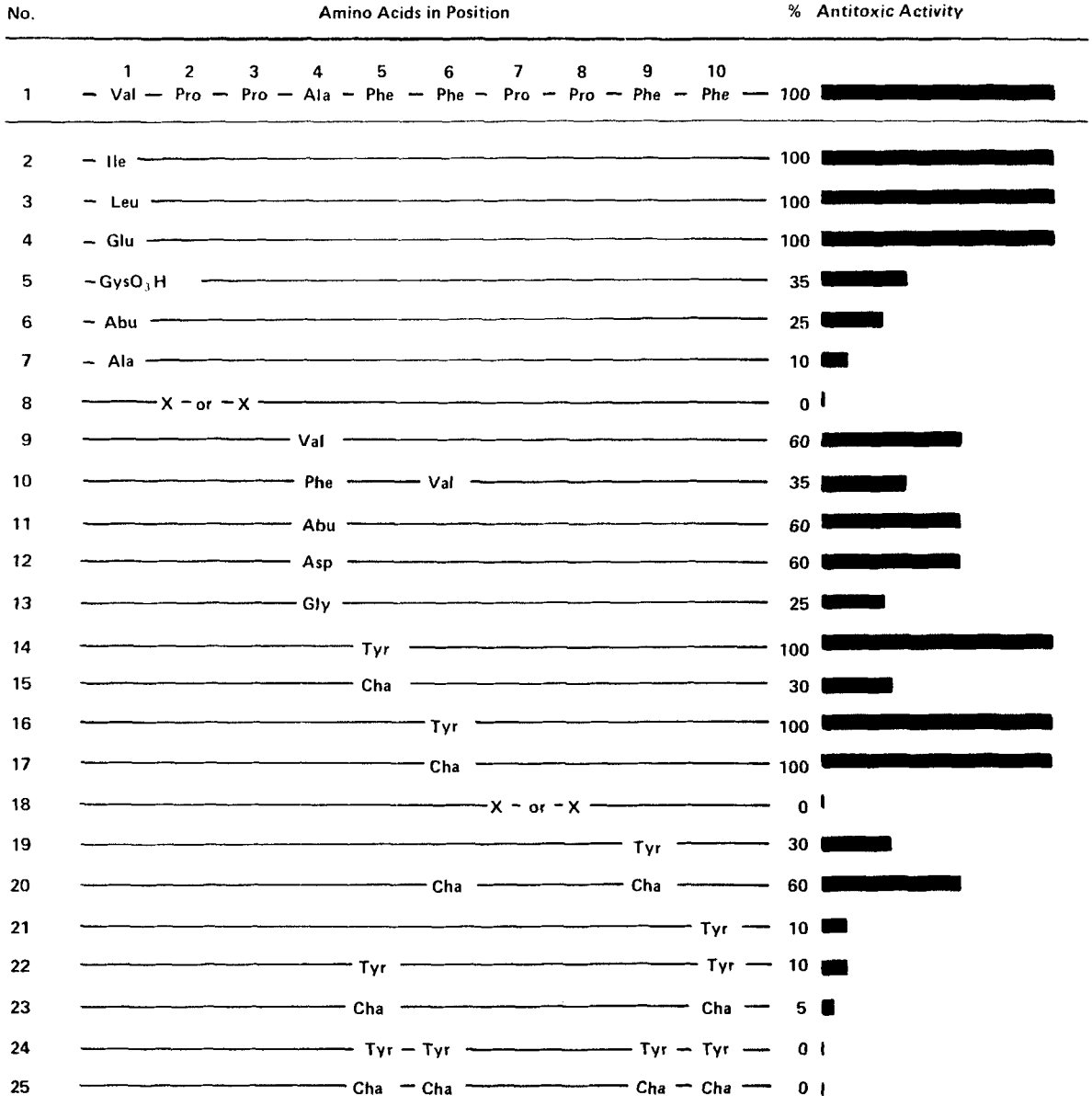


FIGURE 9. Structure and antitoxic activity of several analogs of antamanide. Antitoxic activities expressed in percent based on antamanide (No. 1 = 100%).

the biological activity include the amino acids in Position 1 and 10. Shortening of the hydrophobic side chain in Position 1 (Number 7 in Figure 9) or replacing phenylalanine 10 by amino acids even as similar to phenylalanine as tyrosine (Number 21 in Figure 9) or cyclohexylalanine (Number 23) severely reduced the protective capacity of antamanide. Less important than phenylalanine 10 is the phenylalanine in Position 9. Finally, phenylalanines 5 and 6 can be replaced by tyrosine without affecting the biological activities. In these two derivatives, the phenolic hydroxyl groups in Position 5 and 6 could be used to form additional derivatives. Etherification, e.g., by 2-iodoacetic acid, yielded the carboxymethyl derivatives of two analogs which exhibited 100% of the biological activity. Being easily soluble in water, as ammonium or sodium salts, these greatly facilitated application during in vivo studies.[97]

In summary, we conclude from Figure 9 that the two pairs of prolines (2, 3 and 7, 8) and the side chains of Positions 1 and 10 are the features most essential for the antitoxic activity of antamanide.

C. Ion-Binding Properties

Antamanide forms complexes with alkali metal ions. This has been shown in 1970[98] in Shemyakin's and in our own laboratories by mass spectrometry (occurrence of an ion antamanide + Na^+), IR spectroscopy (increase of carbonyl absorption at 1630 cm^{-1} in the presence of Na^+), potential measurements with ion-specific glass electrodes, vapor pressure osmometry, ORD spectroscopy, and by measurement of the electrical conductivity, which decreases in ethanolic NaCl solutions when antamanide is added. Since then, the investigations have been extended to complexes including metal ions other than sodium as well as to a series of antamanide analogs.[99] Comprehensive surveys on this topic have been given by Ivanov[100] and by Ovchinnikov et al.[101,102] Most importantly, Ca^{++} ions form the strongest complexes;[103] for example, in acetonitrile-water (96:4), a solvent suitable for complexation studies of antamanides, the association constant of $(AA)_2 \cdot Ca^{++}$ is $1 \cdot 10^5\ M^{-1}$ (cf., $AA \cdot Na^+$, $3 \cdot 10^4\ M^{-1}$; $AA \cdot K^+$, $2.9 \cdot 10^2\ M^{-1}$).

Each complex formation induces a conformational change in the cyclopeptide. This has been established by CD spectroscopy. In nonpolar solvents, like 1,4-dioxane, antamanide and most of the antamanide-like cyclic peptides exhibit a strong negative dichroic absorption centered around 230 nm. On complexation of ions, this negative Cotton effect becomes positive, indicating a conformational change. This effect was used spectrometrically to determine the equilibrium association constants of the complexes.[103] As in the case of valinomycin,[100] the stability of the complexes decreased with increasing concentration of water, probably due to solvation of the amide bonds involved in the metal chelate. In Table 6, a series of association constants (K_A) is given for several antamanide analogs with Na^+ and Ca^{++} ions in two different solvents; for comparison, the antitoxic activities of the compounds have also been compiled. Clearly, in the solvents used, all analogs with biological activity form strong complexes with Na^+ and Ca^{++} very similar to antamanide itself, whereas some of the less- or nonprotecting analogs are weak complexones. There exists no potent antitoxic analog with an association constant less than $10^3/M$. It is possible that Ca^{++} ions, specifically bound in the cell membrane by phospholipids or proteins, might be the target for antamanide.[103] However, there are several analogs with poor biological activity which possess about the same complexing capacity as antamanide or its highly antitoxic analogs. Therefore, the Na^+ or Ca^{++} complexing capacity, if at all, can only be one of the prerequisites necessary for the biological activity. Other features of the molecule will similarly contribute to biological activity. These features involve the structural properties of the peptide backbone as well as those of some side chains. This is strongly suggested by the fact that the enantiomorphic all-D-antamanide, which has an identical complexing capacity to antamanide, exhibits only partial biological activity.[94] Ivanov[100] has put forward the suggestion of a "sandwich"-like complexation of antamanides with a phospholipid-bound cation (Na^+ or Ca^{++}) combined with a more or less specific fit of the cyclic peptide due to the nature of the side chains. However, as mentioned above, it is not necessary to assume that complexation participates in the protecting effect by antamanides; the correct topology of the molecule seems to be mainly responsible for the membrane stabi-

TABLE 6

Association constants K_A (M^{-1}) of Na^+ and Ca^{++} Complexes in Two Solvents, and Antitoxic Activity of Antamanide and Some of Its Analogs

Antamanide (AA) analogs	In 96% ethanol	In 4% H_2O containing acetonitrile		Antitoxic activity (dose in mg/kg, which protects the white mouse against 5 mg/kg phalloidin)
	K_A (Na^+)	K_A (Na^+)	K_A (Ca^{++})	
Antamanide	2.4×10^3	1.1×10^3	1.8×10^4	0.5
[Leu^1]-AA	1.0×10^3	—	—	0.5
[Abu^1]-AA	1.5×10^3	—	—	2.5
[Tyr^9]-AA	—	3.6×10^3	7.5×10^3	2.0
[Tyr^{10}]-AA	—	2.7×10^3	8.4×10^3	5.0
[Cha^5, Cha^{10}]-AA	—	3.8×10^3	8.4×10^3	10—20
[Cha^5, Cha^6, Cha^9, Cha^{10}]-AA	2×10^3	—	—	>20.0
[Phe^4, Val^6]-AA	—	1.8×10^3	1.8×10^4	1.5
[Ala^1]-AA	1.5×10^2	—	—	15.0
[Gly^1, Gly^4]-AA	1.0×10^2	—	—	>20.0
[Val^6, Ala^9]-AA	2.5×10^4	—	—	>10.0

lizing property, whereas the cation-binding ability contributes less or simply represents a less important side effect of certain cyclic decapeptides which by chance exhibit a suitable cavity and therefore give rise to complexation.

The rate constants for complex formation (k_1) of Na^+ and Ca^{++} with antamanide were determined using the temperature-jump relaxation technique.[104] The values are lower by orders of magnitude ($k_1 \sim 10^5/M/sec$) than those for similar complexones like monactin, valinomycin, and crown ethers ($k_1 \sim 10^7$ to 10^8).

D. Molecular Shapes of Antamanides

1. Uncomplexed Antamanides

As deduced from CD curves, the molecule of antamanide can adopt different conformations depending on the nature of solvent or the presence of complexed metal cations. In earlier studies, a conformation was proposed for antamanide in nonpolar solvents resembling that of valinomycin under similar conditions, namely, a bracelet like all-*trans* structure with a maximal of six internal hydrogen bridges.[105] In a more recent NMR study, Patel and Tonelli suggested the existence of two *cis* acyl-proline peptide bonds, which, however, could not be attributed unambiguously to one of the two possibilities X-Pro or Pro-Pro, respectively.[106,107] A recent X-ray analysis of the biologically active C-2 symmetrical analog [Phe^4, Val^6]-antamanide, crystallized from methylacetate/*n*-hexane, revealed the conformation shown in Figure 10.[108]

The elongated relatively planar ring contains two *cis*-Pro-Pro peptide bonds. In addition to this, the structure has some other unusual features. Only two intramolecular hydrogen bonds of the rare 5 → 1 (10 → 6) type are formed, whereas the four remaining NH groups are hydrogen-bonded to an array of three water molecules. Antamanide retains one to two molecules of water even after drying *in vacuo* over P_2O_5 at 60°C for 1 week. In fact, the structure can be considered an H_2O-complex of antamanide.

A second crystal species of [Phe^4, Va^6]-antamanide containing 12 water molecules per cyclic peptide has ben obtained by chance from a solution containing calcium nitrate (with some molecules of crystal water), acetone, and acetonitrile.[109] Its conformation was identical to the trihydrate considered above, and the packing was very similar in both crystals with continous channels for the solvent molecules but with *n*-hexane/methylacetate in the previous study[108] and water in this case. These water molecules were hydrogen-bonded to the peptide molecules and to each other to form an uninterrupted column of polar atoms. Since the structure of water surrounding protein molecules is presently of prime interest, an analysis

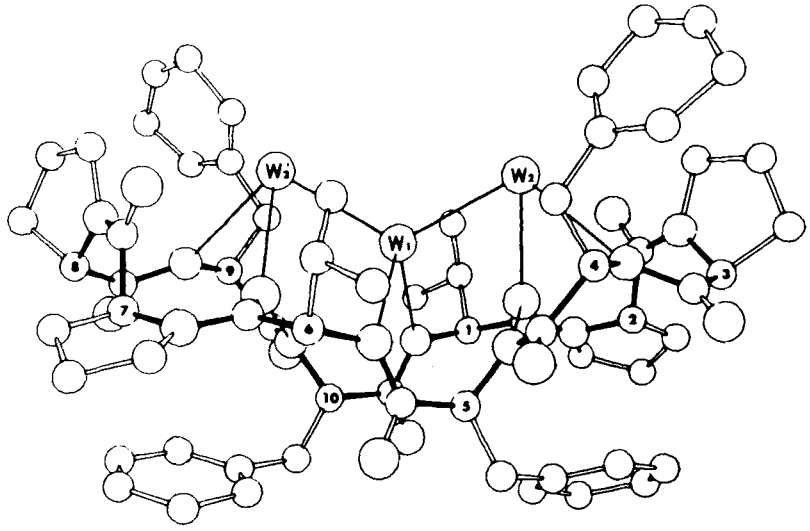


FIGURE 10. Diagram of uncomplexed (Phe⁴, Val⁶) antamanide. The peptide backbone is drawn boldfaced and the C^{α} atoms are numbered 1 to 10. The three water molecules intimately associated with antamanide are labeled W_1 and W_2. Hydrogen bonds are indicated by thin lines.

of the water structure in smaller peptides like antamanide may give further insights into the relationship of water to macromolecular peptides and to membrane constituents and presumably into the mechanism of the membrane-tightening action of antamanides.

In polar media like organic solvents with high amounts of water, antamanide and [Phe⁴, Val⁶]-antamanide exhibit CD spectra different from the hydrates in nonpolar solvents and similar to those of the metal complexes. This supports the argument, deduced also from nuclear magnetic resonance, spectroscopic and ultrasonic measurements in various solvents, suggesting the existence of at least three different conformations of antamanide.[106,110]

2. Metal Complexes

Several attempts have been made to derive the conformation in solution from IR, ORD, and NMR data for the metal complexes of antamanide.[111] Recently the structures in crystals of Li-antamanide⁺[112,113] and Na-[Phe⁴ Val⁶]-antamanide⁺ have been elucidated by X-ray analysis (Figure 11).[112,114] The structures of both complexes agree in several features, namely, in the similar molecular shape of the peptide backbones, two *cis*-Pro-Pro peptide bonds, and two 4 → 1 type intramolecular hydrogen bonds in identical positions. One feature not predictable from spectroscopic data is the pentacoordination of the central ion. It is coordinated by four carbonyl oxygen atoms, two of which also participate in internal hydrogen bonds. The fifth ligand is provided by the nitrogen of a molecule of acetonitrile, which was the solvent of crystallization. The Na⁺ complex of [Phe⁴, Val⁶]-antamanide also resolved by X-ray crystallography was found to be isostructural with the Li⁺-antamanide complex.[112,113] By the introduction of the larger

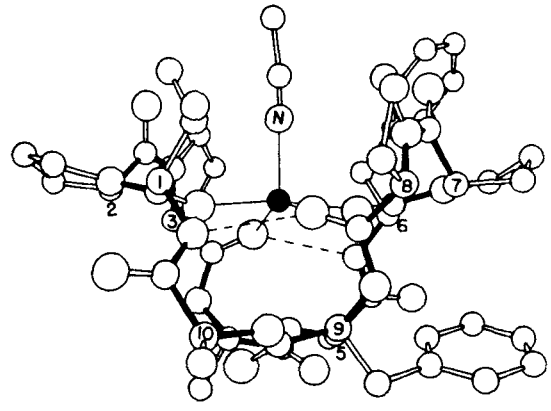


FIGURE 11. Diagram of the lithium antamanide·CH_3CN complex. The peptide backbone is drawn boldfaced; the numbers refer to the C^{α} atoms in the ten residues. Five-coordinated Li^+ is represented by the black dot. One of the ligands is acetonitrile (solvent).

Na^+-ion instead of Li^+ in the upper cavity of the complex, the two internal hydrogen bonds are considerably lengthened but not broken, and it seems unlikely that the cavity can expand further. This would explain the preference of the antamanides for Na^+ over K^+. The close similarity of the X-ray data of the complexes and the data of the various spectroscopical measurements strongly suggests that the conformations of the complexes in solid phase and in solution are highly comparable. A detailed ^{13}C-NMR investigation by Pook et al.[115] is also entirely consistent with the conformations obtained from the crystalline alkali metal complexes.

V. PHALLOLYSIN

A. Historical

In 1891, Kobert[12] detected a hemolytic activity in mushrooms of the species *Amanita phalloides,* which he named phallin. He described it as unstable in alcohol, acids, and at temperatures > 65°C. Since phallin effected lysis of red cells in dilutions as high as 1:125,000, there can be no doubt that Kobert had detected one of the lytic proteins present in most of the *Amanitaceae.* However, it remains uncertain whether the mushroom sample he investigated was in fact from *Amanita phalloides* since he described his "proteid" phallin as also causing hemolysis in bovine erythrocytes. Later on these cells proved to be completely stable to treatment with hemolysin of *Amanita phalloides.* On the other hand, bovine red cells are sensitive to the hemolysin of *Amanita rubescens,* and most probably, Kobert had examined extracts of the latter species. A few years later, this work was carried on by Abel and Ford.[14] These authors extracted tissue from *Amanita phalloides* and achieved a partial purification. They separated the *Amanita* hemolysin from the toxic peptides by various precipitation steps. Their hemolytic fraction was rich in saccharide material as indicated by Fehlings' reagent, orcinol, and the reduction of Ag ions. This strengthened their opinion even more that the hemolysin was a "glucoside" rather than a "toxalbumin", more so, since some agents known to precipitate proteins failed to do so with the hemolysin. Today we know that the hemolytic substance, called phallolysin, is indeed a protein but cannot be precipitated by trichloroacetic acid. Interest in this mushroom toxin decreased quickly when it became evident that phallolysin could not possibly contribute to human intoxication because of its lability during cooking and because it would be inactivated by contact with the acids of the gastric juice if ingested in a crude state. It wasn't until 1967 that phallolysin was "rediscovered" by Fiume,[116] who used a partially purified fraction of phallolysin in cytolytic studies.

B. Occurrence and Isolation

As already mentioned, hemolytic activity is not only present in *Amanita phalloides* but also in other *Amanita* species (Table 7).

According to Seeger and co-workers,[117,118] hemolytic activity is strongest in *Amanita phalloides.* Other species of *Amanita* contain less, or in some cases, no hemolytic activity at all. Hemolytic activities have also been found in many other species than *Amanitaceae.* In all species, it is noteworthy that the hemolytic activity was greater if the samples were collected later in the year. In all cases, the course of hemolysis induced by other extracts was similar to that described for *Amanita phalloides.*

So far, a thorough purification of hemolytic substances has only been achieved for *Amanita phalloides.* The earliest procedures wasted large amounts of the material and were inferior to later preparations. The most efficient purification steps are precipitation with 40% ammonium sulfate,[119,121] ion-exchange chromatography on DEAE-cellulose,[119,121] and gel chromatography on Biogel P30.[119-121] The highest purities obtained were 3000 to 5000 H·U/mg protein, and the highest overall yields were 6 to 22%.[119,120] There is agreement that the stability of phallolysin decreases with increasing purity.

Hemolysin from species other than *Amanita phalloides* must vary in some way, because they differ in their hemolytic activities with respect to human and bovine red cells. Of these, only the hemolytic component of *Amanita rubescens* has been somewhat characterized.[117] In contrast to phallolysin, it is stable at pH 3 to 6, lyses bovine erythrocytes, and has a higher molecular weight (52,000). Its molecular weight is similar to that of one subunit of volvatoxin, which has been described by Lin et al.[122]

TABLE 7

Hemolytic Activities in *Amanitaceae* and in Some Other Genera or Species of Mushrooms[117,118]

		H.U.(S)g^{-1} fresh tissue[a]	
Amanitaceae	*Amanita phalloides*	416—770	—
	Amanita verna	45—345	—
	Amanita virosa	—	—
	Amanita panterina	—	—
	Amanita citrina	0—205	—
	Amanita rubescens	28—500	—
	Amanita muscaria	0—280	Agglutination
Boletaceae	*Xerocomus chrysenteron* (Bull. ex St. Amans) Quel	20—250	Agglutination
Tricholomataceae	*Oudemansiella mucida* (Schrad. ex Fr.) V. Hoehn	40—500	—
Agaricaceae	*Agrocybe semiorbicularis* (Bull. ex Fr.) Fay	67—200	—
Strophariaceae	*Hypholoma fasciculare* (Huds. ex Fr.) Kummer	0—200	—
	Kuehneromyces mutabilis (Schff. ex Fr.) Sing & Smith	0—200	Agglutination

[a] H.U.(S) = hemolytic units according to Seeger.[123]

C. Physical and Chemical Properties of Phallolysin

Unlike all other biologically active components of *Amanita phalloides*, phallolysin is not an oligopeptide but has a molecular weight of around 30,000 dalton as determined by gel permeation.[123] The toxin is undoubtedly a protein though as yet it is uncertain whether polysaccharides are covalently attached to it. Abel and Ford have already described phallolysin as a "glucoside" because sugar-containing material was closely associated with it. Most of the polysaccharide material can be separated by chromatographic procedures or by concanavalin A precipitation.[120] One of our first preparations contained 25% neutral sugars, which can now be reduced to a few percent by further purification.[124] Seeger et al.[121] report a residual amount of 1% glycosides, and it is still unclear whether this is covalently bound to the protein.

Nevertheless, it is certain that the protein is tightly associated with polysaccharide material in the mushroom extracts, and there are indications that the sugar component has a stabilizing effect. Furthermore, the affinity of phallolysin for polysaccharides was a help in understanding the molecular mechanism of its activity.

From electrofocusing studies, Faulstich and Weckauf[120] were able to state that phallolysin consists of two protein species differing slightly in their isoelectric points. The major band, (A), and the minor one, (B), have pI values of 7.8 and 8.2, respectively. Recently, Seeger[123] attributed about 70% of the total hemolytic activity to phallolysin (A) and detected a small amount of a third hemolytic compound (C), with pI = 6.9. It was present in only some samples of *Amanita phalloides*.

Most of the hemolytic activity was destroyed when phallolysin solutions were heated to more than 65°C for 5 min.[12,14,120,123] Phallolysin is sensitive to acidic conditions but less to an alkaline medium. When incubated for 24 hr, it was found to be stable in the pH range of 5.5 to 9.0. Phallolysin also withstands treatment with all proteases and glycosidases,[123,125] and there is evidence that resistance to proteases was not occasioned by inhibition of these enzymes by the phallolysin itself.[123] Phallolysin is irreversibly denatured by sodium dodecylsulfonate and also by 8 *M* urea.[123]

D. Cytotoxicity

Red cells have been used for all assays of the cytolytic activity of phallolysin. Hemolytic

units (HU) have been defined by Seeger[123] and Faulstich et al.[120] According to Seeger,[123] one HU is that amount of phallolysin which completely (100%) lyses a 1% suspension of rabbit red cells in 2 hr at 37°C [HU(S)]. According to Faulstich and Weckauf-Bloching,[100] 1 HU is the amount of phallolysin which lyses a 1.3% suspension of human red cells by 50% in 45 min at 22°C [HU(F)]. There has been no direct match of the two units as yet, but from lethal doses, we estimate that 1 HU(S) ≈ 2.5 HU(F).

1. Susceptibility of Cells In Vitro

In 1967, Fiume[116] stated for the first time that phallolysin exhibited cytopathogenic activity not only on erythrocytes, but also on other mammalian cells. In in vitro tests, he found that KB-cells and human amnion cells are detached from glass walls by the toxin. He did not check the vitality of the cells, but from our experiments, we must assume that they had suffered cytolysis. Villa and Agostoni[126,127] investigated Ehrlich-ascites tumor cells and Yoshida ascites AH 130 cells of rats. These cells were also sensitive; although there is no doubt that the active component in these studies was phallolysin, it is difficult to evaluate or compare the results with others because only crude extracts of *Amanita phalloides* were used.

Extended studies on various mammalian cells were done by Faulstich et al.[125] and Seeger and Lehmann.[128] The results are compiled in Table 8.

The most sensitive cells were the hepatocytes, followed by red and white blood cells, which were equally affected by phallolysin. The lowest sensitivity was found in hamster peritoneal cells. Most interesting was the fact that among these cells, part of the population was totally resistant, even to a large excess of the toxin. Assuming that the cell culture was homogenous, we must consider that there is less sensitivity to phallolysin during certain phases of the reproduction cycle of these cells. A similar behavior but with a much smaller proportion of resistant cells was also found with P_{43}-ascites cells. In some preparations of human amnion cells, there was varied sensitivity, whereas in others, the cell population was homogeneous. Tumor cells found to have a higher sensitivity than the corresponding normal cell were not reported.

2. Toxicity and Antitumor Activity In Vivo

As judged by the rapid lysis of erythrocytes by phallolysin, the protein is highly toxic when applied parenterally.[120,129] For instance, toxicity in the white mouse is higher than that of α-

TABLE 8

Sensitivity of Various Mammalian Cells Against Phallolysin

Cell type	Incubation time (min)	Onset of cytolysis (H.U./ml)[a]	50% Cytolysis (H.U./ml)	% Cells resistent to excess doses	Ref.
Erythrocytes					
Human, A_2, A_1, B, O	15	—	9 (F)	0	125
Mouse	—	—	1.5 (F)	0	130[c]
Rabbit	—	—	3 (F)	0	130[c]
Rat	—	—	6 (F)	0	130[c]
Beagle dogs	—	—	195 (F)	0	130[c]
Bovine, sheep	—	—	>1500 (F)	0	130[c]
EBV-transformed lymphocytes and normal lymphocytes	15	—	9 (F)	0	125
Hela cells	15	10 (F)	21 (F)	0	125
Hela cells	120	1.6 (S)	—	0	128
Isolated hepatocytes	15	3 (F)	5 (F)	0	125
Human amnion cells	15	3 (F)	8 (F)	0[b]	125
Hamster peritoneal cells	15	25 (F)	60 (F)	20—55	125
P_{43}-ascites cells (mouse sarcoma)	120	0.7 (S)	—	4	128

[a] H.U.(F) = hemolytic units defined from Reference 120; H.U.(S) = hemolytic units defined from Reference 123.
[b] In some preparations, approximately 50% of the cells were 2.7 times less sensitive than the rest of the cells.
[c] Estimated as H.U.(F) from Reference 130.

amanitin. The LD_{50} in the white mouse is 580 HU(F) = 200 HU(S) per kilogram of body weight corresponding to 120 μg of protein, assuming use of the purest protein prepared so far. This is only 60% of the dose of α-amanitin expressed in μg/kg body weight or as little as 2% of the dose of α-amanitin when calculated on molar amounts. In addition to this, the sensitivity of mammals increases with body size. For example, the LD_{50} of the rat is 200 HU(F) = 85 HU(S), corresponding to 50 μg protein. For the rabbit, the LD_{50} is even lower than 100 HU(F) or less than 25 μg of phallolysin. This low dose is unexpected since the red cells of the rabbit were reported[130] to be less sensitive than those of the mouse.

In most cases, death occurred by acute hemolysis only a few minutes after the i.v. application. With very high doses of the toxin, hemolysis and the following liberation of high amounts of K^+ ions caused cardiac arrest or kidney damage. From experiments with the isolated guinea pig heart, a direct attack on heart cells must also be considered.[129] In this context, it may be of some interest that volvatoxin[122] had been described as a cardiotoxin before the cytolytic properties of this fungal protein became obvious. Since some rabbits died with convulsions only a few minutes after application and with only a small degree of hemolysis, neurotoxic effects cannot be excluded. When phallolysin was administered i.p., only 20% of the i.v. toxicity was found; in this case, the animals clearly died in pain. As expected, there was no sign of toxicity following oral application of the toxin.[120,129]

Some contradictory results were reported from attempts to prove antitumor activity of phallolysin in vivo. Villa and Agostoni claimed that Yoshida and Ehrlich ascites cells did not develop after inoculation into phallolysin-treated rats. They even postulated that rats treated in this way were insensitive to a second inoculation 2 weeks later, probably via active immunization. On the other hand, Seeger and Lehmann[128] demonstrated only a retarded growth of sarcoma P_{43} in mice when phallolysin was administered simultaneously. None of these animals were saved. The simplest explanation is that the number of inoculated cells had been decreased through direct lysis by phallolysin, thus retarding the first phase of proliferation significantly. This explanation is supported by the fact that repeated treatment with phallolysin in the days following the inoculation did not enhance the effect of the single phallolysin treatment.

E. Mechanism of Action of Phallolysin

Lysis of cells by phallolysin is a rather slow reaction by comparison with the instant damage of membranes caused by melittin[124] or digitonin.[130] The rate of lysis strongly depends on temperature; at 20°C, 1 HU(S) has the same effect as 10 HU(S) have at 10°C. The optimum temperature for the activity is 20°C rather than 37°C, at which lysis is 1.7 times slower. In addition to this and in contrast to the saponins, phallolysin is no longer bound by the cell ghosts after lysis. However, there are contradictory data with respect to the amount of phallolysin released after the reaction for instance with red cells. Seeger et al. reported that the full amount of toxin was available after the reaction, while in our hands, only ~ 50% of the toxin was recovered in the supernatant of destroyed cells.[124] A release of toxin after lysis is consistent with the observation that hemolysis is achieved with extremely small amounts of the toxin, provided the time of incubation is long enough.

It became obvious from many experiments that neither protease activity, phospholipase, nor sphingomyelinase can be attributed to phallolysin. Likewise, Seeger et al.[130] established that phallolysin is neither a detergent capable of releasing phospholipids from membranes. A cholesterol-binding capacity like that of saponin could also be ruled out. Some conclusions about the structures which are involved in the mechanism of lysis could be drawn from experiments in which hemolysis could be inhibited by various additives. Seeger et al.[130] reported that bivalent cations inhibit the process as measured by the time required for 50% hemolysis. The reaction times were prolonged to 1.6 times or > 2.7 times, respectively, of that of the controls by 1 m*M* Ca^{++} or Zn.$^{++}$ Mg^{++} ions were less effective. Faulstich and Weckauf[131] reported an inhibition of the lysis by phosphate ions. A slight inhibition was also demonstrated with ghosts of red cells. The inhibiting compound in these membrane preparations was probably the so-called MN-glycoprotein, which after the isolation from red cell ghosts, was an effective inhibitor.

The most potent inhibitor discovered so far

is wheat-germ agglutinin, which is known to bind to acetyl glucosamine. Since acetylglucosamine is also part of the MN-glycoprotein from red-cell ghosts, we suggest that a glycoprotein or glycolipid containing this amino sugar might be involved at least in the binding of phallolysin to cell membranes, if not in the lysis process itself. Further support for this idea is given by presently running experiments which show that oligomers of acetylglucosamine, obtained by acid hydrolysis of chitin, are potent inhibitors of phallolysin.[124] The fact that exceptionally high concentrations of agglutinins can also cause lysis of cells beside agglutination suggests that both agglutination and lysis of cells are related processes, both depending on saccharide-containing membrane components.

Recently, Petzinger and Seeger[132] published pictures showing pseudopods or protrusions formed during the lysis of isolated hepatocytes which are very similar to those generated by phalloidin. In contrast to the protrusions induced by phalloidin, those occasioned by phallolysin burst and expel cytoplasmic material. It appears unlikely that these pictures describe the general mechanism of phallolysin-induced damage to cell membranes. The protrusions observed are more likely to be a response specific for hepatocytes to various poisons and treatments, since they can also be brought about in other ways, e.g., oxygen deficiency.

VI. TOXIC *AMANITA* PEPTIDES AND BIOLOGICAL SYSTEMS

A. Molecular Toxicity of Amatoxins

The molecular mechanism of the toxicity of amatoxins is the inhibition of RNA synthesis in cell nuclei. This finding followed from the observation of Fiume and Stirpe in 1966 of a significant decrease in the amount of RNA in the cell nuclei of mouse liver 1 hr after administration of α-amanitin[133] due to the blockage of an RNA polymerase.[134,135] The further development of this new field of toxicological enzymology has been amply described in several reviews.[49,50,136,137] Hence, this chapter will be introduced by a brief summary merely outlining the major events from the beginning in 1966 until 1973.

α-Amanitin inhibits in vitro the DNA-dependent ribonucleoside triphosphate, RNA nucleotidyltransferase (EC 2.7.7.6), here shortly RNA polymerase, solubilized from isolated rat-liver nuclei. Evidence that the toxin interacts with the enzyme and not with DNA was first given in 1970 by Novello et al.[138] and later confirmed by Jacob et al.[139,140]

At the same time, Roeder and Rutter[141] succeeded in separating three distinct RNA polymerases, called I, II, and III, from sea-urchin embryos and two polymerases I and II (later also a third one) from rat liver. The enzymes were separated on a DEAE sephadex column eluted with an ammonium sulfate gradient. They suggested that enzyme I was localized in the nucleolus and enzyme II in the nucleoplasm.

Shortly thereafter, Lindell et al.,[142] using the enzymes from both sea urchin and rat liver, demonstrated that only enzyme II is inhibited by α-amanitin and by doses as low as 5 to 10 ng/mℓ. Simultaneously, the corresponding enzyme from calf thymus, called RNA polymerase B, was likewise shown to be sensitive to the drug by Kedinger et al.,[143] whereas the polymerase eluting ahead, form A, was insensitive. The authors also suggested that interaction of the drug with the enzyme causes inhibition of elongation of the polyribonucleotide chain.

RNA polymerase from *Escherichia coli* was found to be completely insensitive to α-amanitin. A 1:1 complex was deduced for rat liver polymerase by Seifart and Sekeris[144] from the evidence that maximal inhibition is caused by a ratio of 30 ng toxin to 60 μg of protein. This was confirmed by Chambon et al.[145]

Radioactive labeling of γ-amanitin by etherification of its phenolic hydroxyl group with ^{14}C-methyliodide was performed by Wieland and Govindan.[61] The labeled amatoxin was first used in binding experiments by Meilhac et al.[146] In 1971, Kedinger et al.[147] found that the amanitin-sensitive RNA polymerase from calf thymus could be separated into two isoenzymes, both of which were equally inhibited by amatoxins.

An RNA polymerase isolated by low-salt extraction from calf thymus and bovine lymphosarcoma ($RNAP_L$) was found by Furth and Austin[148] to possess a lower sensitivity to α-amanitin. This RNA polymerase activity seems closely related to RNA polymerase "C," which is also sensitive to high concentrations of α-amanitin as described by Seifart et al.[149]

1. In vitro Inhibition of RNA Polymerases by Amatoxins

a. Terminology

The terminology of the DNA-dependent RNA polymerases is confusing because the three types of enzymes known to date were differently named by two laboratories: Roeder and Rutter[141] designated the enzymes I, II, and III according to their sequence of elution in an ammonium salt gradient. This terminology has not been adopted by Chambon's group on the basis that future experiments might reveal new RNA polymerases. Therefore, Kedinger et al.[143] proposed to differentiate the three polymerases according to their sensitivity to α-amanitin: A is the completely insensitive form and B is the highly sensitive one, inhibited by 10^{-9} to 10^{-8} *M* α-amanitin. The third form, C, is likewise sensitive to α-amanitin, but at much higher concentrations (10^{-5} to 10^{-4} *M*) of the toxin.[149]

This situation became more complicated when isoenzymes of all forms were detected. In addition to this, one RNA polymerase fraction of yeast was described which was eluted according to form I (or A) but showed sensitivity to high concentrations of α-amanitin,[50,151] while form III (or C) from yeast, like that from *Bombyx mori,* proved virtually resistant to inhibition by the toxin.[152]

A certain confusion also arose with the RNA polymerases of type C (or III) due to varying reports on solubility, localization (cytoplasm, nucleus), and divergencies in catalytic properties. The physical and biochemical nature of all these enzymes, (see References 153 and 154) including $RNAP_L$,[155] allows them to be treated as one family.

It is beyond the scope of this review to balance the advantages of the I, II, etc. terminology against those of the A, B, and C nomenclature. The three types of DNA-dependent RNA polymerases will therefore be designated here as A (or I), B (or II), and C (or III).

b. Sensitivity of Several RNA Polymerases from Different Origins to Amatoxins

DNA-dependent RNA polymerases of the three types have been obtained from different origins. In Table 9, a series of these enzymes has been listed together with their sensitivities to α-amanitin. It is evident from Table 9 that RNA polymerases of type B (or II) are by far the most sensitive ones, although there are differences of three orders of magnitude depending on the source. Generally, the more highly developed eukaryotes are more susceptible to the toxin. The RNA polymerases of type C (or III) require higher concentrations for inhibition apart from four organisms (Table 9) which are insensitive. Enzymes of type A (or I)

TABLE 9

Concentrations of α-Amanitin (ng/m*l*) Causing 50% Inhibition of the Activity of RNA Polymerases from Different Origins (Reliability of Most Values ± 20%)

Cell species	RNA polymerase form		
	A (or I)	B (or II)	C (or III)
Various mammalian cells	Not inhibited	10^1	$1—4 \times 10^4$ [153]
Xenopus laevis	Not detectable	5×10^1 [156]	2×10^4 [157]
Drosophila melanogaster[158]	—	3×10^1	Not detectable
Bombyx mori[152]	—	$1—5 \times 10^1$	Not inhibited at 10^4
Tetrahymena pyriformis[159]	—	5×10^2	—
Saccharomyces cerevisiae	3×10^5	10^3 [160]	Not inhibited
Physarum polycephalum[161]	Not present	5×10^2	Not inhibited at 5×10^3
Mucor rouxi[162]	—	5×10^{2a}	—
Blastocladiella emersonii[163]	*Not inhibited*	*0.5×10^{2a}*	*Not inhibited at 2.5×10^3*
Zea mais[164]	—	10^2	—
Wheat germ[165]	—	$\sim 2.5 \times 10^2$	—

[a] Extrapolation from concentrations causing >50% inhibition.

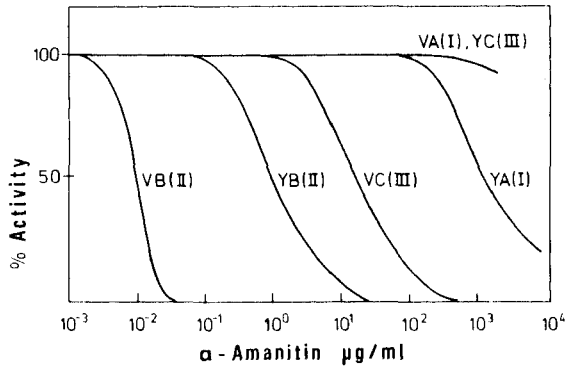


FIGURE 12. Inhibition curves of the different RNA polymerases A (or I), B (or II), and C (or III) of vertebrates (V) and yeast (Y) by α-amanitine.

are totally insensitive with the exception of A (or I) enzymes from yeast which are inhibited by high concentrations of α-amanitin (Figure 12). It is obvious from the table that enzymes of type C (or III) are only inhibited in species where enzymes B (or II) are inhibited by concentrations of 1 to 5 ng/mℓ, however, not in those species where enzymes B (or II) are less sensitive to α-amanitin (inhibition at concentrations of 100 to 1000 ng/mℓ).

RNA polymerase from *E. coli,* like other bacterial enzymes, is completely insensitive to amatoxins. This is also true for RNA polymerase from mitochondria.[166-168] Only one laboratory[169] reports a 50% inhibition of mitochondrial polymerase with 20 μg α-amanitin per milliliter. This is the very concentration at which RNA polymerase C (or III) is inhibited by 50%. The RNA polymerase of plastids has been reported to be resistant to the toxin up to 100 μg/mℓ.[170,171] However, since a small inhibition (< 18%) has been consistently seen at high concentrations of the drug, the authors concluded that nuclei from both peas and spinach may contain a small component of a polymerase which resembles the animal enzyme B (or II).[172]

It may be mentioned here that the activity of RNA polymerases A (or I) and B (or II) in isolated rat liver nuclei, as well as in *E. coli,* is inhibited by relatively high amounts of patulin. Patulin is a mycotoxin from *Byssochlamys nivea* and from several strains of *Penicillium* and *Aspergillus.* The mechanism seems to involve an alteration of the enzymes and concerns the initiation step only.[173]

c. RNA Polymerase B (or II) from Various Eukaryotic Cells

Nucleoplasmic RNA polymerases B (or II) have not only been found in the species compiled in Table 9, but have also been detected in, or isolated from a variety of other cells. Most of them are listed in Table 10.

2. In vivo Effect of Amatoxins on RNA Synthesis

As early as 1954, Wieland and Dose observed that the blood proteins of mice and rats were diminished after amanitin poisoning. They concluded that α-amanitin might interfere with protein synthesis in the liver.[207] However, the mechanism of protein synthesis was not understood at that time, and it took a further 10 years until Fiume and Laschi[208] got back on the track again when carrying out morphological studies of nuclei from mouse livers and kidneys poisoned by amanitin. Parallel to the work on morphological changes, Fiume and Stirpe investigated the RNA content of the poisoned nuclei, which was found to be much lower than in controls.[133] When the incorporation of [^{14}C]-orotate into the RNA of mouse livers was measured, the incorporation rate was found to be reduced to 25% of that of the control.[134,135] Most recently, Romen et al.[394] continued the morphological studies. They investigated the sequence of the nuclear and nucleolar changes by actinomycin D and α-amanitin. According to this study, amanitin starts to condensate the nuclear chromatin, an event which consequently causes a fragmentation of the nucleolus. Following, segregation and degranulation of the nucleolus occurs only 30 to 90 min after the fragmentation. Contrary to this, nucleolar lesions take place with actinomycin D in one step and can be directly correlated to an interaction of this drug with the nucleolar chromatin.

After in vitro assays had established that RNA polymerase B (or II) was exclusively inhibited by amanitin, the in vivo reduction of RNA synthesis was also attributed exclusively to the inhibition of enzymes B (or II). However, detailed in vivo analysis of the effect of α-amanitin in rats by Jacob et al.[139,209] demonstrated that within 1 hr the incorporation of [^{14}C]-orotate into nucleoplasmic RNA (precursors of *m*RNA) as well as into nucleolar RNA (precursors of *r*RNA) was inhibited to an

TABLE 10

RNA Polymerases B (or II) in Various Eukaryotic Cells

Source	Ref.
Mammalian cells	
Rat liver	138
	141
	144
Calf thymus	145
Sea urchin	141
Human placenta	174
	175
Mouse liver	176
Mouse myeloma cells	177
Beef brain nuclei	178
KB cell	179
Hela cells	179
	180
Chick liver	181
Chick oviduct	181
	182
Chinese hamster ovaries	183
Frogs, anura, insects	
Xenopus laevis	156, 184
Rana pipiens (oocytes)	185
Calliphora erythrocephala (*larvae*)	186
Drosophila melanogaster	187
	188
	189
	190
Bombyx mori	152
Lower eukaryotes	
Saccharomyces cerevisiae (= yeast)	191
	192
	193
	160
Physarum polycephalum (slime mold)	161
	194
	195
Blastocladiella emersonii (aquatic *fungus*)	163
Dictyostelium discoideum (*amoeba*)	197
Acetabularia	198
Achlya (algae)	199
Rhizopus	200
Allomyces	201
Tetrahymena pyriformis (protozoa)	159
Higher plants	
Maize leaves	164
Sugar beet, potato tuber	202
Parsley (callus cells)	203
Wheat germ	204
	205
Cauliflower (inflorescences)	206

equally high extent. The suppression of nucleolar RNA synthesis was reported to be overcome after 2 to 3 hr. Niessing et al.[210] also found an almost total halt to the incorporation of labeled orotate into all high-molecular RNA species of rat livers 1 hr after in vivo administration of α-amanitin. Compared to the high molecular weight precursors, the synthesis of low-molecular RNA (4 to 10 S) was less impaired. But here, 5 hr after intoxication, a 120-min pulse experiment revealed that incorporation into ribosomal RNA (extracted from the cytoplasma) was still inhibited by nearly 40%. However, the amount of RNA polymerase A (or I) recovered from liver nuclei of α-amanitin- (or cycloheximide-) treated rats did not differ from that of the controls; a part of this enzyme was found dislocated from the nucleoli to the extranucleolar space of the nuclei.[211]

The inhibition of precursor *r*RNA synthesis in rat liver was measured by Tata[212] as still 70% 4 hr after in vivo amanitin intoxication, whereas the ability of isolated nuclei to incorporate [^{3}H]-ATP into *r*RNA precursor was not influenced at any time. This confirmed earlier as well as contemporary results from other laboratories.[134,135,209,213]

The in vivo effect of α-amanitin on the synthesis of the ribosomal RNA precursor 45 S RNA has also been studied in animals other than rats. A detailed analysis in mice by Hadjiolov et al.[176] has demonstrated that it is the early stage of processing 45S RNA to 32S and 21S RNA which is impeded by poisoning with α-amanitin. This is followed by a cessation of pre-*r*RNA production for at least 3 hr. In these experiments, the labeling of 4S and 5S RNA was also inhibited by 50%. In larvae of *Calliphora*, 45S RNA synthesis is also blocked several hours after administration of α-amanitin[214] and in adult *Aedes Aegyti* 24 hr after addition of the toxin.[215] In the ovaries of *Dysdercus*, as in mouse liver, not only RNA polymerase B (or II) but also the maturation of pre-*r*RNA is affected by the poison.[216] Gel electrophoresis of [^{3}H]-uridine-labeled ribonucleic acids after varying incubation times with 5μg α-amanitin per milliliter of nutrition medium clearly showed an accumulation of labeled 36S RNA species within 20 min, whereas the lower molecular weight-species, 28S and 18S, were entirely absent. Inhibition of *m*RNA synthesis by the drug causing failure of a specific nuclease is thought to be responsible for this effect.

In contrast to the effects in whole animals, in cultured chick embryo fibroblast cells, α-

amanitin (20 μg/mℓ) causes immediate inhibition of RNA polymerase B (or II) activity, but does not inhibit polymerase A (or I) activity of incorporation of [^{3}H]-uridine into ribosomal RNA for several hours.[217] The nucleolar RNA synthesis is not affected by α-amanitin in cells of *Chironomus* salivary glands [218-221] and in oocytes of *Triturus*.[222]

In general, it is reasonable to assume that the in vivo effects observed are, without exception, consequences of the interaction of amatoxins with B (or II) enzymes only. Enzymes A (or I) are probably not directly inhibited by amatoxins. Rather, they are hampered in their function by their dependence on some component formed by enzymes B (or II). This scheme is capable of explaining both the reduced synthesis of ribosomal RNA precursors as well as the slow rate of their maturation in the presence of amatoxins.

Following this idea, some of the effects of amatoxins in vivo would be secondary in nature in that they were brought about by the lack of mediating substances whose synthesis depends on a functioning RNA polymerase B (or II). The suggestion is supported by some observations recently described. The inhibition of enzymes A (or I) is delayed, or less pronounced, in cultured cells,[183] which generally possess a reduced metabolism compared to that of liver cells in vivo. It has also been reported that in vivo applications to rats of cycloheximide, an inhibitor of protein synthesis, leads to a rapid decrease of RNA polymerase A (or I) activity in isolated nuclei.[223,224] Further support for a regulation of *r*RNA transcription in vivo by RNA polymerase B (or II) activity was given by Lindell[395] in experiments on mouse liver nuclei with actinomycin D together with α-amanitin. In summary, these results indicate that the activity of enzymes A (or I) depends on cytoplasmic components and translation processes. Moreover, the results with cycloheximide suggest that the mediating substances in question are proteins rather than nucleic acids.

Assuming that the above theory is correct, the speed with which the transcription by enzyme A (or I) is affected would depend on the half-lives of the proteins which link these two polymerase systems. Thus, in some cases, the suppression of nucleolar RNA polymerase activity only becomes evident several hours after amanitin administration, as in Chinese hamster ovary cells[183] or insect larvae.[214] In others, where the half-lives of mediator proteins must be short, as in rat and mouse livers, there is an almost synchronous decrease of both polymerase activities. Indeed, in rat uteri there are indications of the formation of short-lived RNA and/or protein factors which can activate synthesis of *r*RNA after hormone induction.[225] In line with this result, a polypeptide with a rapid turnover has been postulated by Lampert and Feigelson[226] which has been proposed to regulate ribosomal RNA synthesis. They found that in liver nuclei isolated from rats 3 hr after administration of cycloheximide, the transcription resistant to α-amanitin was normal when assayed with an exogenous template as poly d(A-T) but strongly reduced with endogenous DNA. Since the total number of polymerase A (or I) molecules remained unchanged after cycloheximide, the authors suggest that treatment with this substance inhibits the synthesis of a rapidly turning over polypeptide which determines the proportion of polymerase A (or I) molecules which transcribe the endogenous DNA template.

3. Subunit Composition of Amatoxin-sensitive and -Insensitive RNA Polymerases

On SDS gel electrophoresis, all the RNA polymerases of form B (or II) exhibit very similar patterns consisting of at least five bands. For example, one of the isoenzymes from calf thymus (CT form B II) is composed of protomers of 180,000, 140,000, 34,000, 25,000, and 16,500 dalton,[136] and according to a more recent analysis, 180,000, 145,000, 36,000, 25,000, 20,000, 18,500, 16,000, 15,000, 12,000 and 11,000.[165] In this polymerase, the subunit of 140,000, called SB3, has been identified by radioactive affinity labeling as the amatoxin-binding part, so one would expect to meet with an analogous protein of about 140,000 dalton in each of the RNA polymerases sensitive to low concentrations of α-amanitin. This proves to be true. From table 11, in which only the subunits heavier than 100,000 dalton are registered, it is apparent that at least all the vertebrate B (or II) enzymes possess one subunit with a molecular weight of about 140,000 dalton. All the B (or II) heavy subunits differ distinctly from those of RNA polymerase A (or I). Since the A (or I) enzyme

TABLE 11

Subunits (mol wt > 100,000 dalton) of RNA Polymerases from Various Eukaryotes (in Thousand Dalton)

Origin	RNA polymerases form A (or I)		B (or II)			C (or III)		Ref.
Rat liver	170	126	214		140			136
				180	140			
Calf thymus	197	126	214		140			136
				180	140			
Mouse myeloma	195	117	240		140	155	138	227
			205		140			
				170				
Xenopus laevis						155	138	227
HeLa, KB cells			220	(170)	140			179
Human placenta			200	(180)	150			174
Hen liver, oviduct			214	(180)	140			181
				180	140			
Tadpole liver			190	170	150*			228
Bombyx mori						155	136	227
Drosophila melanogaster				174	137			190, 191
Saccharomyces cerevisiae	185	137		170	145	160	128	160, 229
Physarum polycephalum	185	135		175	145			194
Mucor rouxii	210	125		185	140	170	145	162
Dictyostelium			(190)	170	150			196
Zea mais			200		160			230
Wheat germ			220		140			205
Parsley			200	180	140			231

* The molecular weight of this subunit is rather 140,000 for it proved similar to the corresponding subunit from rat liver enzyme.

from yeast shows a certain sensitivity to α-amanitin,[150] one might expect to find a subunit corresponding to the amatoxin-binding one. There is a divergency from the general subunit pattern of forms A (or I) insofar as the molecular weight of the second heavy subunit (137,000) is distinctly greater than that of the corresponding subunits in the other A (or I) enzymes. The second subunits of many C (or III) polymerases (also amanitin-sensitive) have corresponding molecular weights.

It is not yet sure whether RNA polymerase A (or I) from yeast is the only one sensitive to high concentrations of amatoxins since not all of the enzymes of type A (or I) investigated to date have been assayed with doses as high as 0.1 to 1.0 mg/ml of the drug. RNA polymerases C (or III), which are less sensitive than the B (or II) type to the toxin, show patterns of subunits different from A (or I) as well as from B (or II). In mouse myeloma enzymes where all the three classes have been compared by coelectrophoresis (Table 11) of their subunits,[227] it is apparent that the subunit of 138,000 dalton in enzymes C (or III) differs from that of 140,000 dalton present in enzymes B (or II); nevertheless, one might speculate that the subunit of 135,000 to 145,000 dalton might be the receptor of amatoxins for the type C (or III) enzymes also, particularly since the corresponding subunit of RNA polymerase C (or III) from yeast, which lacks amatoxin sensitivity, is definitely smaller.

4. Analysis of Amatoxin Binding to RNA Polymerases

a. Binding to RNA Polymerase B (or II)

The binding of amatoxins to RNA polymerases B (or II) is very tight. The first evidence for this was obtained using O-[^{14}C]methyl-γ-amanitin, which on ultracentrifugation through a glycerol gradient, migrated together with the enzymes.[146] Similarly, on electrophoresis in polyacrylamide gels using enzymes from calf thymus and from rat liver, the labeled toxin was associated with the fraction of RNA polymerases B (or II).[232]

This strong interaction of the toxin with the

RNA polymerases B (or II) has been confirmed and quantitated by Sperti et al.[233] by equilibrium dialysis. They stated a dissociation constant, K_D, of 3.6×10^{-9} *M* at 4°C for O[^{14}C]methyl-γ-amanitin. A more detailed study of the toxin complex was performed by Cochet-Meilhac and Chambon[53] using O[^{3}H]methyldemethyl-γ-amanitin and RNA polymerase B (or II) from calf thymus. They found evidence for a 1:1 complex, and with a special filter technique, they established a K_D of 6.6×10^{-10} *M* for the above toxin at 20°C and of 6.4×10^{-9} *M* at 37°C. A similar value (4.4×10^{-9} *M*) was obtained for the inhibition constant K_i in an enzyme assay at 37°C, thus indicating that binding of the toxin is closely related to inhibition of RNA polymerase activity.

The inhibition is of a noncompetitive type. Amatoxins affect neither the binding of DNA nor that of the nucleoside triphosphates. RNA release is not influenced by amatoxins either. In fact, amatoxins most probably block the formation of phospho-diester bonds in the initiation step as well as in the elongation steps.

The toxin-enzyme interaction depends on temperature and salt concentration (Table 12). Interaction was stronger at 5°C ($K_D = 1.7 \times 10^{-10}$ *M* and became weaker at 37°C ($K_D = 6.4 \times 10^{-9}$ *M*). The complex was strengthened by a factor of 3 by 1 *M* $(NH_4)_2SO_4$, while dimethylsulfoxide (3%) weakened it by a factor of 2.

Cochet-Meilhac and Chambon also determined the dissociation rate constants k_2 and the association rate constants k_1 of the enzyme using various amatoxins (Table 13). They obtained ratios k_2/k_1 which were equal to the equilibrium constant K_D as obtained by the equilibrium experiments. The half time of dissociation of the complexes can be calculated from the k_2 value, which for [^{3}H]O-methyl-demethyl-γ-amanitin at 20° and 37°C is 1.6 hr and 7 min, respectively. Furthermore, by relating the rate constants to the structural features of the toxins, evidence for the molecular binding

TABLE 12

Equilibrium Dissociation Constants (K_D), Dissociation Rate Constants (k_2), Half-life Times (t ½), and Association Rate Constants (k_1) of Calf Thymus RNA Polymerases B (or II) Complexes with Two Amatoxins at Various Temperatures[53]

	T (°C)	K_D (*M*)	k_2 (s^{-1})	t½ (h)	k_1 ($M^{-1}\,s^{-1}$)
6′-O-Methyl-demethyl-γ-amanitin (A10)	5	1.7×10^{-10}	4.5×10^{-6}	43	3.2×10^{4}
	10	2.2×10^{-10}	1.2×10^{-5}	15.5	5.4×10^{4}
	20	3.9×10^{-10}	1.2×10^{-4}	1.6	1.4×10^{5}
	37	6.4×10^{-9}	1.8×10^{-3}	0.11	1.9×10^{5}
6′-O-Methyl-γ-amanitin (A3-OMe)	10	—	7.7×10^{-6}	25	—
	20	7.2×10^{-10}	4.0×10^{-5}	5	—
	37	2.6×10^{-9}	5.1×10^{-4}	0.38	—

TABLE 13

Inhibition Constants (K_i) and Dissociation Rate Constants (k_2) of Various Calf Thymus RNA Polymerase B- (or II)-amatoxin Complexes at 37°C[53]

No. (Tables 2, 3)	Toxin and side chain in Position 3	K_i (*M*)	k_2 (s^{-1})	k_1 ($M^{-1}\,s^{-1}$) calculated
A1	α-Amanitin-$CH(CH_3)$-$CH(OH)$-CH_2OH	3.1×10^{-9}	1.2×10^{-4}	4×10^{4}
A3	γ-Amanitin-$CH(CH_3)$-$CH(OH)$-CH_3	4.3×10^{-9}	4×10^{-4}	—
A10	O-Methyl-demethyl-γ-amanitin-$CH(CH_3)$-CH_2OH	10.0×10^{-9}	9.8×10^{-4}	9.8×10^{4}
A5	Amanullin-$CH(CH_3)$-CH_2-CH_3	9.1×10^{-9}	14.4×10^{-4}	15.8×10^{4}

mechanism could be gained. From Table 13, it is evident that changes in the side chain in position 3 are reflected by the values of the kinetic constants and by those of K_D. For maximal binding, four carbon atoms and at least one hydroxyl group is necessary (A1, A3). The lack of one methyl group (A10) or of the hydroxyl group (A5) leads to weaker binding, i.e., an increased K_D. This means that the binding involves both hydrogen bonding as well as hydrophobic interaction. The rate of dissociation seems to be predominantly determined by hydrogen bonding, as the loss of a methyl group accelerates the dissociation about twofold (A10 against A3), while the loss of hydroxyl (A5) increases the rate of dissociation by a factor of about four. Most interestingly, the in vivo toxicity is reduced for O-methyl-demethyl-γ-amanitin (A10) and is absent for amanullin (A5), thus indicating that the in vivo toxicity is predominantly determined by the dissociation rate constant k_2. For further data on the influence of the side chain in Position 8, see Table 3.

b. The Amatoxin-binding Site of RNA Polymerase B (or II) from Calf Thymus

Using a radioactively labeled amatoxin, [^{3}H]-6′-amanin, Brodner and Wieland were able to determine the binding site of RNA polymerase B (or II) from calf thymus.[234] The labeled toxin binds to the enzyme as strongly as α-amanitin, which enabled one to monitor the enzyme during chromatographic procedures by following the radioactivity. When a pure enzyme preparation had been obtained, as proved by the correct SDS gel electrophoresis pattern, the amatoxin was covalently fixed to the protein via its carboxylic group using the water soluble 1-ethyl-3-(dimethylaminopropyl) carbodiimide. Gel electrophoresis, in the presence of SDS, of the affinity labeled RNA polymerase exhibited a pattern very similar to that of the unpoisoned enzyme, showing the following high molecular weight subunits distinctly: SB1, SB2, SB3, and an additional one, possibly SB0 previously found in rat-liver enzyme B.[136] The radioactivity profile had its main peak coincidental with the 550 nm absorption peak of subunit B3 (SB3) on a stained parallel gel. Thus B3, to which a molecular weight of 140,000 is attributed, is most probably the binding site for all amatoxins to all RNA polymerases of B (or II) class.

c. An Additional Protein with Amatoxin Affinity

During the isolation of the [^{3}H]amanin-RNA polymerase complex as described above, a protein with a strong amatoxin-binding property was observed eluting from a phosphocellulose column at 0.23 -M- ammonium-chloride before the enzyme (0.39 *M*).[235] The novel amatoxin-binding protein (ABP) coprecipitates with [^{3}H]-amanin using ammonium sulfate and is not dissociated from the toxin during chromatographic separations. Both observations indicate a high affinity of this protein to amatoxins, being comparable to that of polymerase B (or II). By SDS gel electrophoresis, it has been characterized as being composed probably of two subunits of 100,000 and 10,000 to 15,000 dalton, different from any of the subunits of RNA polymerases B (or II) and C (or III). A possible function for ABP in the cell has not yet been revealed.

d. Binding to RNA Polymerases C (or III)

As yet, no binding studies have been made on the RNA polymerases form C (or III) which show a considerably lower affinity for amatoxins.

5. RNA Polymerases from Mutant Cell Lines Resistant to Amatoxins

a. Mutated Enzymes

Mutant cell lines resistant to α-amanitin have been selected from Chinese hamster ovary (CHO),[236] BHK-T6 hamster cells,[237] rat myoblasts,[238] mouse myeloma MOPC 104E,[239] as well as from short-term cultures of human diploid fibroblast explants.[240] At least some of these mutants contain an altered form of DNA-dependent RNA polymerase B (or II) as indicated by its insensitivity to α-amanitin.

In CHO cells, where detailed experiments have been conducted,[241] the drug sensitivity of the different lines varied widely but could be correlated well to the sensitivity of polymerase B (or II) activity in each of the mutant cell lines. The polymerases were separated by chromatography on DEAE Sephadex of extracts prepared by sonication.

In Figure 13, the sensitivities to α-amanitin of RNA polymerases B (or II) from wild-type and from three mutants together with one type hybrid cell are presented. The most sensitive enzyme (50% inhibition at about 4 ng/m*l*) stems

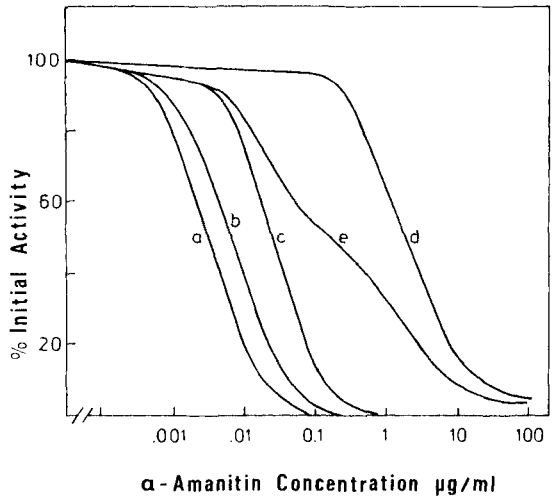


FIGURE 13. Sensitivity of wild-type and mutant RNA polymerases B (or II) from Chinese hamster ovary cell lines to α-amanitin. (a) Parent cell line ($A_2$2C), (b) Ama 39, (c) Ama 6, (d) Ama 1, and (e) Ama 6 × Ama 1 hybrid. (From Ingles, C. J., Guialis, A., Lam, J., and Siminovitch, L., *J. Biol. Chem.*, 251, 2729, 1976. With permission.)

from the parental cells, the next, requiring a two- to threefold higher concentration, comes from the mutant Ama 39 (selected at 0.75 μg α-amanitin per milliliter medium). The enzyme next in sensitivity shows 50% inhibition at an eight- to tenfold higher concentration of the drug than the wild-type enzyme and comes from the mutant Ama 6 (selected at 1.0 μg/mℓ). The most resistant enzyme (over 800 times more) was isolated from Ama 1, one of the first mutants described.[236] Ama 6 × Ama 1 hybrid cells contain both of the respective polymerases as seen by the biphasic shape of the inhibition curve (Figure 13). The monophasic inhibition curves of the mutant activities indicate that CHO cells appear to be functionally hemizygous for this gene and that the mutant cells possess only the mutant form of the enzyme.

In contrast to CHO cells, the inhibition of polymerase B (or II) from the rat myoblast mutant[238,242] Ama 102 was biphasic. In these cells as well as in human diploid fibroblasts,[240] both sensitive and resistant forms of RNA polymerase are present.

In order to explain that only about 30% of the total RNA polymerase B activity extracted from Ama 102 cells (and also from another cell line L6) showed a reduced sensitivity towards α-amanitin, one may suggest that the chromosome set of each cell contains three to four alleles of the gene coding for the α-amanitin-binding polymerase subunit, one of which is mutated. In the human fibroblast cell lines, the corresponding number of alleles is most likely two, although a strictly biphasic inhibition curve could not be seen because the difference in α-amanitin sensitivity was too small between mutant and wild-type enzymes. However, such a biphasic curve has been observed for a mouse myeloma cell line MOPC 104 E Ama^R.[239] In this case, the ratio of resistant to sensitive polymerase activity is 1:1, again suggesting the existence of one wild-type and one mutated allele.

It was demonstrated with CHO cells that the resistance of mutant cells is due to a lower affinity of their B (or II) enzymes to amatoxins. The binding of amatoxin to wild-type and mutant enzymes from mouse myeloma cells was directly determined by Wulf and Bautz[239] using [^{3}H]O-methyl-demethyl-γ-amanitin as originally described by Cochet-Meilhac and Chambon.[53] A 100-fold lower affinity was found for the resistant enzyme. In another study,[243] the equilibrium dissociation constant K_D for the parental CHO polymerase was 3.8×10^{-11} *M*; the values for the enzymes from resistant mutants Ama 39 and Ama 6 were 8.5×10^{-11} and 29×10^{-11} *M*, respectively, i.e., 2.2 and 7.6 times less sensitive. An enzyme more than 600 times less sensitive than that of the wild-type has been isolated from Ama 1. This mutant survived in the presence of 6 μg α-amanitin per milliliter (conditions where the wild-type cells are killed completely). Half maximum inhibition was achieved with 2.4×10^3 ng/mℓ while the corresponding concentration for the wild-type was only 3.9 ng/mℓ. On SDS gel electrophoresis, the mutant and wild-type enzymes separated into subunits comparable with those of calf-thymus RNA polymerase B (or II) isoenzymes. Since both enzymes showed the same marked preference for denatured DNA template, which is characteristic for polymerases B (or II), and since they were equally inhibited by anti-RNA polymerase B (or II) serum, it can be concluded that Ama 1 cells possess a mutated form of the enzyme. The mutation probably involves structural changes in the gene coding for the amatoxin-binding subunit SB3 of RNA polymerase B (or II). However, one cannot exclude the possibility that mutations concerning the primary

structure of other subunits could induce an alteration in subunit SB3 causing a diminished affinity for the toxin. A thorough understanding of the mechanism that is regulating the sensitivity of RNA polymerase B (or II) will require the elucidation of the amino-acid sequence of the polypeptide chains of all subunits of both the mutant and the wild-type enzyme.

b. Regulation of RNA Polymerase Activity

Some mutant cell lines cannot maintain their resistance for long if the toxin is withdrawn from the culture medium. From a resistant mutant rat myoblast cell line (Ama 102) grown for many generations in the absence of α-amanitin, a RNA polymerase B (or II) was purified of which only 30% was resistant to 0.1 μg/mℓ α-amanitin.[242] When these cells were grown in the presence of α-amanitin (3 μg/mℓ) for 4 days, the polymerase B (or II) activity became completely resistant, representing a threefold increase in resistance. A detailed investigation of the time course of this activation revealed that the enzyme resistance was reached within 24 hr of culturing the cells in 3μg/mℓ α-amanitin and then remained constant. On removal of the drug, resistance dropped back to 30% within 10 to 40 hr. The authors suggested that the synthesis of RNA polymerase B (or II) in rat myoblasts is regulated autogeneously, the enzyme possibly acting as its own repressor (and, correspondingly, α-amanitin being the derepressor).

In order to detect an increased enzyme synthesis, levels of total and resistant RNA polymerase were assessed both enzymatically and immunologically in mutant CHO hybrid cells which also manifest this regulation. The cells were grown in either the presence or absence of 3 μg/mℓ α-amanitin for 48 hr.[244a,244b]

The results indicate that the total enzyme activity remained constant (3540 to 3590 cpm of [^{3}H] UMP incorporated), while the amanitin-resistant activity, which was 20% (1030 cpm) of the total activity in cells grown in absence of the toxin, increased to 95% (3340 cpm) in its presence. A corresponding change in the total enzyme mass could not be immunologically detected (320 to 390 ng of enzyme in both cell types).

The presence of such low levels of (sensitive) wild-type enzyme in cells grown with α-amanitin could be due to a rapid degradation of the amanitin-bound enzyme, e.g., through proteolysis. Alternatively, it is conceivable that α-amanitin interferes in the synthesis of the wild-type polymerase B (or II) by combining with its toxin-binding subunit SB3 during, or shortly after, translation, thus preventing the assembly of the different subunits. However, a rather trivial error should be experimentally excluded, namely, that only those RNA polymerase molecules which are actively synthesizing RNA are recovered in the extraction procedure.[245]

6. Transcription of Cellular Genes as Revealed by the Use of Amatoxins

a. Transcriptional Role in Mammalian Cells

α-Amanitin has been utilized in studies of the function of RNA polymerase in order to selectively inhibit specific RNA polymerases in intact cells and in isolated nuclei and nucleoli. Extended periods of exposure to α-amanitin are necessary with intact isolated cells, since they are not freely permeable to the drug. However, studies of RNA polymerase function in isolated nuclei have been unambiguous, as nuclei are easily permeable to the toxin, and endogenous RNA polymerase activities show the same sensitivities to α-amanitin as do the purified enzymes. Furthermore, isolated nuclei synthesize defined RNA species that appear similar to those synthesized in intact cells.

The role of RNA polymerases A (or I), which are located in the nucleoli, as producers of the precursor of ribosomal 18S and 28S RNAs has been clearly recognized by differential inhibition. As early as 1970, Blatti et al.[246] showed that the RNA synthesized in isolated nuclei from rat liver in the presence of α-amanitin had a high content of guanylic acid and cytidylic acid similar to DNA and characteristic of ribosomal RNA. As further proof, the RNA synthesized in this system as well as that of isolated nuclei from *Xenopus laevis* under analogous conditions[247] was specifically hybridized to ribosomal DNA. These and later results summarized in Reference 227 also rule out a direct involvement of RNA polymerases C (or III) in the synthesis of the large *r*RNAs.

RNA polymerases B (or II) are located in the nucleoplasm. This localization was made visible by Moore and Ringertz[248] by autoradiography in human fibroblasts fixed with ethanol-ace-

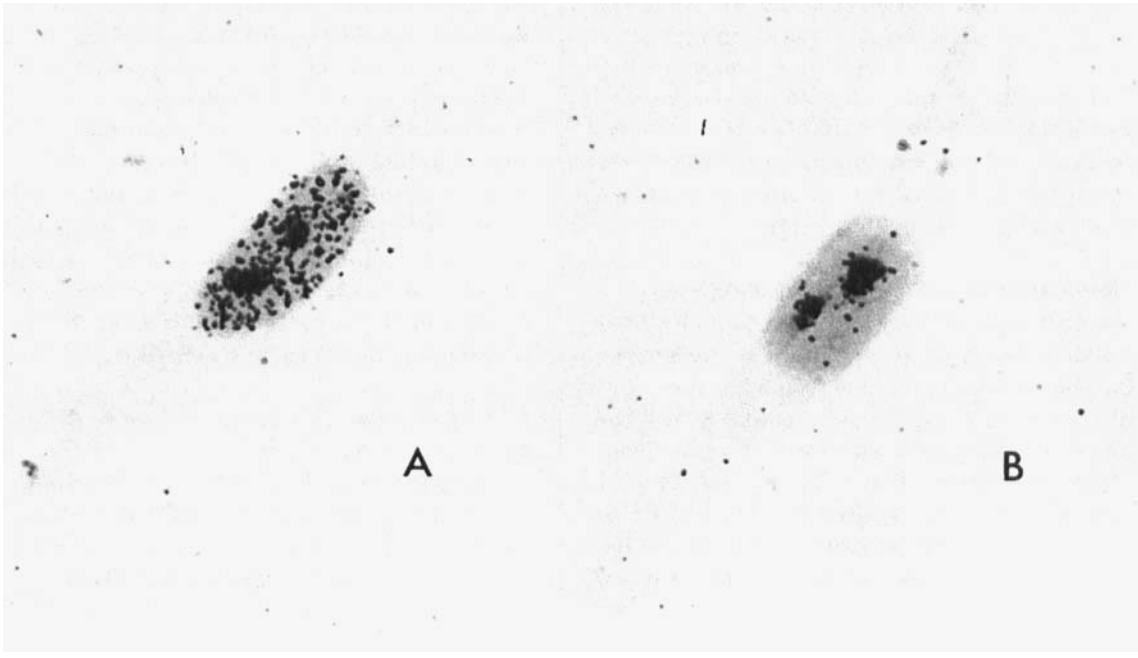


FIGURE 14. Autoradiographs of human fibroblasts grown in culture and tested for enzymatic incorporation of (^{3}H) UTP at 0.4 *M* ammonium sulfate. (A) Without 5.0 μg/mℓ α-amanitin and (B) with 5.0 μg/mℓ α-amanitin. (From Moore, C. P. M. and Ringertz, R., *Exp. Cell Res.*, 76, 223, 1973. With permission.)

tone at 4°C. As seen in Figure 14, in absence of the toxin, the whole nucleus (nucleolus and nucleoplasm) has incorporated [^{3}H] UTP into RNAs, while after 10 min preincubation with 5 ng/mℓ α-amanitin, only the nucleolus remained active. The high sensitivity of RNA polymerases B (or II) to small amounts of amatoxins enabled several laboratories referred to in Reference 227 to show that the products of nucleoplasmic RNA synthesis consist of heterogenous nuclear RNAs (*hn*RNA), the precursors of *m*RNAs.

RNA polymerase C (or III) is also present in the cell nucleus. As Weinmann and Roeder[249] have shown, isolated nuclei and nucleoli from mouse myeloma cells (MOPC 315) continue to synthesize RNA when incubated with the tritium-labeled nucleoside triphosphates. Only when using nuclei were newly synthesized 4.5S RNA (precursor to 4S RNA, i.e., *t*RNA) and 5S *r*RNA species detected by electrophoretic analysis (Figure 15). The synthesis of these products was completely inhibited by high amounts (400 μg/mℓ) of α-amanitin. Since the inhibition curve of the synthesis of low molecular RNAs was identical to that of solubilized RNA polymerase C (or III)(50% inhibition at approximately 30 μg α-amanitin per milliter), it follows that the genes for pre-4S RNA and 5S *r*RNA are transcribed by RNA polymerase C (or III). Polymerase(s) C (or III) catalyzing low molecular weight RNA synthesis in He La nuclei has (have) also been recognized by Udvardy and Seifart,[250] who using better resolving gels, found four low molecular RNA species, whose synthesis was inhibited by high concentrations of α-amanitin.

b. Transcriptional Role in Yeast

Yeast RNA polymerases also show a differential sensitivity towards inhibition by α-amanitin.[150] However, the corresponding yeast enzyme species A (or I) and C (or III) show a pattern of α-amanitin sensitivity which is the reverse of that of the vertebrate enzymes. Yeast polymerase A (or I) can be completely inhibited by very high concentrations of the drug, whereas yeast polymerase C (or III) is practically insensitive (see Figure 12). Thus, one could not predict whether the polymerases which were chromatographically and substructurally analogous (Table 11) would show analogous transcriptional behavior. Schultz[251] studied RNA synthesis in isolated yeast nuclei using

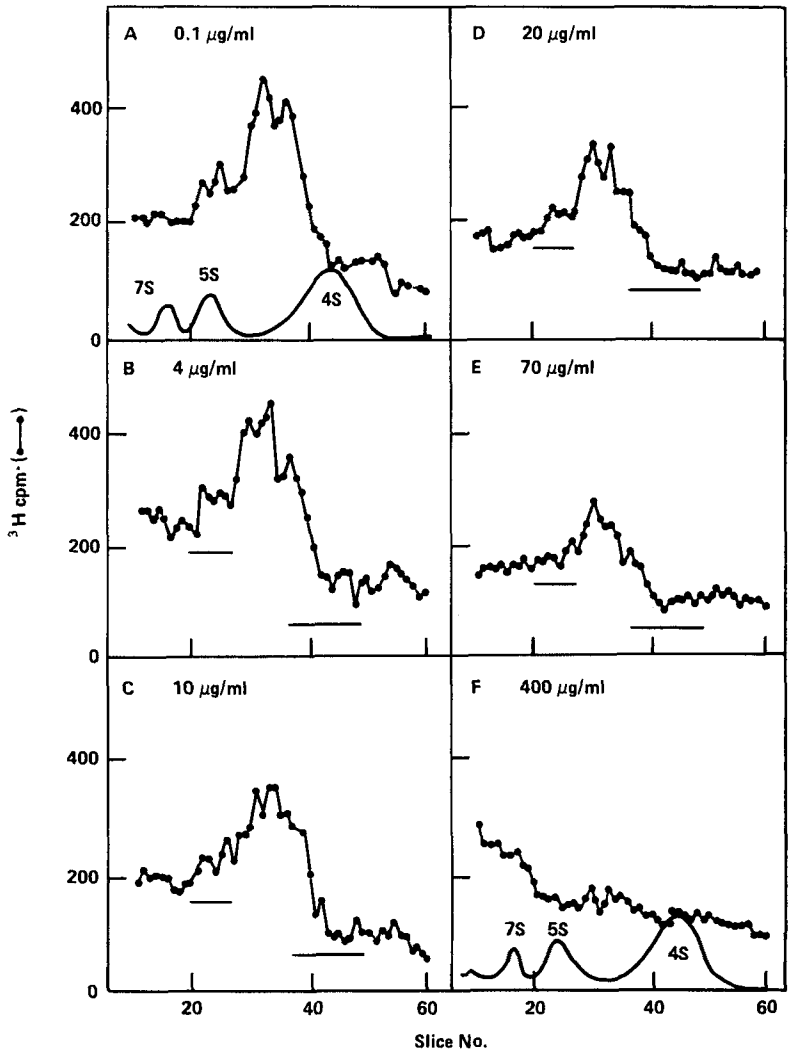


FIGURE 15.. Effect of increasing concentrations of α-amanitin (as indicated in each panel) on the synthesis of low molecular weight RNA released from mouse myeloma cell nuclei. RNA synthesis is analyzed after incorporation of (^{3}H) UTP by SDS-polyacrylamide gel electrophoresis. (From Weinmann, R. and Roeder, R. G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 1790, 1974.)

the differential α-amanitin sensitivities of the three classes of RNA polymerases and examining the RNA products formed in the presence of increasing concentrations of α-amanitin by gel electrophoresis.

In yeast as in HeLa cells, among the RNA species of low molecular weight (less than 5.8 S) apart from precursor *t*RNA (pre-4S, 4.5S) and 5S *r*RNA, two additional RNA species were present, probably a second precursor (~5.4 S) and *t*RNAs (4 S). In addition, a large amount of RNA was found heterogenous in size and ranging from 7 S to greater than 25 S (heterodisperse RNA). The synthesis of all four classes of low molecular weight RNA is unaffected by α-amanitin in concentrations sufficient to inhibit more than 85% of the polymerase I activity.[150] Therefore, in yeast as well as in vertebrates, it is RNA polymerase C (or III) which is responsible for the formation of the low-molecular RNA species.

The heterodisperse RNAs whose production is reduced to about 65% in the presence of a high concentration of α-amanitin seem to be products of RNA polymerase A (or I) and C (or III). In yeast, the latter enzyme also appar-

ently catalyses the synthesis of intermediate and high molecular weight RNAs.

By analogy, the role of yeast RNA polymerase II is surely that of transcribing genes for *hn*RNA.

7. Amatoxins in Virus Research

Amatoxins have been widely used in virus research as a means of indicating whether viral growth requires a cellular function, which ultimately depends on RNA polymerase B (or II) activity. The following presentation does not claim to be a complete survey of the literature published in this field but rather a discussion of the matter via several typical examples.

a. Riboviruses

As early as 1966, Fiume et al.[252] reported that replication of some riboviruses (polio type 2, parainfluenza type 3) is not influenced by α-amanitin. Since then it has been confirmed that this toxin does not affect the growth of most riboviruses, as a matter of fact, the replication of the ribovirus genome is carried out by virus-coded (virus specific) RNA-dependent RNA polymerases, which are amatoxin resistant. Nevertheless, inhibition of growth of *myxo* viruses and of *oncorna* viruses by α-amanitin has been observed. The multiplication of the influenza virus in chick fibroblasts is inhibited by α-amanitin (50 μg/mℓ) only when the toxin is present in the early stages.[253,254] This early step is susceptible even to actinomycin D. Since the inhibition by amanitin did not occur in Chinese hamster ovary cells resistant to amatoxins, a host-cell-specific RNA (perhaps a primer) seems to be necessary in the early stages of viral RNA replication (most probably for the onset of synthesis of RNA complementary to virion RNA).[255,256]

The only other RNA viruses showing dependence on amanitin are the RNA tumor viruses. As early as 1971, Zanetti et al.[257] found that the replication of Rous sarcoma virus in chicken-embryo fibroblasts could be inhibited by 0.5 μg of α-amanitin per milliliter to an extent depending on time and duration of application of the drug. The effect of the toxin could not be observed before 24 hr, suggesting that the event inhibited by the toxin was not an early one. The first step in oncornavirus replication, the reverse transcription of viral RNA into proviral DNA, is indeed insensitive to α-amanitin. Subsequently, more detailed assays in isolated nuclei[258,259] and in whole cells[260] indicated that α-amanitin inhibits the viral RNA synthesis which takes place on the proviral DNA. Hence, this phase is most probably carried out by host RNA polymerase B (or II).

b. Deoxyriboviruses

The DNA of deoxyriboviruses which multiply entirely in the cytoplasm of infected cells (pox viruses, iridoviruses) is transcribed into RNA by virion associated DNA-dependent RNA polymerases, which are insensitive to amatoxins.[261,262]

The most studied iridovirus, frog virus 3 (FV3), causes a strong early inhibition of host cell RNA synthesis. By using α-amanitin as a discriminating agent,[263] Campadelli-Fiume et al. found that RNA polymerase B (or II) is the enzyme activity impaired,[264] and later determinations employing a radioactively labeled amatoxin revealed that the diminished synthesis of RNA in infected cells results from a reduced content of active RNA polymerase B (or II).[265,266]

The replication of those deoxyriboviruses which multiply in the cell nucleus is sensitive to amatoxins. The drug has been mainly applied in cell-free systems, i.e., in isolated nuclei, in order to find out whether virus genome transcription is mediated by host cell polymerase B (or II) or by virus-coded polymerases according to the general, but not entirely validated, assumption that only the host enzyme is susceptible to amatoxins. Rather conclusive evidence that host polymerase B (or II) is the enzyme responsible for transcription of *Herpes simplex* virus DNA is found in the observation that in whole HEp-2 cells, synthesis of RNAs for α-, β-, and γ-polypeptides could be inhibited by β-amanitin (which enters more rapidly after treating cells with DEAE-dextrane for a short time)[267] and, furthermore, that genome transcription was entirely unaffected by amanitin in mutant cells which possess an amatoxin-resistant RNA polymerase B (or II)[268] (see Reference 269).

The DNA of adenovirus is also transcribed in the cell nucleus by the host RNA polymerases. Ledinko's observation in 1971[270] that a relatively low concentration of α-amanitin (0.25

μg/mℓ, a dose which did not affect cells) was able to reduce adenovirus 12 yield by 84% in 3 days in human embryonic kidney cultures suggested that RNA polymerase B (or II) played a key role. During the following years, numerous additional papers appeared on this subject.[271] In addition, participation of RNA polymerase C (or III) in the replication process of adenovirus has been reported by Price and Penman[396] and confirmed by the observation of a biphasic inhibition curve of α-amanitin- poisoned RNA polymerase activity in nuclei isolated from infected cells. Inflection points were found at about 0.02 μg toxin per milliliter (50% inhibition of polymerase B [or II]) and 20 μg/mℓ (50% inhibition of form C [or III]).[272]

8. Effects of Amatoxins on Various Cell Species

a. Uptake by Diffusion

In in vivo toxicity experiments, generally, doses of 0.1 to 0.4 mg α-amanitin per kilogram body weight can be considered as absolutely lethal. Only the rat needs a dose > 2 mg/kg body weight. Therefore, concentrations of 0.2 μg to approximately 0.8 μg/mℓ are sufficient to cause nuclear lesions in hepatocytes of the various animals within 30 min.[273] For rats, which apparently do not reabsorb the poison in the kidney tubules[72] and hence excrete it rapidly, the actual liver-damaging concentration is most probably also below 1 μg/mℓ. Some cell species cultivated in vitro exhibit a similar sensitivity; Chinese hamster ovary cells,[236,241] rat myoblast cells[238] and human diploid fibroblasts[240] have been cultivated in the presence of 0.2 to 3.0 μg α-amanitin per milliliter for several days or weeks in order to obtain resistant mutants, conditions under which the Ama+ parent cells would scarcely survive. Inhibition of mouse ova developing to blastocysts in cultures has been reported to occur with 0.1 to 1.0 μg α-amanitin per milliliter.[397]

Comparable concentrations of the toxin also have been stated to inhibit the growth of cultures of Epstein-Barr virus-transformed lymphocytes in the authors' laboratory.[274] Here, several natural as well as chemically modified amatoxins have been investigated. After 48 hr, a 50% inhibition of growth was observed with 3×10^{-6} *M* α-amanitin (approximately 3 μg/mℓ). This relatively high concentration suggests that the toxin penetrates rather slowly into these cells. Penetration was easy into hepatocytes in situ; already after 30 min perfusion of a rat liver, the concentration of the toxin inside the cells was determined to be as high as in the medium. Lipophilic amatoxins like γ-amanitin or amanullin penetrate more easily into cultured lymphocytes than α-amanitin. This is indicated by an inhibition capacity which is 1.5 and 3 times, respectively, higher. Similarly, some lipophilic ethers of α-amanitin (A17, A18 in Table 3) proved to be three to four times more potent inhibitors of growth than the mother compound (A1). On the other hand, the cationic aminohexylether (A19) was completely ineffective up to a concentration of 10^{-5} *M*. This indicates that growth-inhibiting capacity does in fact strongly depend on the rate of penetration.

Macromolecular derivatives of amatoxins like β-amanitin-bovine serum albumin[67,68] or β-amanitin-γ-globulins did not have an inhibitory effect on the lymphocyte cultures. Phallotoxins did not affect the cells, even in concentrations as high as 10^{-4} *M*.

It is difficult to define lethal doses of amatoxins for cultured cells. The killing effect of the toxins depends on their concentration as well as on the duration of their action. Again, this indicates that the rate of permeation into the cell is an important factor governing toxicity. As early as 1966, Fiume et al.[252] exposed human amnion and KB-Eagle cells to various concentrations of α-amanitin. They found similar correlations between concentrations of toxin, first appearance of morphological changes, and time of complete death of the cells. At concentrations of 10 or 20 μg/mℓ, the first cytopathological effects, fragmentation of nucleoli, were already apparent after 6 to 8 hours, complete death occurring within 3 to 4 days. At 2 μg/mℓ, the first effects were not observed before 1 to 2 days, and all cells were killed after 4 to 6 days. A dose of 1 μg α-amanitin per milliliter caused no cytopathological effects until after 4 days and never totally killed the cells. In a more recent paper,[275] a dozen various cell types have been compared (Table 14). The toxicity is given in terms of a 25% killing rate within 24 hr.

In order to obtain clear effects of the inhibition of RNA polymerase B (or II) by amatoxins

TABLE 14

Toxicity of α-Amanitin for Different Cell Species

Cell species	Toxin[a] (μg/mℓ)
Macrophages	2.5
HeLa-cells	3
KB-cells	5
HEp-2-cells	10
Neoplastic cells from a methyl-cholanthrene-induced sarcoma in Fisher rats	5
Madin-Darby bovine kidney cell line	10
Virus-transformed mouse embryo fibroblasts	5
Virus-transformed human embryo fibroblasts	2.5
Normal human embryo fibroblasts	5
Baby hamster kidney cells	2.5
Green monkey kidney cells	10

[a] Concentration of toxin given is that at which 25% of the cells were killed after 24 hr.

in CHO cells, the drug (5 μg/mℓ) had to act on continuously growing cell cultures for almost 10 hr.[183] In cultures of chick fibroblasts, a diminution of RNA polymerase B (or II) activity to 30% was seen by Hastie and Mahy within 1 hr (to zero after 4.5 hr) with, however, concentrations as high as 20 μg/mℓ of α-amanitin.[217] Even higher concentrations (100 μg/mℓ) were used in experiments with chick fibroblasts in which the disaggregation of nucleoli and the biological activity of the fractions formed was studied by Paweletz.[276] A similarly high concentration also inhibited RNA synthesis and protein synthesis in rabbit zygotes within several hours.[277]

Rat hepatoma cells in culture also seem to be nearly impermeable to α-amanitin, since there was almost no inhibition of a cortisol-induced increase of tyrosine amino-transferase activity in cells added with the toxin 20 min prior to the addition of the hormone.[213] In transformed rat fibroblasts, inhibition of RNA synthesis did not occur at a concentration of 2 μg α-amanitin per milliliter. However, addition of amphotericin B, a membrane-active polyene antibiotic not affecting cell growth by itself, resulted in an almost complete stop of *m*RNA synthesis.[278] In cultures of HEp-2 cells, the same antibiotic, by itself, caused an inhibition of RNA synthesis to 30% at a concentration of 2 μg/mℓ after 6 hr contact, thus frustrating an analogous experiment with the toxin.[267] However, a pronounced effect was found when cells were pretreated with DEAE dextran (+ glucose) and exposed to the acid β-amanitin. A dose of 10 μg/mℓ caused a 60% inhibition of RNA synthesis after only 2 hr incubation. The more lipophilic O-methyl-γ-amanitin also seems to penetrate slightly better since it caused maximum inhibition within 2 hr among other amatoxins tested. Destruction of the membranes of human fibroblasts with ethanol-acetone at 4°C facilitated the penetration of the toxin which inhibited the RNA synthesis almost completely after only 10 min incubation.[248]

Salivary gland cells of *Chironomus pallidivittatus*,[219] *Ch. thummi*,[220,221] and *Ch. tentans*[218,219] have been incubated with α-amanitin concentrations of 1 to 20 μg/mℓ for 60 min and thereafter analyzed by autoradiography and gel electrophoresis of the labeled RNAs. In all cases, a retraction of the puffs of the polytene chromosomes and a strong inhibition of ^{3}H-incorporation into the bands were noted, while the incorporation of ^{3}H into nucleoli remained intact. This is in agreement with analytical data which showed an 80 to 90% reduction in the amount of labeled *hn*RNA.[218] A faint residual radioactivity over whole chromosomes indicated that amatoxin-resistant RNA synthesis was still going on most probably via RNA polymerase C (or III), which is much less sensitive to amatoxins. Similar effects of the autoradiographic pattern of the salivary glands of *Drosophila hydei* were also observed after injection of 4 to 500 ng α-amanitin into midthird instar larvae.[279] Isolated nuclei from salivary glands of the Diptera *Rhynchosciara americana* still possessing the stage specific morphological pattern of the chromosomes incorporated [^{3}H]-UTP in the presence of α-amanitin only into the micronucleoli, whereas the drug arrested RNA synthesis in DNA puffs.[281]

In plant cells (callus cells of parsley), the specific inhibition by α-amanitin of the synthesis of high molecular RNAs (except 32 S) has also been found following incubation with 17 μg/mℓ α-amanitin for 8 hr.[203] An inhibition of the growth of *Avena* mesocotyls by amatoxins (and phalloidin) has also been described.[282]

Varying sensitivity to α-amanitin in cultured chick embryo cells, depending on the cell cycle as well as by transformation by Rous sarcoma

virus, has been reported by Dinowitz et al.[398] Changes were due neither to the appearance of an amanitin resistant polymerase nor to a difference in levels of the enzyme. Rather, the altered sensitivity was attributed to a factor regulating the transcription by RNA polymerase B (or II).

b. Uptake by Pinocytosis

An enhanced penetration of amatoxins covalently bound to proteins into cells which readily take up proteins has been demonstrated by Fiume and Barbanti-Brodano.[275,283] The conjugate formed from β-amanitin and bovine serum albumin caused death in 50% of mouse peritoneal macrophages within 24 hr in the presence of 20 ng/mℓ of the toxin, fixed on 0.8 μg of the vehicle, whereas a 50-fold greater concentration of free α-amanitin was necessary to achieve the same effect. The effect on lymphocytes was much less drastic; more than 50% remained viable after incubation with 100 μg of the conjugate per milliliter (equals 2.6 μg amatoxin). The transformation of cells by phythemagglutinin was also reduced by the same factor in the presence of an equal concentration.[283]

The conjugate inhibits RNA polymerase B (or II) in vitro and hence may either be active in this manner or by releasing β-amanitin inside the cell as a result of proteolytic degradation of the albumin by lysosomal enzymes. The enhanced toxicity of the β-amanitin-serum albumin conjugate already mentioned was initially attributed to a slower rate of glomerular filtration.[284] However, more recently, it has been shown to be due to a specific uptake by the sinusoidal cells of (mouse) liver.[69] Damage and necrosis of hepatocytes do not occur before the sinusoidal cells have been badly damaged or have actually disappeared.

In contrast to mice, rat cells of proximal convoluted tubules of the kidney are not attacked by α-amanitin. However, 5 mg/kg of the protein-conjugated amanitin, corresponding to approximately 150 μg toxin, produced nuclear lesions after 24 to 48 hr comparable to those following normal amatoxin poisoning in liver, while injection of 30 mg/kg (0.9 mg) caused death of the rats within 24 hr. From these experiments, Fiume's group concluded that the cells of rat kidney are susceptible to the action of amanitin but that the toxin is unable to penetrate them unless coupled to albumin and forced to enter by pinocytosis.[284]

o. Amatoxins in Physiological Research

a. Induction of Protein (mRNA) Synthesis by Hormones

The mechanism of the hormonal induction of *de novo* synthesis of enzymes in liver has been a subject of controversy. Control at the level of transcription, translation from *m*RNA, or transport of *m*RNA from the nucleus to the cytoplasm are candidates for consideration. Through the use of α-amanitin, it has been demonstrated in several experiments that the hormonal induction consists of an augmented synthesis of *m*RNA. Sekeris et al.[285] showed for the first time that the induction of tyrosine transaminase in rat liver by cortisol is completely inhibited for over 7 hr when adrenalectomized animals are treated with α-amanitin (0.6 mg/kg) followed by cortisol 1 hr later. The enzyme activity in the livers of rats subjected to cortisol alone rose by threefold within 7 hr, whereas it remained constant when α-amanitin was present. A similar potent inhibition by α-amanitin (1.0 mg/kg body weight) was observed when tyrosine transaminase (and other enzymes) was induced by i.p. injections of dibutyryl cyclic AMP. Without application of hormones or cyclic AMP, treatment with α-amanitin caused a gradual decrease of enzyme activity in the liver of rats due to an inhibition of synthesis. The data permitted the estimation of the half-life of the enzyme as being approximately 3 hr.[286]

The in vivo induction by β-ecdysone (ecdysterone) of DOPA decarboxylase, an enzyme present in the integument of many insects involved in the sclerotizing process, was also inhibited by the fungal toxin when 1 μg was injected into the posterior part of ligated larvae of *Calliphora erythrocephala* 24 hr after pupation of the head.[287] In apparent contrast to larvae, the induction of DOPA decarboxylase by ecdysterone was not inhibited by α-amanitin in adult female *Aedes aegypti*.[288]

Incefy and Kappas observed an in vivo inhibitory effect of α-amanitin on the induction by etiocholanone or allylisopropylacetamide of the enzyme aminolevulate synthetase in chick-embryo liver.[289] A strong inhibitory effect on the nucleoplasmic RNA polymerase was also noted

in chick embryo liver cells in culture. Here, concomitantly, nearly a 20-fold increase of δ-aminolevulinate synthetase was first induced by allylisopropylacetamide, which was then inhibited by 90% during 19 hr incubation in presence of 1.0 μg α-amanitin per milliliter of the medium.[290] As a consequence, the drug also stopped prophyrinogenesis. In hepatocyte cell cultures where cyclic AMP in the presence of hydrocortisone stimulates tyrosine transaminase, α-amanitin of the same concentration[291] and even at a tenfold higher concentration[213] failed to inhibit the induction due to the hindrance to permeation through the cytoplasmic membrane.

Another isolated system in which α-amanitin caused the inhibition of hormonally induced protein synthesis was described by Corradino.[292] In organ-cultured duodena from chick embryos, 1,25-dihydrocalciferol gave rise to a *de novo* synthesis of the intestinal calcium-binding protein. Thus, the increment of ^{45}Ca uptake induced in controls (137%) was reduced to 106% when α-amanitin (0.5 μg/mℓ) was present in the incubation medium.

These findings indicate that DNA transcription by RNA polymerase B (or II), i.e., synthesis of *m*RNA, is required for the induction of enzymes, though an increase in the activity of RNA polymerase A (or I) as induced by the hormones was also reported.

α-Amanitin also proved useful for detecting how the different RNA polymerases increased, e.g., by growth hormone or tri-iodothyronine, in livers of hypophysectomized rats. In these experiments, each of the hormones caused its own pattern of raised polymerase levels.[293]

In immature chickens, both forms A (or I) and B (or II) RNA polymerase are also stimulated by estradiol as measured in isolated oviduct nuclei as well as by the endogenous polymerase activity associated with nuclear chromatin. Form B (or II) represented 90% of the endogenous enzymes as specified by the use of α-amanitin.[294]

In one case, where hormones inhibit protein synthesis, α-amanitin had an synergistic activity. Arnstadt and Stohr[401] reported that the capacity of dexamethasone to block the growth of lymphoma cells in culture in phase G_1 was enhanced by α-amanitin.

b. Indication of Protein Factors Mediating Hormonal Induction

Estradiol causes a marked increase in RNA biosynthesis in the uteri of immature rats 2 to 3 hr after in vivo administration[295] as well as an increased ability in vitro of uterine nuclei from pretreated animals to incorporate nucleoside triphosphates into RNA.[296] The hormone-induced increase in RNA polymerase activity was ascribed to form A (or I) by Raynaud-Jammet et al.[225a] In fact, the increase in transcription due to estradiol was observed only in nuclei incubated in the medium of low-ionic strength (where RNA polymerase A works optimally) and is maintained in presence of α-amanitin. The authors also described that RNA synthesis by RNA polymerase A (or I) in a medium of high-ionic strength did not increase under the influence of estradiol. These results led the authors to envisage the possible existence of a factor activating RNA polymerase A which does not operate in a medium of high-ionic strength and of which estradiol promotes the activity. More recently, the effect of estradiol on the activities of DNA-dependent RNA polymerases in nuclei of rat uterus after in vivo administration has been reinvestigated systematically in the same laboratories.[225b] Now, an early increase of RNA polymerase A (or I) activity of 50% at 1 to 2 hr attaining 100% after 6 hr was stated whether measured in low or high-ionic strength medium. Enzyme activity measured under high-ionic strength conditions mainly reflects the number of enzyme molecules engaged in transcription. RNA polymerase B (or II) activity, measured under low-ionic strength in presence of Mn^{2+}, also increased by 70% already within 2 hr but remained constant during the first 6 hr when measured at high-ionic strength. From these results, it is concluded that estradiol causes an increase in the number of RNA polymerase A (or I) engaged in transcription while the number of molecules of RNA polymerase B (or II) remains constant. The early increase of its activity observed at low-ionic strength would reflect the synthesis of longer RNA chains either by an increase in template capacity or by an activation of RNA polymerase B (or II) molecules already tightly bound to DNA, or both.

An enhanced RNA polymerase A (or I) activity above that of the controls is maintained for 2 hr in surviving uteri from estrogen-treated an-

imals on incubation at 37°C in tissue culture medium. The addition of cycloheximide (25 μg/mℓ) or of α-amanitin (1 μg/mℓ) suppresses that part of the enzyme activity due to hormonal induction. Thus, it appears that a protein (half-life ~ 30 min) activating *r*RNA synthesis may be formed which is itself dependent on a short-lived *m*RNA synthesized by an α-amanitin-sensitive RNA polymerase of the B (or II) type.[297] A similar protein factor mediating the inhibition of *r*RNA synthesis by α-amanitin in vivo has already been discussed.

α-Amanitin was also used to demonstrate the existence of a long-lived intermediate which acts as a mediator of triiodothyronine-induced enzyme induction in rat liver. This intermediate was still active in inducing the synthesis of α-glycerophosphate dehydrogenase after 16 hr and malic enzyme even after 70 hr.[399]

c. Further Processes Mediated by RNA Synthesis

The regulation of active Na^+ transport by mineral corticoids is another process mediated by the stimulation of DNA-dependent RNA synthesis. By making use of the selective inhibition of RNA polymerase B (or II) by α-amanitin, Chu and Edelman[298] found that aldosterone increased the activity of RNA polymerase A (or I) more than that of B (or II) in the nuclei of kidney cells of adrenalectomized rats.

The masculinizing effect of androgens on newborn female rats which becomes apparent in the course of life by severe dysfunctions of the genital organs has not yet been fully understood. Since several studies have localized this effect to the brain, the antagonistic activity of several inhibitors has been studied by Salaman and Birkeit.[299] Testosterone propionate was intracerebrally injected in different doses into 4-day-old rats. α-Amanitin at 0.1 mg/kg or *N*-hydroxyurea (5000-fold higher doses) markedly inhibited the masculinization which must, according to these results, be dependent on the synthesis of a specific RNA in the brain.

d. Amatoxins and Memory

One of the most prominent functions of the brain, memory, seems to be dependent on RNA synthesis. From studies with drugs known to influence RNA and protein synthesis, it must be concluded that the molecular basis of the consolidation of memory involves the synthesis of specific proteins or peptides.[300] α-Amanitin has been used in this context in several experiments with rats[301] and mice.[300] After the drug had been injected into the cerebral ventricles (intracerebroventricularly [i.c.v.]) of the animals, the inhibition of RNA polymerase B (or II) in the brain nuclei was tested in vitro. The results demonstrate that α-amanitin is more than 100-fold more toxic by i.c.v. application than by the usual forms of application in both animal species. Moreover, the i.c.v. lethal doses, LD_{50} for rats and mice, 10 and 2 μg/kg body weight, respectively, reflect the higher sensitivity of the mouse as already stated by i.v. or i.p. administration, where the LD_{50} values are 4 mg/kg and 0.35 mg/kg, respectively. Accordingly, a 40% inhibition of brain nuclear RNA polymerase B (or II) is achieved with lower doses in the mouse (15 μg/kg) than in the rat (50 μg/kg). A 100% inhibition was observed in the mouse with 0.3 mg/kg within 1 hr of the administration of the toxin, whereas a similar effect in the rat required 6 hr and a dose as high as 10 mg of α-amanitin per kilogram of body weight.

In rats, i.c.v. administration of α-amanitin did not affect the RNA polymerase B (or II) of liver nuclei. Consequently, the toxin does not pass the blood-brain barrier.[301] In mice, i.c.v. administration of 0.3 mg toxin per kilogram of body weight inhibited the RNA polymerase B (or II) of the liver by 95% 1 hr after administration. Hence, the toxin can pass the blood-brain barrier,[302] a fact which may well be considered one of the factors responsible for the tenfold higher sensitivity to α-amanitin of mice than rats.

An exceptionally small amount of RNA polymerase A (or I) has been found in the brains of both animal species. In both species, a distinct effect on passive avoidance retention was observed. Mice treated with α-amanitin 2 hr before or immediately after training in an electrical platform test or in darkness avoidance demonstrated a retention deficit 4 hr later compared with saline-injected controls. The effect was seen only at maximal inhibition of brain RNA polymerase B (or II). Furthermore, in an active avoidance test, the animals showed a significant loss of memory under the influence of the drug.[300] Intraperitoneal administration had no effect on any of the reactions.

In rats where the toxin does not leave the brain, injection of only 0.05 mg/kg (inhibition of RNA polymerase B [or II] to 40%) 6 hr before training for a passive avoidance task gave rise to an impaired performance on retesting even after 7 days.[301] More recent studies by the Italian scientists[402] revealed that in rats i.c.v. poisoned with α-amanitin, memory consolidation is impaired when RNA synthesis in the brain has decreased, i.e., after 6 hr, where protein synthesis is still normal, which is affected only after 12 to 24 hr.

e. Circadian Rhythm of RNA Polymerase Activity in Rats

An influence of light or darkness on the activity of RNA polymerases in vivo has been observed in rat liver by Glasser and Spelsberg using α-amanitin as an analytical tool.[303] The shifts of the two principal RNA polymerases were complementary; the activity of RNA polymerase A (or I) rose during the 12 dark hours and fell from noon to the evening, whereas polymerase B (or II) activity rose with the daylight and dropped during the dark hours. The authors attempted to correlate these findings with other biochemical processes which are subject to diurnal variations; however, a definite conclusion from these and related phenomena could not be drawn.

B. Molecular Toxicology of Phallotoxins

It is most probable that the phallotoxins play no role in the lethal mushroom poisoning of humans. This is suggested by several facts. First, all symptoms described for fatal human cases are those also observed with experimental intoxications of α-amanitin alone. Second, amatoxins are eightfold more toxic than phallotoxins; however, the amount (by weight) of amatoxins in *Amanita phalloides* mushrooms is only half that of phallotoxins, thus, the toxic efficiency of amatoxins in mushroom tissue must be fourfold that of the phallotoxins. Finally, phallotoxins are not resorbed by the gastrointestinal tract, at least not in any of the experimental animals investigated so far.

After parenteral application, phalloidin causes a series of lesions in the mammalian liver, which have been the object of many studies. Despite being under investigation for many years, the molecular mechanism of phalloidin action remained undiscovered. Only recently have Wieland and co-workers[51] succeeded in identifying the actin of liver cells as a target protein of the toxin. Since then, most of the experiments and deliberations aim at the understanding of those molecular processes in the cell, which depend on actin and hence may be disturbed by the binding of the toxin to this protein.

1. Hepatocytes

a. Liver Specificity and Rate of Uptake

Unlike the amatoxins, the phallotoxins possess a high specificity for the mammalian liver. In rats, for instance, 2 hr after application of 2 mg of a radioactively labeled phallotoxin, 57% of the radioactivity was found in the liver as opposed to only 9.4% in the skeletal muscle and 2.7% in the kidneys.[304] Related on the basis of dry weight of the different tissues, the liver takes up an amount of phallotoxins (supposed 1.0) which is distinctly higher than that of skeletal muscle (0.02), spleen (0.06), or lungs (0.12); however, the concentration of the toxin in the kidney (0.56) is comparable to that in liver. The relatively high concentration in the kidney may be a consequence of the excretion of the toxin in urine.

Liver specificity of phallotoxins is closely connected with the rapid uptake of the toxins by hepatocytes *in situ* as well as in vitro. In perfused rat livers at 37°C, the organ has taken up 70 to 90% of the toxin only 10 min after its addition to the perfusion medium (Figure 16).[304-306] Similarly, in isolated rat hepatocytes, the uptake is completed after 30 min.[307] This differs totally from the uptake of amatoxins which are resorbed about 20 times more slowly.[306] The mechanism of the rapid uptake of phallotoxins into hepatocytes *in situ* and in vitro is unknown.

The rate of uptake is strongly temperature dependent. For example, perfusion of a rat liver at 27°C instead of 37°C lowers the rate of uptake by 50%. This suggests that the toxin is in fact incorporated by the cells rather than bound to the cell surface. This is confirmed by experiments where unlabeled phallotoxin did not exchange with labeled toxin bound in a perfused rat liver.[306] Though it is well established nowadays that phallotoxins bind predominantly to the actin of liver cells, the amount of

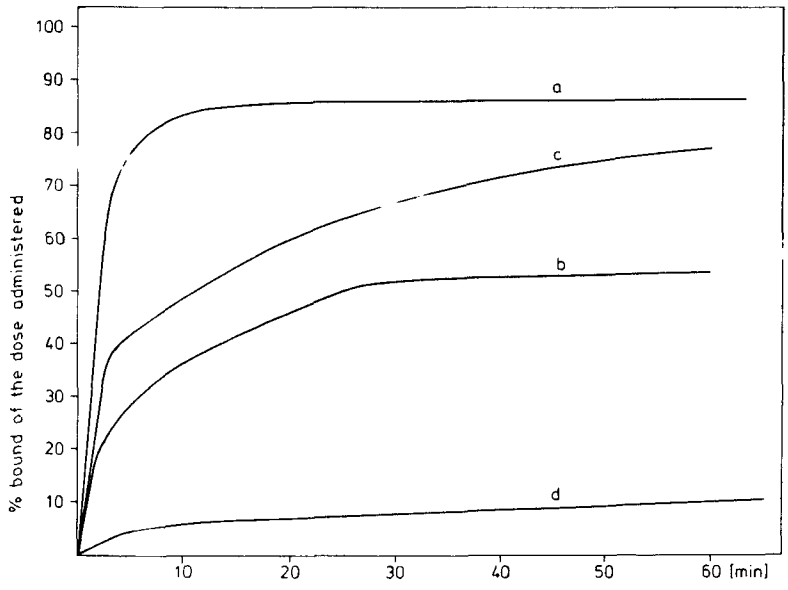


FIGURE 16. Rates of uptake at 37°C by the perfused rat liver of (a) (^{3}H)-demethylpalloin (P9) without antamanide, (b) (^{3}H)-demethylphalloin (P9) with antamanide, (c) nontoxic (^{3}H)-seco-demethylphalloin, and (d) the rate of uptake of (^{3}H)-O-methyl-dehydroxymethyl-α-amanitin (A10), for comparison.

phallotoxins taken up by isolated hepatocytes or by perfused rat livers does not seem to be determined by the amount of actin present in the cells or in the liver tissue.[307] Incorporation is not limited by saturation but may be as high as 350 μg toxin per gram of liver, depending on the toxin concentration in the perfusion medium, whereas the binding capacity of homogenates of liver tissue or hepatocytes was determined to be as low as 15 μg toxin per gram of tissue. It is uncertain whether the toxin which is overincorporated remains free or is adsorbed to structures such as intracellular membranes.

b. Vacuoles in Cells of the Perfused Liver

Morphologically, one of the most prominent alterations caused by phallotoxins is swelling of the liver caused by the development of vacuoles in liver cells (Figure 17). These vacuoles were described in vivo in livers of phalloidin-intoxicated mice as early as 1938,[308] shortly after phalloidin had first been obtained in the crystalline state.[1]

One of the earliest events leading to symptoms of intoxication in perfused rat liver is the formation of vacuoles occurring only 2 min after resorption of the toxin.[309] We suppose that due to the binding of phallotoxins to the actin filaments associated with the plasma membrane, a structural change in the membrane is induced. The release of a small amount of Ca^{++} and a change in the light scattering of perfused liver lobes observed by Jahn,[310] also 2 min after application of phalloidin to the medium, may be due to such a transformation of the plasma membrane. Concomitantly, the plasma membrane seems to relax, permitting the formation of vacuoles as a consequence of extracellular fluid being pressed into the hepatocytes. It is reasonable to discuss whether vacuolization of the liver cells by itself is responsible for most of the other symptoms described for phalloidin intoxication, e.g., swelling of the organ, efflux of K^+ ions, and release of enzymes.

Furthermore, it is a reasonable assumption that invagination of the plasma membrane and penetration of blood components into hepatocytes are the predominant perturbations causing death in the rat and mouse. As early as 1938, Vogt[308] observed the two crucial symptoms of fatal phalloidin poisoning: the liver weight had increased by up to 125% and red cells had penetrated into the dilated interspaces of the cells. Twenty years later, Matchinsky and Wieland determined the blood content in poi-

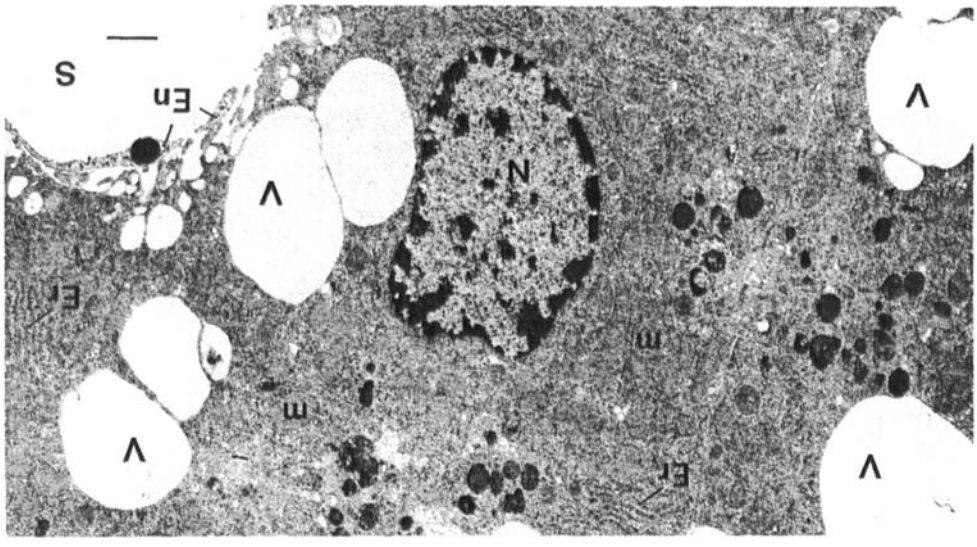


FIGURE 17. Electron micrograph of a section of an isolated rat liver after 90 min perfusion with phalloidin (0.5 mg per 10 g liver) at 27°C. The parenchymal cells contain large vacuoles (V). Stacks of rough endoplasmic reticulum (Er) are still present. N, nucleus; En, endothelia cell; S, sinus; and M, mitochondria (1:8000). (Courtesy of W. Jahn.)

soned livers as being five times higher than in normal livers[311] and postulated that the animals "bleed to death into their own liver." Indeed, these livers absorb more than 50% of their own blood volume. Accordingly, Tuchweber et al.[312] found that up to 13 erythrocytes had penetrated a single hepatocyte via the vacuoles. These workers compared the blood lakes in these livers with liver peliosis. Apart from the erythrocytes, they observed fibrin clots and platelets aggregated in a mosaic pattern in the dilated sinusoids. Therefore, it is most likely that phalloidin death results solely from a hemodynamic dysfunction. Support for this assumption comes from the course of phalloidin intoxication in rats with a two thirds hepatectomy.

c. Origin of Vacuoles in Hepatocytes

It is now widely accepted that the phalloidin-induced vacuolization of liver cells begins at the sinusoids, and in a few cases, at pericellular space.[313] Electron microscopy gave direct evidence for this by showing vacuoles opened to the space of Disse. Vacuolization sets in with a large number of vesicles along the plasma membrane migrating into the cytoplasm; they fuse into vacuoles which become larger and larger.[313] Vacuolization proceeds from the periphery to cells in the central parts within the liver lobules.[314] As early as the beginning of vacuolization, the endothelial lining is disrupted and the space of Disse is dilated.[309,312]

There is a great deal of evidence that vacuoles are a type of endocytotic invagination from the sinusoids. Dolora et al.[315] observed that the inulin space had been dilated twofold by phalloidin. Jahn[316] showed that the vacuoles of hepatocytes in perfused rat livers contained dextran or albumin, present in the perfusion medium, rendering it unlikely that the vacuoles had originated from an increased permeability of water into the cells under the influence of the toxin.[317] Finally, the vacuoles contained erythrocytes and fibrin.[312-314,318] Similar vacuoles in liver cells can be produced by various toxins or treatments.[312,314] Hence, their formation cannot be phalloidin-specific as already stated by Miller and Wieland.[314] For example, Jahn[316] and Frimmer[319] produced vacuolization in the perfused liver by raising the posthepatic pressure mechanically. Both procedures, pressure or phalloidin, produced vacuoles which resembled each other histologically.[316] Also, the typical effects, e.g., liver swelling and efflux of K^+ ions, were similar. However, vacuolization induced by pressure could be reversed easily, while that induced by the toxin could not.[316] In a morphological study, Jahn[320] was able to attribute

this difference in behavior to the presence of a microfilamentous web surrounding the vacuoles in the pressure-treated cells but not in those induced by phalloidin. Evidently, the web of microfilaments has a function in the elimination of vacuoles. In parallel with this, the pressure-injured liver, on reducing the pressure, releases part of the water resorbed from the perfusion medium, in contrast with the phalloidin-poisoned liver which does not.

As for the mechanism of vacuolization, we assume that the structural change of the plasma membrane produced by phalloidin renders the cell surface highly sensitive to even slight variations in pressure. Hence, the low presinusoidal pressure which is normally withstood by the elasticity of the membranes suffices after phalloidin treatment to cause severe damage of hepatocytes by invaginations. The extreme sensitivity of the cells is made manifest by the fact that even a by-pass of the liver (portocaval shunt) did not save rats from phalloidin death. These animals could withstand higher doses of phalloidin but died with their livers soaked with blood, as is typical of phalloidin intoxication.[35]

d. Swelling of the Liver and Subsequent Stages

Swelling of the liver was described in vivo as well as during perfusion as one direct consequence of the vacuolization of hepatocytes. Increases in liver weight between 100 and 200% have been reported. In the perfusion experiments with increased posthepatic pressure, liver swelling depends on the perfusion pressure (and consequently also on the viscosity of the perfusion medium),[316] but swelling of the phalloidin-treated liver was essentially independent of the perfusion pressure.[319] However, it did become more severe when the perfusion medium contained red cells. It has been suggested that it is this factor which accounts for the early death of phalloidin-poisoned animals.

In later stages of the intoxication with phalloidin, Kupffer cells have been reported to be affected by vacuoles, too.[309,312] Another consequence of vacuolization and liver swelling is the rupture of the plasma membranes, as documented by electron microscopy.[312] As a consequence, cytoplasmic, lysosomal, and mitochondrial enzymes are released into the perfusion medium. These effects will be discussed later.

e. Protrusions in Isolated Hepatocytes

Hepatocytes are susceptible to phallotoxins not only in vivo or *in situ*, but also in vitro in the form of isolated cells obtained from the organs by the usual methods, such as treatment with collagenase[321] followed by mechanical separation. However, isolated hepatocytes develop protrusions or pseudopods instead of vacuoles (Figure 18).[322,323] Most probably, these protrusions are occasioned by the same structural change in the plasma membrane which, under the perfusion pressure, produces the vacuoles in the hepatocytes *in situ*.

The strong correlation between the two cytotic processes is evident from the observation that in the late stages of rat-liver perfusions with phalloidin, protoplasmic vesicles are also found to be constricted from the damaged cells *in situ*.[313] The vesicles are found in the sinusoids and contain protoplasmic material similar to the protrusions of isolated hepatocytes. Furthermore, isolated hepatocytes from poisoned livers already suffering from vacuolization of the cells develop pseudopods after being released from the tissue.[324] The strongest evidence for this comes from perfusion experiments with an EDTA-containing medium, which loosens the tissue so extensively that the cells develop protrusions rather than vacuoles.[325]

As with vacuolization in liver cells, pseudopods on isolated hepatocytes can also be induced by other agents. For example, the cytolytic (glyco)-protein phallolysin (Section V) causes similar protrusions at low concentrations which, however, become disrupted.[132] Likewise, maltreatment of the cells such as O_2 or substrate starvation can cause peudopodic protrusions.[326] However, phalloidin-induced protrusions of hepatocytes develop within 10 min in cells kept under optimum living conditions, whereas those protrusions occasioned by starvation occur only after 30 min.

Protrusions in isolated hepatocytes have been produced by micromolar phalloidin concentrations.[323] The sensitivity of the cells depended on their isolation procedure; use of hyaluronidase together with collagenase lowered the sensitivity of the cells by a factor of 25 to 200.[322] This treatment also decreased the vitality of the cells, which were reported to die within 2 hr. In our hands, the phalloidin-treated hepatocytes, though badly deformed in shape, showed no se-

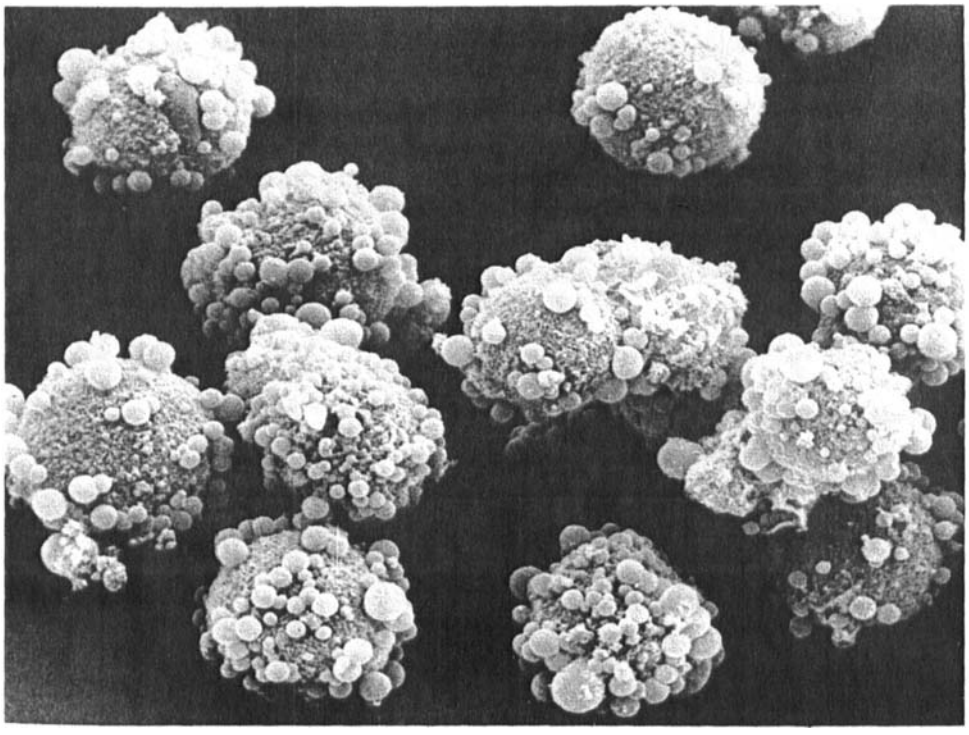


FIGURE 18. Isolated rat hepatocytes after phalloidin treatment. (Courtesy of M. Frimmer.)

rious alterations in metabolism (respiration, glucagon response, etc.).[327] The development of phalloidin-induced protrusions can be inhibited by phalloidin-specific antagonists and used to quantitate antiphalloidinic activities.[323,328,329]

Surprisingly, even with extremely high doses of the toxin, a certain portion of isolated hepatocytes resisted the treatment in all populations under investigation. So far, there is no explanation for this phenomenon. A similar observation was made by Miller and Wieland,[314] who observed single cells in perfused livers which had suffered neither vacuolization nor loss of glycogen.

f. Bile Flow, Microvilli

As early as 1960, Matschinsky et al.[330] observed that bile flow abruptly ceases shortly after the administration of phalloidin to perfused rat livers. The effect was confirmed by many other workers, and there is agreement today that bile secretion stops about 6 min after application of the toxin. Nontoxic secophalloidin does not affect the bile flow, and a radioactively labeled analog of this nontoxic derivative is excreted rapidly[306] (~30% of the amount resorbed per hour). Certainly, the cholestasis by phalloidin also accounts for the retarded clearance of bromosulphalein and bilirubin, as reported by Wieland.[311]

Morphologically, the bile canaliculi in phalloidin-perfused rat livers appear dilated. Parallel with this, the microvilli in the bile canaliculi disappear. Similar morphological changes were also observed in vivo in livers of the white mouse.[312] Since disappearance of the microvilli in the canaliculi, dilation of the bile ducts, and inhibition of bile excretion occur simultaneously, it is reasonable to assume that these processes are interdependent or are links of a causal chain. Loss of microvilli in the bile ducts can also be brought about in rats after continuous application of sublethal doses of toxin according to Gabbiani et al.[331] Once again, the bile flow significantly decreased simultaneously with the loss of microvilli.

Together with the microvilli in the bile canaliculi, the microvilli directed towards the Disse space also disappear.[314] As with vacuolization, this effect could also be produced without phallotoxin by congestion of blood in the perfused rat liver as soon as 5 sec after raising posthe-

patic pressure by ligation. Again, as for vacuoles induced by raised pressure, the effect was reversible.[309]

In isolated hepatocytes, microvilli cover the whole surface of the cells. These microvilli are also affected by phalloidin, becoming shorter and broadened.[322] According to Miller and Wieland,[314] the sinusoidal microvilli of the mouse liver are less affected than those of the rat liver. This difference may perhaps explain why the mouse ($LD_{50} \sim 2.0$ mg/kg) is less sensitive to phalloidin than the rat (1.0 mg/kg).

Loss of microvilli in bile ducts and dilation of bile canaliculi seem to be interdependent processes. Hence, it is reasonable to assume that the loss of sinusoidal microvilli and the vacuolization of the liver cells of the sinusoids are also related processes. Smoothening of microvilli possibly relaxes and widens the membrane, which consequently undergoes invagination even with the small pressure gradients occurring *in vivo* or with low perfusion rates.

g. Release of Potassium Ions and Enzymes

In 1967, Frimmer et al.[317] found that rat livers perfused with phalloidin released K^+ ions. The efflux was as great as 60 mmol K^+ per gram of liver, and the K^+ ion gradient of the hepatocytes was equilibrated within 1 hr after intoxication. Together with cholestasis, K^+ efflux was the first evidence of phalloidin intoxication at that time.

However, in a careful kinetic study, Jahn[310] noticed that the onset of K^+ efflux (12 min after intoxication) was subsequent to some other effects, including Ca^{++} efflux (3 min), increase in light scattering of peripheral liverlobes (2 to 3 min), increase in O_2 consumption (2 to 3 min), and swelling (6 min). Therefore, K^+ efflux must be a secondary effect. This was strongly supported by experiments with 4,7-phenanthroline, which reverts the K^+ efflux but not the underlying lesions caused by the toxin. The phalloidin-intoxicated liver takes back 85% of the K^+ ions from the medium under the influence of this drug. The K^+ ions are released again if the phenanthroline is washed out. A similar reversal of K^+ efflux was observed with EDTA.

Coincidently, Jahn and Frimmer found that K^+ release from perfused livers could be similarly affected by pressure, the release being 20 mmol K^+ per gram[316] to 60 mVal K^+ per gram[319] depending on the conditions. As with vacuolization, the flux of K^+ ions was reversed when the perfusion pressure was reduced. Since vacuolization can also be induced by pressure, K^+ efflux is most probably a result of vacuolization. This is in line with the following experimental data. Firstly, in liver perfusions, the pressure-induced K^+ efflux is inhibited at pH values < 7.0 as is vacuolization; however, the uptake of phalloidin is insensitive to pH changes. Secondly, K^+ release as well as vacuolization, as indicated by uptake of dextrans,[309] are strongly temperature dependent; for instance, when induced by pressure at 8 to 10°C instead of 27°C, both effects decrease concomitantly to 25% or lower. Further support for a direct relationship between vacuolization and K^+ release is the fact that isolated hepatocytes, which do not suffer vacuolization when subjected to phalloidin, also release only minimal amounts of K^+.[332]

Perfused rat livers subjected to phalloidin also release various enzymes into the perfusion medium. The cytoplasmic enzymes prevail among these enzymes. For example, glutamate pyruvate transaminase is released in larger amounts than glutamate dehydrogenase and cytoplasmic malate dehydrogenase more than the corresponding mitochondrial enzyme.[333] Lysosomal enzymes like β-glucuronidase and acid phosphatase are likewise released, but any direct action of phallotoxins on the lysosomal membrane was ruled out.[334] In rabbits, the increase of serum glutamate-oxalacetate transaminase (3150 U/m*l*) was as high as is usually observed under fatal amanitin intoxication.[31] In summary, the enzyme release also appears to be a secondary effect, probably a direct consequence of vacuolization. Correspondingly, isolated hepatocytes under phalloidin treatment released no cytoplasmic, mitochondrial, or lysosomal enzymes.[332]

h. Endoplasmic Reticulum and Protein Synthesis

In 1963, after the first radioactively labeled phallotoxin had been prepared, Rehbinder et al.[305] studied the distribution of the toxin in different fractions (600 g, 8,500 g, and 18,000 g) obtained by centrifugation of homogenized rat livers. Although the highest binding capacity was found in the 600-g fraction at first, during

the following washing steps the 8,500-g fraction retained relatively the largest amount of radioactivity. This fraction was considered to contain predominantly microsomes, but morphological evidence or assays of key enzymes were not given.

From this observation, it was concluded that the primary site of phalloidin action might be the endoplasmic reticulum (ER). It was further supposed that the large vacuoles in the parenchymal cells might arise from dilated ER. This is certainly not true; however, it may be that definite plasma membrane areas in contact with the ER are favored by the toxin and hence develop vacuoles or protrusions, while others do not. Some indication for this is also given by a comparison of the distribution of pseudopods on isolated and phalloidin-treated hepatocytes.

Apart from the vacuolization effect, the ER of hepatocytes, as well as that of endothelial cells, is affected during phalloidin intoxication. Electron microscopy reveals that the parallel stacks of rough endoplasmic reticulum (RER) present in normal hepatocytes gradually disappear.[312,313] RER is decomposed in favor of smooth endoplasmic reticulum (SER) as well as free ribosomes.[312,313,334] The SER forms vesicles or, in many electron micrographs, surrounds mitochondria.[312,334] Biochemical evidence for the changed structure of ER was given by Hohmann and Frimmer,[335] who measured the enzyme constants of glucose-6-phosphatase, a typical enzyme of ER. They found that K_m was unchanged, while V_{max} was increased, a result pointing towards a different arrangement of the enzymes in the membrane after treatment with phallotoxins.

As early as 1960, van der Decken et al.[336] found an inhibition of protein synthesis in slices and homogenate fractions of livers after phalloidin intoxication. Incorporation of [^{14}C]-leucine into proteins was inhibited by 10 to 60% with 10^{-5} to 10^{-6} *M* of the toxin. The inhibition was observed only if the guinea pigs or mice used were intoxicated in vivo or if microsomal fractions were preincubated with NADPH. Inhibition by phalloidin was stronger in the total microsomal fraction than in ribosomes alone. A final evaluation of these data is difficult since NADPH can inhibit protein synthesis on its own. However, it is likely that phalloidin can affect the synthesis of proteins by modification of the ER structure. Gravela et al.[337] reported that there was no inhibition of amino acid incorporation with a liver cell-free system. However, in isolated rat hepatocytes, there was a 75% inhibition in the presence of 2×10^{-5} *M* phalloidin.[400] Onset of the inhibition was observed 30 min after administration of the toxin, at exactly the time where the disaggregation of polysomes induced by phalloidin had been observed previously.[337] It is difficult to decide whether both the polysomal disaggregation and the following inhibition of membrane-bound protein synthesis are primary effects of phalloidin or only represent secondary or tertiary lesions.

i. Energy Metabolism

Another consequence of phalloidin intoxication of liver cells *in situ* is their depletion of ATP and glycogen.[311] Inorganic phosphate and glucose, the products of their breakdown, were found 5 to 10 times increased in the perfusion medium of rat livers.[338] The decrease of ATP and glycogen began only 30 min after addition of the toxin, while it took 40 min after i.p. application in in vivo experiments with mice.[339] A full depletion of glycogen in perfused livers was not observed earlier than 2 hr after intoxication.[311] Hence, the energy depletion is certainly not a primary effect of phalloidin, but rather represents a consequence of other lesions like vacuolization or loss of K^+ ions.

Glycogen depletion has been attributed to an inhibited synthesis of glycogen rather than to a breakdown of the polymer.[311] In 1956, Hess[340] reported on an inhibition of oxidative phosphorylation at the level of the cytochromes when isolated liver mitochrondria were incubated with 10^{-5} *M* phalloidin. However, it is unlikely that this effect can account for the ATP and glycogen depletion described above.

j. Antagonists, Noxae, and Tolerance

Most of the phalloidin effects discussed here can be counteracted by a series of drugs or other treatments. There are good reasons for distinguishing between such substances. First, those which protect the liver from the toxins without doing remarkable harm to the hepatocytes, such as antamanide and silybine, will be called antagonists (Table 15). Secondly, substances preventing phalloidin action by produc-

TABLE 15

Approximate Concentrations (mg/100 ml) of Some Protective Agents Against Phalloidin (0.5 mg/100 ml) in Perfused Rat Liver and With Hepatocytes

Protective agent	Perfused rat liver (50% inhibition of liver swelling)	Isolated hepatocytes (50% protection from the development of protrusions)
Antamanide	>2	—
Carboxymethyl Tyr^5 antamanide	<0.5	2[a]
Silybin hemisuccinate	9	—
Disilybin[b]	1	9
Thioctic acid	—	150
Rifampicin	—	1.3
Evan's blue	Protective effects qualitatively stated	
Ethacrynic acid	Protective effects qualitatively stated	

[a] Phalloidin, 8.0—9.0 mg per 100 ml.
[b] A mixture of oligomers.

ing lesions which probably impair the process of the rapid uptake of the toxin will be discussed under Noxae, Section VI.B.1.j. (2). Both terms will be distinguished from the intrinsic tolerance against phalloidin, e.g., that observed in newborn rats or in animals with regenerating livers.

(1) Antagonists

Antamanide (Section IV), when administered i.p., not perorally, counteracts all effects of phalloidin known so far; it reduces the in vivo toxicity of phalloidin in that a dose of 0.5 mg/kg body weight protects the white mouse against twice the LD_{50} of phalloidin (equals 5 mg toxin per kilogram). In perfused rat livers, pretreatment with antamanide reduces swelling caused by phalloidin to 25% of the value without the antagonist.[10,90,329] Likewise, antamanide prevents the development of protrusions in isolated hepatocytes.[323] Preliminary results of experiments with isolated liver cell membranes suggest that antamanide even inhibits the binding of the toxin and the enlargement of microfilamentous structures.[341] From experiments with radioactively labeled toxins in white mice,[90] perfused rat liver,[306] and isolated hepatocytes,[307] we know that antamanide reduces the amount of toxin bound by the tissue. In addition, the rate of phalloidin uptake is decreased as well as the binding capacity in perfused rat livers.[305,306]

The simplest explanation for these antagonistic effects, a competition of antamanide with phalloidin for one receptor, could be ruled out.[90] Therefore, we assume that antamanide strengthens a complex involving a still-unknown membrane structure associated with cell actin. Since antamanide in nonpolar solvents complexes cations selectively ($Ca^{++} > Na^{+} >> K^{+} > Mg^{++}$), it is possible that complex formation with specifically bound Ca^{++} ions could be involved in the membrane stabilization by antamanide. Possibly the small amount of Ca^{++} released by phalloidin in the perfused rat livers[309] represents the species of Ca^{++} which is shed by phalloidin action and which can be stabilized by antamanide.

Antamanide acts as a protective agent only, not as a curative one. Consequently, for full antamanide protection, the peptide has to be administered before and certainly not later than the toxin. When applied 10 min after the toxin, protection can be achieved by much higher doses but disappears 30 min after application of the toxin. These figures reflect fairly well the time consumed for the uptake and onset of the first phalloidin effects. Therefore, antamanide can partly prevent these changes but is not capable of reversing them.

The same seems to be true for a group of biologically active components of the milk thistle *Silybium marianum* GAERTN., called silymarin.[342] Silymarin reduces the LD_{90} of phallo-

idin in mice to a LD_0 when administered in doses of 15 mg/kg (body weight).[343] A dose of 9 mg of a water-soluble derivative, silybin hemisuccinate, per 100 mℓ of perfusate reduced the swelling of the rat liver to 50%[344] According to Weil and Frimmer,[345] 30 mg silymarin per 100 mℓ perfusion medium inhibits the phalloidin-induced K^+ ion efflux by 100% The protective action for these compounds was also absent when applied 30 min after the toxin.[339] It appears to us that the partial protective capacity observed for a short time after application of the toxins has been overinterpreted by some authors as a "curative" effect. A ninefold more effective inhibition was given by disilybin, probably a mixture of silybin oligomers.[346] In in vivo experiments with mice, 5 mg/kg disilybin reduced the LD_{95} to LD_0.[347]

As with antamanide, the protection by silymarin derivatives in vivo lasts for only a finite time due to the excretion of the protecting compounds in the bile. The maximum protection period of disilybin was found to be 26 hr.[347] This period was only 3 hr for antamanide and some of its water-soluble derivatives.

Although the mechanisms of protection exhibited by silymarin and antamanide seem to agree with each other to a large extent, they are not fully identical. This is concluded from the observation that disilybin protects mice from a fatal intoxication by amanitin,[344] whereas antamanide has no effect in this case.

In 1971, Floersheim[348] reported that the antibiotic rifampicin had a highly protective effect against phalloidin in vivo. This was confirmed by Frimmer et al.[329] by registering the development of protrusions in isolated liver cells. The 100%-protective dose was as low as 100 μg rifampicin per milliliter, this being comparable with the high-protection capacity of a water-soluble antamanide. However, in vivo, there is no full protection of the white mouse against 5 mg/kg phalloidin with less than 20 mg of the antibiotic per kilogram of body weight.* As for the mode of action of this substance, we assume that the uptake of the toxin is reduced by the antibiotic in this case also.

(2) Noxae

In the past, an abundance of substances has been described which increase the resistance of mice, rats, or rabbits to phalloidin. Today, most of them are regarded as drugs which damage or simply cover those surface structures of hepatocytes which are responsible for the extraordinarily rapid uptake of the toxin. For a review of these substances, see Frimmer et al.[329]

Some of the earliest observations in this field were made by Floersheim,[349] who found that pretreatment of mice with carbon tetrachloride or Na^+-cinchophen protects the animals against phalloidin. The doses needed to protect against LD_{95} of phalloidin were 200 μg/kg and 400 μg/kg, respectively. Without any doubt, protection in these cases was achieved by severe damage of hepatocyte structures, which are involved in resorption of the toxin or in development of its action. Morphological evidence for such lesions by carbon tetrachloride and Na^+-cinchophen has been given by many authors. Kroker and Frimmer[35] reported a decreased binding of phalloidin to isolated hepatocytes after treatment with carbon tetrachloride, but the spectrophotometric method used in these experiments is not unambiguous. An inhibitory mechanism similar to those of carbon tetrachloride and Na-cinchophen may also underlie the protective activity of choleretica.[351] Deoxycholate (DOC) slows down the uptake of phallotoxins in perfused rat liver. The 92% uptake after 30 min is reduced to 63% by the drug. In this case, DOC presumably acts on the plasma membrane unspecifically as detergent rather than in a specific way by inducing the bile secretion and so enhancing the elimination of the toxin. This is suggested by the amount of toxin excreted in the bile which is comparably low either with or without DOC (1.3 and 0.7%, respectively).

The mechanism of the protective activity of other agents, like 4,7-phenanthroline or EDTA, requires careful interpretation because they may counteract only secondary or tertiary effects of phalloidin. For instance, 4,7-phenanthroline inhibits the K^+ efflux by phalloidin in the perfused rat liver. Apart from a protective effect, 4,7-phenanthroline also stimulates the perfused rat liver to take back 83% of the K^+ ions released due to the phalloidin.[310] However, when 4,7-phenanthroline is washed out, K^+ ions flow out again, indicating that the lesions induced by phalloidin still exist. Similar effects

* Our own experiments.

were reported for EDTA. Possibly, the protection of K^+ efflux by thioctic acid could also be explained in a similar way.[352]

Jahn observed that Evan's blue, although causing K^+ release by itself, suppressed the K^+ efflux caused by phalloidin.[353] The protection was not observed when albumin was present in the perfusate. It was stated that the dye was 30 to 40% resorbed in the liver if albumin was absent from the perfusate and hence not in competition with the liver proteins. In this case, the occupation of the surface structures which are involved in the resorption of the toxin may protect the liver from phalloidin. This is supported by the observation that Evan's blue likewise inhibits the uptake of indocyanine green. Similarly, the protection of isolated hepatocytes from phalloidin by trypsin[354] may be explained by assuming that the cell surface is covered by trypsin or that a change in the membrane structure is induced by binding the enzyme. After washing out the enzyme, the cells again reacted to phalloidin by developing the characteristic pseudopods.[355]

(3) Tolerance to Phallotoxins

Tolerance to phalloidin can be achieved by prolonged administration of sublethal doses of phalloidin. This was first observed in 1938 by Vogt,[308] who treated mice with semilethal doses at 5-day intervals. Thereafter, the mice survived sixfold lethal doses of phalloidin. The effect was confirmed by Szabados[356] and Floersheim.[357] Under the permanent treatment with phalloidin, Gabbiani et al.[331] observed that the hepatocytes of rats developed an hyperplasia of microfilamentous material. Liver cells isolated from these phalloidin-treated animals incorporated only 50% of the toxin of normal hepatocytes.[307] Accordingly, tolerance to phalloidin in pretreated animals can be explained by a slow resorption rate of the toxin being somehow connected with the high content of actin in these cells. In the same laboratories, an extensive development of tight junctions between rat hepatocytes was also observed within 2 to 13 days under daily administrations of 0.5 mg phalloidin per kilogram.[404]

A natural tolerance to phalloidin was observed in newborn rats. Rats survive a five-[358] to tenfold[318] lethal dose of phalloidin (10 to 29 mg/kg) up to the age of 18 days. According to Siess et al.,[318] phalloidin also causes lesions in the livers of newborn rats, although to a smaller degree. Electron microscopy showed these lesions to be indistinguishable from those in adults. It is noteworthy that there were signs of repair only in the livers of newborn rats and not in adult rats after phalloidin intoxication. The tolerance of newborn rats (and mice) is puzzling and there is, as yet, no valid explanation.

It is well established that drug-metabolizing enzymes are located in liver cells and that these enzymes are not yet developed in newborn rats. These two facts prompted the theory that phallotoxins might not be toxic by themselves but only after toxification by microsomal enzymes. Indeed, conversion to a toxic metabolite could have explained the liver specificity of phalloidin as well as the tolerance of newborn rats.[358] However, this theory had to be abandoned after Puchinger and Wieland extracted more than 95% of a radioactively labeled phallotoxin unmetabolized from the homogenates of poisoned rats.[359] Accordingly, a phenobarbital treatment of 11- to 16-day-old rats could not induce higher sensitivity of these animals to phalloidin.[360] Further proof of the fact that the toxic activity of phalloidin is independent of the metabolizing enzyme system was given recently by Guenther and Nebert, who found that phalloidin neither induces P_{450}-dependent enzymes nor requires protein synthesis for its lethal activity in mice.[361] Finally, no metabolites of phalloidin could be detected in rat livers, neither in the perfusion medium nor in the bile after application of low doses of toxin.[304]

Tolerance to phalloidin was also observed by Frimmer and Schischke[362] and Tuchweber et al.[363] in rats after ectomy of two thirds of the liver. Both laboratories agree that the tolerance reaches an optimum 3 to 4 days after the operation. After the fifth day, tolerance became weak[362] and disappeared totally after 2 weeks.[363] However, there is no agreement as to whether or not there are morphological changes induced by phalloidin in the cells of the regenerating liver. A further kind of tolerance to phallotoxins was recently described by Agostini et al.[364] for rats with liver carcinoma induced by diethylnitrosamine. These rats tolerated ten times the lethal dose. Rats pretreated with D-galactosamine showed a similar tolerance against the toxin.[390]

In trying to make a synopsis of the various kinds of tolerance, it is tempting to seek a common basis in the fact that most of the less-sensitive hepatocytes are cells in the proliferating state. This is true for the hepatectomized rats as well as for the rats with liver carcinoma. This may also prove correct for the hepatocytes of newborn rats whose livers increase in weight or those which just left the state of growth.

A more general explanation of the effect of tolerance should also account for the tolerance following phalloidin treatment; Agostini et al.[364] reported an increase in filamentous material in the carcinoma cells similar to that after long-term administration of sublethal doses of phalloidin. Thus, it might well be that a phalloidin insensitive cell, proliferating or not, is one rich in microfilaments, i.e., cell actin. This view is supported by the observation that lymphocytes with a high content of actin, take up phalloidin slowly, while HeLa-cells with an actin content lower than that of hepatocytes, incorporate the toxin rather rapidly.

2. Cells Other than Hepatocytes

The specificity of phallotoxins for hepatocytes is so marked that only a few experiments have been done with other cells.

As a consequence of phalloidin intoxication, damage in sinusoidal cells of the rat liver has been reported once.[318] Here, the endothelial cells had developed the typical vacuolization (very similar to that of the surrounding hepatocytes).

Specific damage of sinusoidal cells was achieved by Barbanti-Brodano et al.[87] These authors prepared a bovine serum albumin derivative of phalloidin which was preferentially resorbed by sinusoidal cells. It remains open as to whether the phallotoxin acts as the protein derivative itself or only after release of the toxin in the cell. In any case, the vacuoles observed 8 hr after application represented damage by phallotoxins. For endothelial cells, it was also observed, more distinctly than in hepatocytes, that phalloidin modifies the ER. In these cells, vacuoles were formed from the dilated spaces of rough endoplasmic cisternae. Furthermore, it is important to note that the vacuoles of the sinusoidal cells reverted and disappeared after 24 hr. Again, this indicates that vacuolization per se cannot be a lethal lesion.

The protein-phalloidin conjugate also caused vacuolization in other protein-consuming cells, e.g., those of the proximal tubules in the kidney and in macrophages.[87] The RER was dilated in tubule cells, forming vesicles of various size. Instead of vacuolization of RER, there was an increase in the SER in some cases. Macrophages in culture were killed by the protein conjugate. Although a concentration of 25 μg of free phalloidin per milliliter produced no dead cells, only a quarter of this amount, conjugated to 388 μg of albumin, was sufficient to kill all the cells in the culture.

The molar concentrations of phalloidin conjugate needed to kill 25% of the macrophages are rather high. Barbanti-Brodano reported a concentration as high as 1.3×10^{-4} *M*.[87]

Also, with free phalloidin, high concentrations were necessary to inhibit the growth of Epstein-Barr virus-transformed lymphocytes ($>10^{-4}$ *M*).[274] Despite these high concentrations necessary for growth inhibition, lymphocytes also take up phalloidin at low concentrations. For example, at 10^{-6} *M*, phalloidin lymphocytes resorb 0.4 μg/100 mg wet tissue.[307] However, to suppress the growth of the cells requires a 100-fold higher toxin concentration in the medium.

The rate of phalloidin uptake in lymphocytes is very slow compared with hepatocytes and HeLa cells.[307] We related the amount of toxin taken up after 30 min by the cell suspensions to the binding capacity of the cell ghosts or homogenates, which probably represents the amount of actin present in the cell.

It is evident from Table 16 that those cells which contain relatively high amounts of binding capacity in the homogenate, probably due to actin, incorporate the toxins very slowly (26 and 29% in lymphocytes and pretreated hepatocytes, respectively), while other cells with a low binding capacity in the homogenate incorporate considerably more toxin than could be bound by actin present in these cells. These results demonstrate that the incorporation of the toxin is not paralleled by the amount of actin in the cell; on the contrary, a high content of actin in a cell apparently inhibits the incorporation of phallotoxins.

Blood platelets are known to be rich in actin; this fact prompted some experiments with these cells. Behnke[365] observed no increase in the number of microfilaments or any change of

TABLE 16

Uptake of Labeled Phallotoxin by Various Intact Cells and by the Corresponding Ghosts after 30 min Incubation with 1—2.5 × 10^{-6} *M* Toxin at 37°C

Cell species	Toxin concentration	Toxin bound by intact cells (μg per 100 mg wet tissue)	Toxin bound by ghosts (μg per 100 mg wet tissue)	$\frac{\text{Toxin bound by ghosts} \times 100}{\text{Toxin bound by intact cells}}$
Lymphocytes	10^{-6} *M*	0.33	1.25	26
HeLa cells	2.5×10^{-6} *M*	1.1	0.73	151
Rat hepatocytes	2.5×10^{-6} *M*	1.53	0.97	158
Hepatocytes of rats pretreated with phalloidin	2.5×10^{-6} *M*	0.83	2.82	29

shape, aggregation, or contraction of platelets; thus, neither the morphology nor the function of platelets is affected by the toxin.

3. Isolated Plasma Membranes and Microfilaments

Up until now, isolated membranes have only rarely been used in experiments with phalloidin. In those studies performed, the membranes were obtained exclusively from rat-liver tissue.

In 1969, Hegner et al.[366] investigated whether the K^+ efflux from hepatocytes could be due to a phalloidin-induced inhibition of cation-dependent ATPases in the membrane. They found that the toxin affected neither the Mg^{++}-dependent nor the Na^+/K^+-dependent ATPases; likewise, the activity of K^+-dependent *p*-nitrophenylphosphatase was insensitive to phalloidin.

Govindan et al.[341] isolated rat-liver plasma membranes according to Ray[367] and found that there was a distinct increase of filamentous structures which were only occasionally present in the controls (Figure 19) in the preparations from phalloidin-intoxicated rats. An increase in filamantous material also appeared after in vitro poisoning of plasma membranes obtained from healthy animals. The filaments in these preparations consisted predominantly of cytoplasmic actin which, when centrifuged, accompanied the membrane particles. It is uncertain whether the filaments stick to the membrane material or are only randomly associated. In SDS-gels of the membrane preparation, the actin band is about tenfold that of control preparations.[368] The increase was not observed with the nontoxic phalloidin oxide (P21 [*S*]) and could be inhibited by treating the rats or the membrane preparation with antamanide before intoxication. Thus, it is evident that the increase of microfilaments represents a specific effect of phalloidin. In subsequent experiments with radioactively labeled toxin,[369] the centrifugation procedure was refined with the result that the specific binding capacity increased together with the amount of filaments observed by electron microscopy in one fraction. There can be no doubt that the filamentous material isolated with the plasma membrane fraction in these studies is the microfilamentous material later described by Gabbiani et al. as being formed in rat liver after prolonged in vivo

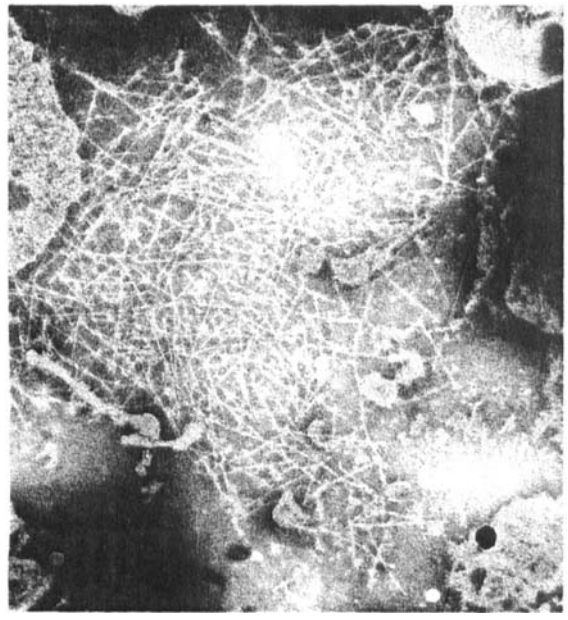


FIGURE 19. Electron micrograph of membrane fragments of the liver of a phalloidin-poisoned rat. (Courtesy of A. M. Lengsfeld.)

administration of phalloidin.[331] SDS-gel electrophoresis of liver filaments indicated a strong band of molecular weight of approximately 40,000 dalton, indistinguishable from that of rabbit-muscle actin.

A similar membrane preparation of rat liver was used to investigate the binding properties of phallotoxins by Lutz et al.[370] They found that the ^{3}H-demethylphalloin dissociated with a K_D of 3×10^{-8} *M*. Despite its high affinity, the labeled compound exchanged rapidly and specifically with unlabeled toxin ($T_{1/2}$ = 180 sec). Antamanide had no effect on the binding of the toxin. Recently in our laboratory, the K_D of the same labeled toxin complexed with muscle actin was determined to be 3.6×10^{-8} *M*.[371] The similarity of the two dissociation constants renders it likely that the phallotoxin binding site in the rat-liver plasma membrane is predominantly actin and that this is isolated together with the membrane particles.

4. Phallotoxins and Muscle Actin

a. Stabilization of Filamentous Actin

One of the outstanding features of muscle actin is that it can exist in at least two forms, a monomeric form (G-actin) and a polymeric, fi-

lamentous form (F-actin). Phallotoxins bind to polymeric or oligomeric actin only, thus stabilizing the filaments. It was this stabilizing effect leading to the formation of abundant microfilaments in liver cell preparations of a phalloidin poisoned rat which lead to the discovery of the target protein.

Identification of the filaments as actin had been hampered by the stabilizing capacity of phalloidin; the treatment commonly used to identify F-actin, 0.6 *M* KI,[372] failed because phalloidin rendered the filaments too stable to undergo depolymerisation by chaotropic ions.[373] However, subsequently, an analogous result was obtained for actin isolated from rabbit muscle. Since then, muscle actin, which is more easily available than cell actin, has become the protein of choice for the study of the various consequences of phalloidin interaction with actins.

The resistance of phalloidin-complexed actin to 0.6 *M* potassium iodide was quantitated by viscosimetric measurements. At a concentration of 3.6×10^{-5} *M*, F-actin and its complex with phalloidin both exhibit specific viscosities η of ~ 1.2. On addition of 0.6 KI, η of a solution of F-actin decreased to 0.03,[374,375] whereas the viscosity of the phalloidin-actin complex remained unchanged. The viscosimetric assay using KI proved useful in determining what may effect the destabilization of actin filaments, e.g., cytochalasin B.[376] Furthermore, this assay made a correlation between the stabilizing effect of various toxins and their in vivo toxicity possible. Löw and Wieland[374] found that the neutral toxins (phalloidin) as well as the acidic toxins (phallacidin) or toxic derivatives of phalloidin, such as the (*R*)-configurated sulfoxide, could stabilize F-actin against KI. Other nontoxic derivatives of phalloidin, e.g., (*S*)-configurated phalloidin-sulfoxide or seco-phalloidin, failed to do so. These data suggest the likelihood that the in vivo toxicity of phalloidin and the stabilization of actin filaments are, at least, related events. Antamanide did not counteract the toxins in this system, indicating that antamanide protection against phallotoxins is on a molecular level, more complex or different from that of actin.

A protein capable of depolymerizing actin filaments by forming complexes with the monomers is pancreatic DNAase I.[377] The enzyme is inhibited by G-actin and this inhibition may be significant under physiological conditions.[378] In recent experiments, the activity of DNAase I was first inhibited by about 36% by G-actin and then fully restored by the addition of phalloidin.[379] The result confirms the observation of Lindberg and Lazarides that only monomeric actin binds to DNAase I. Most recently, this complex has been obtained in crystals.[380] Phalloidin probably destroys the 1:1 complex of the two proteins by forming a stronger complex with the actin polymer.

Another system for studying the stabilizing effect of phallotoxins has been the assay of the ATPase activity of actin first detected by Asakura.[381] This ATPase activity is induced in actin filaments under certain conditions or by drugs which break or loosen the interaction of actin protomers. The subsequent reassembly of the filaments is accompanied by the splitting of ATP and incorporation of ADP into actin as the essential nucleotide. ATPase activity can be produced mechanically by sonic vibration as well as by acidic pH or by addition of cytochalasin B.

According to Dancker et al., the ATPase activity induced by sonic vibration can be completely inhibited by phalloidin (see Figure 22).[375] The ATPase activity induced by protons with maximum activity at pH 4.7 was also inhibited by the toxin at pH values between 6 and 8. At pH values lower than 3.5 and in the absence of toxin, the structure of F-actin is so loosened that denaturation occurs (no ATPase activity). Phalloidin stabilizes the structure of the fragments, thus maintaining the enzymic activity even at low pH values.[382]

Addition of cytochalasin B decreases the specific viscosity η of F-actin solutions by a still unknown mechanism. The viscosity drops with this alkaloid, although actin is not depolymerized; in fact, the actin monomers decrease. According to Löw and Dancker,[383] cytochalasin B affects actin filaments only if the filaments are not stabilized by 0.1 *M* KCl.

Phalloidin counteracts the drop in viscosity of actin solutions caused by cytochalasin B.[376] Together with the drop in viscosity, cytochalasin B induces ATPase activity due to an enhanced repair reaction of the filaments. Phalloidin also counteracts this ATPase activity. In preparations of liver cell membranes, cytochal-

asin B seems to diminish the number of filaments induced by phalloidin.[384]

It is well established that monomeric actin is denatured more rapidly than polymeric actin. Therefore, it is reasonable to assume that in an equilibrium of the two forms, the actin monomers and terminal protomers will be predominantly affected. This is supported by the fact that at pH < 3, breaks in the filament occur so frequently that denaturation becomes faster than the ATP-splitting repair process (see above). If this concept is correct, phalloidin should also prevent heat denaturation of actin. Indeed, deVries et al.[385] found that by heating actin to 70°C for 3 min, it is largely denatured as estimated by turbidity; under the same conditions, the presence of 1 equivalent of phalloidin reduced this process to only 20% (Figure 20). As with the results in viscosimetric studies,[374] nontoxic seco-phalloidin had no protective activity. Toxic phalloidin R-sulfoxide exerted a full protection whereas the "nontoxic" (*S*)-sulfoxide protected only partially. This is in agreement with the fact that the affinity of the (*S*)-compound to actin is distinctly lower than that of the (*R*)-diastereomer.

It could be that not only denaturation but also digestion of F-actin by subtilisin need monomers and filament terminals to start on. This was suggested by the finding of deVries and Wieland,[386] who detected that phalloidin suppresses the proteolysis of actin by subtilisin, probably by consolidation of the double helical structure.

Recently, phalloidin has been used to prepare columns of Sepharose 4B with trapped actin filaments for the separation by affinity chromatography of myosin, heavy meromyosin, and heavy meromyosin subfragment 1 by Grandmont-Leblanc, and Gruda.[403] The columns stabilized with the toxin did not show any leakage of protein even after 2 weeks and remained active for months.

b. Acceleration of Actin Polymerization

Dancker et al.[375] studying the polymerization process of actin by measuring the light scattering, found that 1 equivalent of phalloidin increased the polymerization rate by a factor of 7 (Figure 21). The polymerization was started by addition of Mg^{++} ions. Even in the presence of KI at a concentration as high as 0.5 *M*, a slow phalloidin-induced polymerization occurred. The accelerated polymerization can be

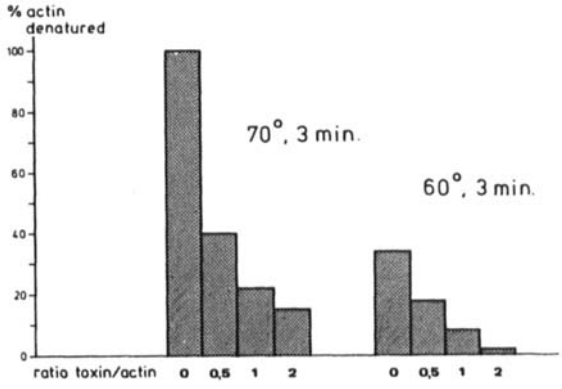


FIGURE 20. Protection of rabbit muscle F-actin from heat denaturation by phalloidin as followed by light scattering. (From deVries, J. X., Schafer, A. J., Faulstich, H., and Wieland, Th., *Hoppe Seyler's Z. Physiol Chem.*, 357, 1139, 1976. With permission.)

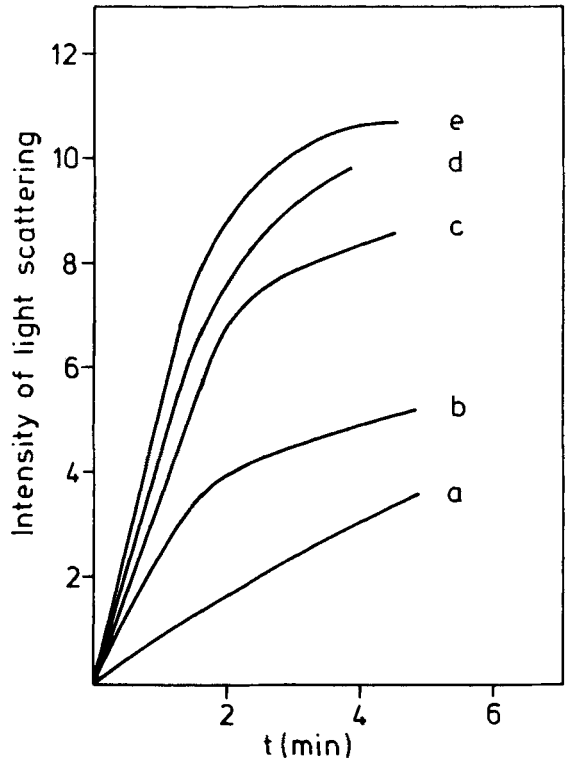


FIGURE 21. Rate of polymerisation of rabbit muscle G-actin ($3 \times 10^{-5} M$) (a) without phalloidin, (b) with 0.1 equivalents (c) 0.2 equivalents (d) 1.0 equivalents, and (e) 1.7 equivalents of the toxin. (From Dancker, P., Löw, I., Hasselbach, W., and Wieland, Th., *Biochim. Biophys. Acta*, 400, 407, 1975. With permission.)

related to the stabilizing capacity of phallotoxins. It is reasonable to assume that the toxin binds not only to actin filaments but also to oligomers, which are the nuclei of the polymerization reaction. According to the model of Wegner and Engel,[387] the nuclei are actin trimers. However, formation of trimers is limited by the rapid dissociation of dimers. Hence, the strong acceleration effect of phalloidin would consist of phallotoxins binding to the smallest kind of actin aggregates, the dimers. The stabilization of actin dimers could efficiently increase the number of the trimer nuclei and thereby the rate of polymerization.[307]

c. Mechanism of Interaction Between Actin and Phallotoxins

Direct evidence for the interaction of phallotoxins with actin was obtained from difference UV spectroscopy[388] (see Figure 22). The difference spectrum shows two peaks at 305 and 295 nm and a shoulder at 287 nm. As for the question whether the chromophoric system of the tryptophan and tyrosine side chains of the protein or that of the tryptophylthioether moiety of the toxin are disturbed by this interaction, it is evident that at least the absorption band at 305 nm must be attributed to the toxin. Apparently, the 300-nm absorption band of the toxin (Figure 6) is shifted 5 nm to longer wavelengths by interaction of the toxin chromophore with lipophilic regions of the protein. A difference spectrum was also obtained from the toxic (*R*)-sulfoxide of phalloidin (P21 [*R*]) as a single peak at 300 nm. The long wavelength and the altered shape of this spectrum give further support that it is the aromatic part of the toxins rather than those of the protein which predominately contributes to the difference spectrum.[385] Unexpectedly, the nontoxic (*S*)-sulfoxide (P21 [*S*], Table 5) exhibited a similar difference spectrum. However, the affinity of the (*S*)-sulfoxide is many times lower than that of the toxic sulfoxide as shown by a dilution series.[385] Direct evidence for the low affinity of the (*S*)-sulfoxide was given in an exchange experiment; addition of small amounts of phalloidin to the complex of actin + (*S*)-sulfoxide instantly turned the difference spectrum of the sulfoxide into that of phalloidin with the characteristic two peaks at 305 nm and 295 nm. In a corresponding experiment, the difference spectrum of actin + (*R*)-sulfoxide remained unchanged.

Phallotoxins form 1:1 complexes with each protomer of polymeric actin. Evidence for this ratio first came from the viscosimetric studies where full protection of F-actin filaments against KI-induced depolymerization was achieved only by equimolar amounts of phallotoxins.[376] Likewise, ATPase activity induced by sonic vibration was not completely inhibited at molar ratios of toxin per actin lower than 0.8 to 1.0[375] In difference spectrophotometry, the maximum optical density at 305 nm was almost attained with 1 mol toxin per mole of actin. Addition of excess toxin, 2 to 6 mol/mol actin, increased optical density$_{305}$ only by 6%.[388] Final

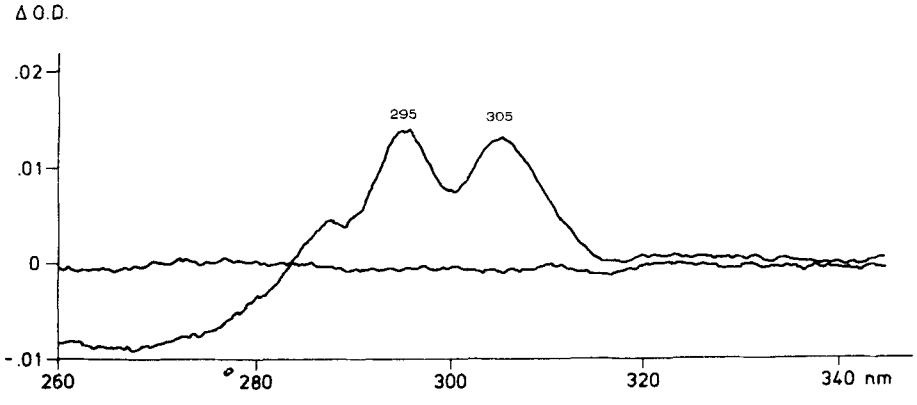


FIGURE 22. Difference ultraviolet spectrum of equimolar amounts (1×10^{-5} *M*) of rabbit muscle actin and phalloidin. (From Wieland, Th., deVries, J. X., Schafer, A., and Faulstich, H., *FEBS Lett.*, **54**, 73, 1975. With permission.)

proof for the 1:1 complex of phallotoxins and actin was recently given by equilibrium dialysis experiments.[371]

Although maximal protection of actin filaments was achieved with equimolar amounts of toxin, the protective activity was in many cases higher than would be expected from the corresponding toxin concentration at molar ratios <1. For example, ultrasonic ATPase was inhibited by 50% with only 0.15 to 0.25 equivalents of toxin (Figure 23).[375,376] DNAase I activity, partially inhibited in the presence of G-actin, was totally restored when only 0.26 equivalents of toxin were added, based on the actin present in the incubation.[379] In kinetic studies of actin polymerization, a 40% increase in the acceleration rate was achieved with only 0.1 equivalents of toxin.

This cooperativeness in phalloidin action was quantitatively treated by Dancker et al.[375,376] using binomial functions. They found that the dose-response curve of ATPase inhibition by phalloidin obeys the function $A = 100 \times (1 - P)^3$, where A is the % ATPase activity and P is the concentration of phalloidin. Hence, a single phalloidin molecule can extend its stabilizing effect over three actin protomers. We do not know as yet whether the cooperative stabilization involves the two axial neighbors of a protomer or one axial neighbor and one equatorial one as in the trimer nucleus of polymerization. Likewise, it remains open whether cooperativity is exerted by an allosteric effect or, at least partially, by a bifunctional binding of the toxin between protomers. A bifunctional arrangement of the toxin complex is suggested by some structure-activity relations of phallotoxins.

More detailed information on the complex of phallotoxins with muscle actin was obtained by the determination of the equilibrium dissociation constants.

By equilibrium dialysis, we determined a K_D value of 3.6×10^{-8} *M*, or lower for ^{3}H-demethylphalloin.[371] At the same concentration, labeled $^{45}Ca^{++}$ or [^{14}C] ADP, both of which are tightly bound components of the filaments, are half dissociated from the toxin-actin complex. Since Ca^{++} and ADP only dissociate from monomers and not from the polymers, Ca^{++} and ADP can be used as markers for the presence of G-actin. In conclusion, the dissociation of the toxin from the polymers occurs concomitantly with the depolymerization of the filamentous form at a protein concentration of 3.6×10^{-8} *M*.

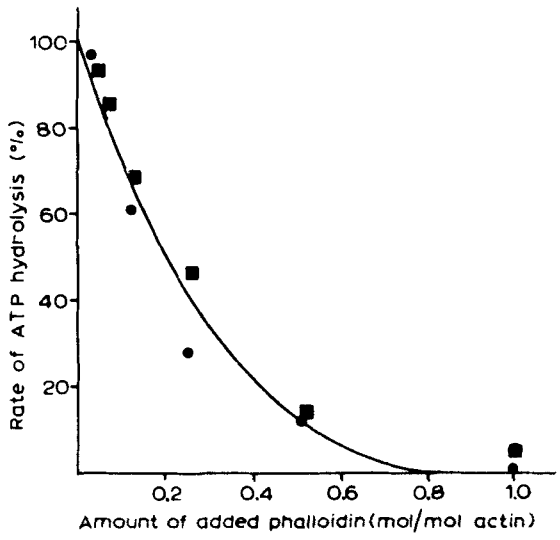


FIGURE 23. Inhibition by phalloidin of ATP splitting during sonic vibration of 3×10^{-5} *M* rabbit muscle F-actin. (From Dancker, P., Löw, I., Hasselbach, W., and Wieland, Th., *Biochim. Biophys. Acta*, 400, 407, 1975. With permission.)

When the dissociation of ^{45}Ca and [^{14}C] ADP was followed in the absence of the toxin, half dissociation was found at 10^{-6} *M*. This value is close to the critical concentration of actin under these conditions, i.e., the concentration of F-actin, where half of the protein is depolymerized to monomers or, in other terms, the concentration of monomers which is in equilibrium with the filaments.

According to this, phalloidin may be defined as a drug, which in equimolar concentration, decreases the critical concentration of actin from 10^{-6} *M* to 3.6×10^{-8} *M*, i.e., by a factor of 30. The presence of 2 equivalents of toxin by mass action decreases the concentration of actin monomers by a factor of 100.

Furthermore, the experimental arrangement of equilibrium dialysis allowed us to directly compare the affinities of various phallotoxins by measuring those concentrations of the toxins necessary to exchange 50% of bound ^{3}H-demethylphalloin. For example, for the nontoxic (*S*)-sulfoxide P21 (*S*), we determined that $K_D = 1.2 \times 10^{-5}$, which is more than 300 times higher than that for phalloidin; K_D of the (*R*)-sulfoxide, 3.2

$\times 10^{-7}$ *M*, is about 9 times higher than that of the mother compound, $K_D = 3.6 \times 10^{-8}$ *M*.[389]

In summary, we can assume that the phallotoxin + actin complex, which stabilizes the polymeric form of the protein, can account for all the lesions observed in the liver cells. As for the molecular mechanism, we can consider two possibilities. First, that the binding of phalloidin to actin filaments changes the affinity of the filaments for other cell components. This is not the case with the interaction of actin filaments with myosin since the ATPase activity of actomyosin was not influenced by the drug.[375] However, viscosimetric studies demonstrated that phalloidin-treated actin filaments no longer bind to troponin and tropomyosin.[375] Similarly, the interaction of actin filaments with actinin, being essential for the structure of microvilli in the plasma membrane could be impaired by the toxin. Disturbed interactions as cited above could interfere with regulatory processes in the cell or with structural features of the plasma membrane.

The second mechanism considered here is more conclusive than the first. It takes into account that the low concentration of actin monomers in a poisoned cell might become crucial by seriously delaying the intracellular translocation of actin. We assume that a translocation of microfilaments in the cell occurs exclusively by the *de novo* formation of microfilaments, wherever needed, from a pool of monomers. If this assumption is correct, the velocity of the nucleation step of the new filament as well as that of the following propagation step depend on the concentration of monomers (c_1). Following a recent suggestion by Wegner and Engel for muscle actin,[387] the increase in filamentous actin was found to be $dc_p = K \cdot (k_1 c_1 - k_2) \cdot c_1^2$, where $k_1 = 5 \times 10^3$ [M^{-1} sec^{-1}] and $k_2 = 3 \times 10^2$ sec^{-1} (rate constants of polymerization and depolymerization, respectively). The formula indicates that the increase of filamentous actin is $\sim [c_1]^{1-2}$. Since the concentration of monomers under physiological conditions is 10^{-6} *M* and since this concentration is decreased to 3.6×10^{-8} *M* in the presence of 1 equivalent of toxin, the formation of new filamentous material at places of actin function is decreased by a factor >30. This means that cellular processes which, for example, might require 15 min for the synthesis of new microfilaments or filaments, will last 8 hr or longer in the presence of 1 equivalent of phalloidin.

The equation further illustrates the toxicological significance of excess phalloidin. For example, in the presence of 2 equivalents of toxin, the formation of filaments would be decreased by a factor >100 and the process mentioned above would require 25 hr or longer.

Further experiments are needed to elucidate whether proliferating or transformed cells can compensate for the lack of monomeric actin by *de novo* synthesis of this protein in a way similar to that of hepatocytes poisoned with sublethal doses of phalloidin.

The actin concentration in liver cells as determined by radioactively labeled toxin is 1 mg/g liver tissue. It corresponds well with the uptake of 20 μg of labeled phallotoxin per gram of liver tissue in a perfused rat liver, assuming a 1:1 complex. Further support for this actin concentration was given by the in vivo uptake of phallotoxin in the mouse which is 25 μg/g liver tissue, corresponding to about 50% of the LD_{50} which is 2.5 mg/kg body weight. Since even a slight excess of the toxin in the liver cells can delay the mobility of actin with high efficiency, a steep dose-response curve of the in vivo toxicity in the mouse would be expected, and indeed, the LD_{90} for mice was found to be only 3 mg/kg, whereas $LD_{50} = 2.5$ mg/kg.

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