Fifty years of amanitin

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Abstract. Pharmacokinetic studies have provided new insights into human *Amanita* poisoning, but it appears to be impossible to treat this intoxication by immunotherapy. New synthetic analogs have revealed structure-activity relationships that were unknown so far. The main toxin, α-amanitin, is in constant use as a tool in molecular biology and in biological research. First experiments have been reported in which amanitin bound to polymers could be internalized into tumor cells via a receptor-mediated endocytosis.
Key words. Amatoxin; peptide synthesis; structure-activity relationship; tool of biological research; receptor-mediated endocytosis.

It was in 1940 that Rudolf Hallermayer described in his Ph.D. thesis the crystallization of 'amanitin', derived from the poisonous green mushroom *Amanita phalloides*, known as the 'death cap'. He achieved this in the laboratory of Heinrich Wieland at the Ludwig-Maximilian University in Munich, crowning a 10-year effort by the Munich investigators [34].

First experiments designed to elucidate the chemical nature of amanita toxins go back to the beginning of this century, when William W. Ford in Baltimore, Maryland, started an investigation of the toxins of an equally poisonous white mushroom, most probably *Amanita virosa*, the 'destroying angel'. Ford gave the name 'amanitatoxin' to the heat-stable factor. He described experiments to enrich it in a publication with Hermann Schlesinger [17]. In retrospect, it is amazing that the crude methods of alkaloid chemistry in use at the time allowed Ford to obtain, albeit in miniscule yield, a preparation that killed a full-grown guinea pig (250–400 g) with a subcutanous dose of 0.4 mg. In the light of current knowledge, this indicates a content of about 10% pure toxin in his preparation. Apart from these experiments, there was very little useful knowledge available in this field before the start of the work in Munich. A. Bertho, H. A. Raab, J. Kimmig and J. Renz, F. Lynen, and U. Wieland advanced the research, and in 1937 Feodor Lynen and Ulrich Wieland succeeded in crystallizing the fast-acting poison phalloidin. This work was the subject of a review three years ago [37]. However, 'amanitatoxin', which alone can result in lethal poisoning, was not at that time enriched enough for crystallization to be possible. It was primarily Hans A. Raab who struggled in the thirties to obtain this substance in its pure form. He has described in detail the early history of amanitin research with its many wrong turns [26]. Finally, 50 years ago, the breakthrough occurred with preparation of pure 'amanitin'. Its step-by-step enrichment was monitored by toxicity testing in the white mouse. In this animal the LD_{50} for α-amanitin is about 0.5 mg/kg body weight. Other animal species are more or less sensitive. The LD_{50} in the guinea pig, for example, is 0.05–0.1 mg/kg, and the dose for human beings is probably as low as or even lower than this. On the other hand, albino rats are about 5–10 times more resistant than mice.

Amanitin poisoning

As is known from fatal mushroom poisoning of humans, amatoxins are toxic agents which develop their effects slowly. Not until 8–10 h after the meal, at the earliest, is there a severe gut reaction, which is followed by a pseudo-recovery with hardly any symptoms. On the third day, however, necrosis of the liver becomes obvious. We know from pharmacokinetic studies that the specific action of α-amanitin on the liver is based on a physiological transport system for bile acids and xenobiotics, present in hepatocytes, through which amatoxins quickly find their way into the liver. There they are bound to RNA-polymerase II and thus inhibit the formation of m-RNA. The three-day latency period is due to slow degradation of the reservoir of m-RNA.

The cellular concentration of RNA-polymerase II, as well as the dissociation constant of the complex of α-amanitin and the enzyme, are in the nM range [8]. Therefore, even though there is extensive inhibition of transcription, a considerable part of the amatoxin is in the dissociated form and is excreted into the bile. The poison returns to the intestine and is reabsorbed and transported to the liver. The prognosis in amanitin poisoning seems to be determined by how long this enterohepatic circulation of amanitin maintains a toxic concentration of amanitin in the liver cells.

Besides compensation for the loss of water and electrolytes, as well as replenishment of blood glucose, the most important therapeutic measure is the disruption of the enterohepatic circulation of the toxin. This can be accomplished through aspiration of the duodenal juice, administration of a suspension of activated charcoal, or administration of silymarin, the active principle of the thistle *Silybum marianum*, in the form of silibinin, its soluble hemisuccinate. As has been demonstrated in the rat, this active principle can completely suppress the uptake of radioactively labeled amanitin into liver cells [11]. But if therapy is started too late, for instance on the third

day, the prognosis is unfavorable. Through the lack of liver function and massive liver necrosis, the patients suffer an encephalopathy as well as kidney failure. Death occurs on the 5th to 12th day.

Experiments were undertaken to attempt to treat amanitin poisoning, similarly to the treatment of snake bites, with an immune serum specific for amatoxins. However, in mice, amanitin became more poisonous when bound to its immunoglobulin. It was approximately 50 times more toxic when complexed with the Fab-fragment of a monoclonal antibody specific for amatoxins. The experimental animals died of kidney failure without detectable lesions of the liver. Evidently, the immunocomplexes of amanitin were at least partly filtered in the glomoruli and resorbed by cells of the proximal kidney tubule, with fatal consequences[12].

The structure of amatoxins

Only the most important milestones in the chemical and toxicological exploration of the active principles of *A. phalloides* will be presented here. A detailed description can be found in a monograph[36]. The amatoxins form a family. With the aid of a simple method of electrophoresis on filter-paper developed at that time with Edgar Fischer[38], it was found that a crystalline preparation of *Amanita* toxin consisted of two components: a neutral one, now called α-amanitin, and an acid one, migrating towards the anode, which was called β-amanitin. Later on, further 'amatoxins' were isolated: γ- and ε-amanitin, as well as amanin and the non-poisonous component amanullin from *A. phalloides*, and amaninamide from *A. virosa*. The structural formulae of these compounds are presented in figure 1, and their inhibition constants for RNA-polymerase in table 1.

For analytical studies a color reaction became essential to visualize the components of *A. phalloides* on paper and thin-layer chromatograms. After spraying with cinnamaldehyde, followed by exposure to an atmosphere of hydrogen chloride, amatoxins develop a red-violet color. Lignin also contains components related to cinnamaldehyde. They give a similar, but blue color, reaction on paper containing wood pulp (newspaper). By dropping concentrated hydrochloric acid onto a dried drop of mushroom juice, the presence of amatoxins can be recognized[35]. This does not hold true for the poisons amaninamide and amanin (IV and V in table 1), which lack the hydroxylgroup on the indole ring and therefore do not give a positive reaction.

Difficulties were encountered in the elucidation of the basic peptide structure, due to the unusual 6-hydroxytryptophan unit (B1 in fig. 1), which during total hydrolysis is completely destroyed. Ulrich Gebert opened the sulfur bridge and converted the 6-hydroxytryptophan unit into octahydrotryptophan by exhaustive catalytic hydrogenation, which simultaneously eliminated the hydroxyl group. Then he selectively split the relatively labile peptide bond between γ-hydroxy-isoleucine (no. 3) and octahydrotryptophan. From the linear octapeptide thus obtained, he elucidated the sequence of the L-amino acids by step-wise Edman degradation. Heinz Faulstich recognized the nature of the sulfur bridge as that of an (R)-sulfoxide. The structure of the amatoxins so elucidated was verified by X-ray crystallography of β-amanitin in

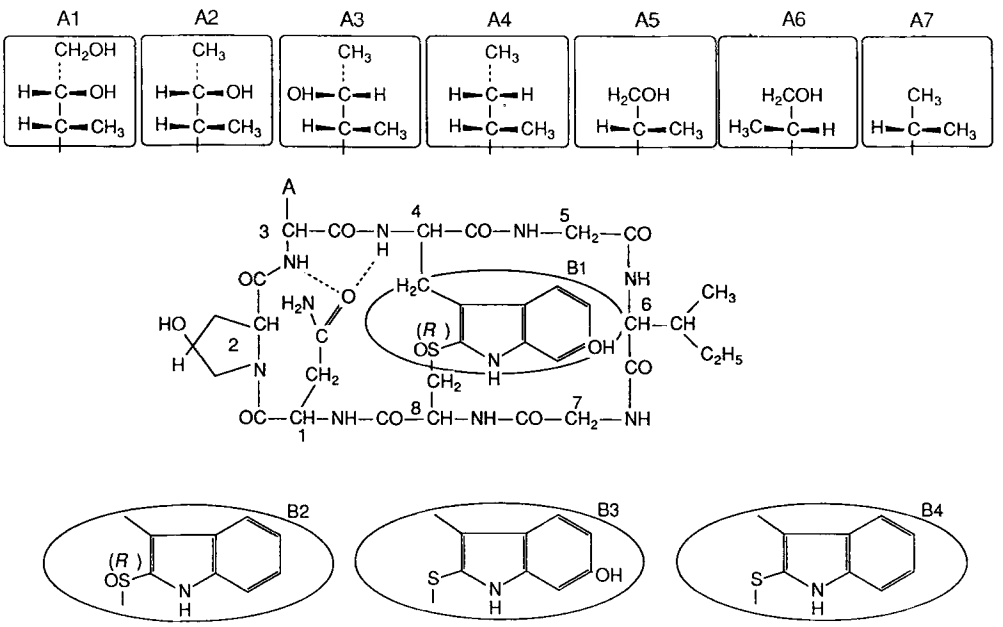


Figure 1. Naturally occurring and synthetically obtained amatoxins. For details see table 1.

Table 1. Natural and synthetic amatoxins and their inhibitory effects (K_i) on RNA polymerase II from *Drosophila*

	Name	Molecule parts (from fig. 1)	K_i (M)
I	α-Amanitin	A^1B^1	3×10^{-8}
II	β-Amanitin as I, but in pos. 1 aspartic acid instead of asparagine	A^1B^1	3×10^{-8}
III	γ-Amanitin	A^2B^1	3×10^{-8}
IV	Amaninamide	A^1B^2	3×10^{-8}
V	Amanin as IV, but in pos. 1, aspartic acid instead of asparagine	A^1B^2	3×10^{-8}
VI	S-Deoxo-α-amanitin	A^1B^3	3×10^{-8}
VII	S-Deoxo-[4(S)-hydroxy-Ile]^3-amaninamide	A^2B^4	3×10^{-8} expected
VIII	Ile^3-amaninamide (R)-sulfoxide (S)-Sulfoxide	A^4B^2	 10^{-6} 5×10^{-6}
IX	S-Deoxo-Ile^3-amaninamide	A^4B^4	10^{-6}
X	Amanullin	A^4B^1	10^{-6}
XI	S-Deoxo-[4(R)-hydroxy-Ile]^3-amaninamide	A^3B^4	10^{-6}
XII	[4(R)-Hydroxy-Val]^3-α-amanitin	A^5B^1	10^{-7}
XIII	S-Deoxo-[4(R)-hydroxy-Val]^3-amaninamide	A^5B^4	3×10^{-7}
XIV	S-Deoxo-[4(S)-hydroxy-Val]^3-amaninamide	A^6B^4	10^{-5}
XV	S-Deoxo-Val^4-amaninamide	A^7B^4	3×10^{-6}

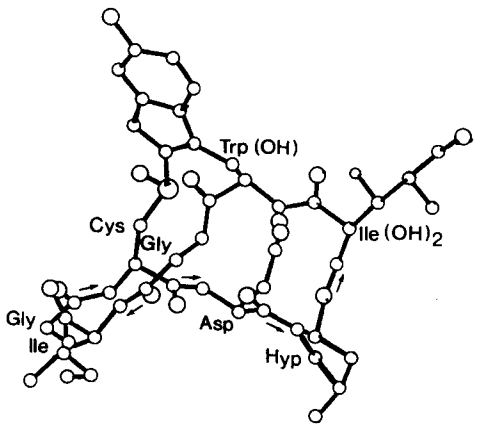


Figure 2. Molecular model of β-amanitin according to Kostansek et al.[22].

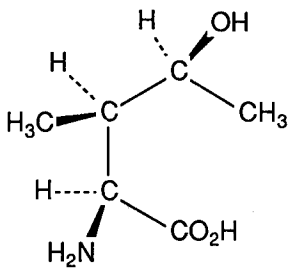


Figure 3. Structure of 4-(S)-hydroxy-L-isoleucine.

the laboratory of W. N. Lipscomb[22] (fig. 2). As was later found, the backbone is identical in all synthetic analogs, including those which are biologically inactive.

Syntheses of amatoxins

After elucidation of the formula, the next task was to prove the structure of the basic skeleton by synthesis of a highly toxic amatoxin and, beyond that, to explore by synthesis of analogs the relationship of structure and activity. Although in the meantime the methodology of peptide synthesis had been well developed, considerable difficulties were encountered in such special cases. So far, no total synthesis of the highly toxic amatoxins has succeeded. The main reason is that 4-(S)-hydroxy-L-isoleucine (fig. 3) has three asymmetric centers and furthermore tends to form a stable γ-lactone. This tendency to lactonization not only destabilizes the neighboring amide bond, but also leads to racemization at the α- and/or γ-C atom during synthesis. Therefore, in the first synthetic approaches these building blocks were replaced by L-isoleucine. Similarly the cumbersome synthesis of 6-hydroxytryptophan and the sulfoxidation were omitted, since Angeliki Buku had shown that amaninamide, a toxin from *A. virosa* without a 6'-hydroxy group (IV in table 1), as well as the semi-synthetically obtained thioether (VI in table 1) exhibited full toxic effects[4]. Therefore, a first aim was the synthesis of S-deoxo-Ile-3-amaninamide (IX in table 1) as a simplified model of an amatoxin. When synthesizing the bicyclic peptide, Giancarlo Zanotti formed the thioether bridge between the indole part of tryptophan and the sulfur atom of the cysteine part through an intramolecular 'Savige-Fontana' reaction[28]. By oxidation with singlet oxygen, tryptophan is activated in the form of a 3a-hydroxy-1,2,4,4a,8,8a,-hexahydropyrrolo[3–6]indole-2-

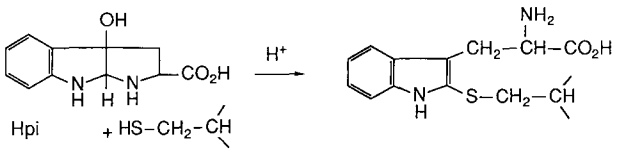


Figure 4. Formation of a thioether from Hpi by the 'Savige-Fontana' reaction.

carboxylic acid residue (Hpi) which, under H^+-catalysis, reacts smoothly with the thiol group of the cysteine side-chain to form the thioether (fig. 4).

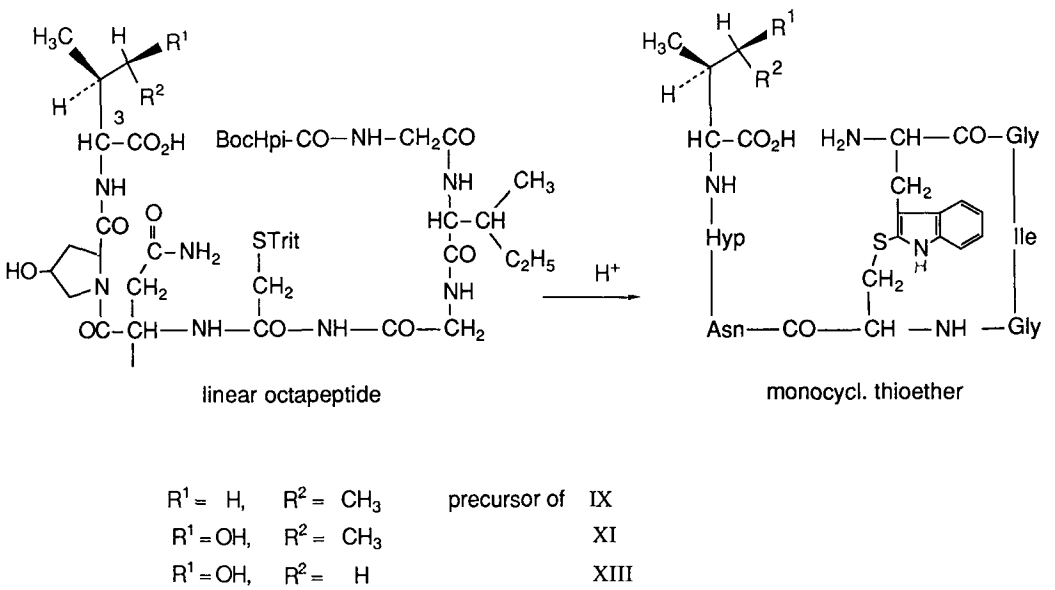
In this way the mono-cyclic thioether could be obtained from the linear octapeptide (fig. 5). The remaining cyclization was achieved by the mixed anhydride method. Unfortunately, it was shown that compound IX possessed an inhibitory activity about 50 times lower than that of the natural toxins[43]. Its biological activity corresponds to that of the naturally occurring amanullin (X, table 1) which likewise contains an isoleucine building block in position-3. Thus the importance of the γ-fixed, (S)-configurated hydroxyl group on the isoleucine in position 3 became evident.

As the next step, the synthesis of S-deoxo-[γ-(S)-hydroxy-Ile]^3-amaninamide (VII in table 1) was attempted. However, the synthesis yielded only a small amount of the (R)-isomer XI[44], while the desired (S)-isomer VII resisted ring closure by lactonization at the carboxyl end. The isomeric compound XI had only 2% of the inhibitory action of the natural amatoxins. The synthesis of a highly effective amatoxin has still not been accomplished.

Structure-activity relationships

The importance of the side chain in position 3 for biological effectiveness had already become evident from an earlier finding. In 6'-O-methyl-α-amanitin (I in table 1, methylated in molecular part B1), the side chain could be shortened to the aldehyde by oxidation with periodic acid. This compound had only very weak inhibitory action, probably owing to a change in conformation. However, after reduction of the aldehyde to the 3(R)-4-hydroxyvaline side chain, affinity for RNA-polymerase was regained to a considerable extent (XII in table 1)[39]. Recently, the diastereoisomeric S-deoxoamaninamides with 3(R)- and 3(S)-4-hydroxyvaline could also be synthesized[46]. The 3(R)-4-hydroxyvaline-amatoxin XIII, corresponding in configuration to the isoleucine derivative and the degradation product XII, likewise exhibited high affinity for the RNA polymerase. On the other hand, the diastereoisomeric 3(S) compound XIV was practically inactive.

As well as the side chain No. 3, the other side chains of α-amanitin were also probed for structure-activity relationships. The hydroxyl group on proline-2 is indispensable for toxic effects. The naturally occurring proamanullin (proline instead of hydroxyproline in X) has 10^3-times less inhibitory activity than α-amanitin. Furthermore, it could be shown that isoleucine-6 cannot be replaced by amino acids with shorter side chains, such as alanine, without causing a thousand-fold diminuation of the affinity for RNA polymerase. A similar loss of activity occurred when the adjacent glycine residue 5, obviously necessary for positioning of isoleucine-6, was replaced by alanine. An interesting effect can be observed



R^1 = H,	R^2 = CH_3	precursor of	IX
R^1 = OH,	R^2 = CH_3		XI
R^1 = OH,	R^2 = H		XIII

Figure 5. Cyclization of a linear amanitin peptide under formation of the thioether bridge.

at the sulfoxide bridge. The (S)-sulfoxide, obtained by A. Buku, has ten-fold less affinity for RNA-polymerase II than the (R)-compound, although no essential difference could be demonstrated in the 'backbone' of the two sulfoxides[41]. Of course, it is possible that the crystal structure does not reflect small differences of conformation of a peptide in solution, that are important for its biological activity.

This premise is supported by a further finding: an analog possessing L-α-aminobutyric acid (Abu) instead of asparagine in position-1 likewise showed no measurable inhibition of RNA polymerase II[45], probably owing to the presence of a nonpolar side chain. This compound was subjected to high resolution nuclear-magnetic resonance (NMR) analysis. This efficient and well-developed method for determining the structure of peptides in solution demonstrated that the conformation of the Abu-analog was in good agreement with the conformation of active amatoxins. Only by refined NMR analysis (NOE effects) was it shown that the side chain of isoleucine (No. 3) in the Abu-amatoxin protrudes from the molecule at an angle different from that found in amatoxins containing asparagine. Evidently, lack of the intramolecular hydrogen bridge (see fig. 1) prevents the important side chain No. 3 from assuming an optimal position. The consequence is a weaker linkage to RNA-polymerase II. It is clear that even minute deviations in the spatial structure of biologically active agents can lead to unexpectedly large differences in activity.

Mechanism of inhibition

The discovery of the molecular mechanism of the action of amatoxin originated with Luigi Fiume and his colleagues in Bologna, Italy. These investigators found in the mid-1960s that the RNA-content of cell nuclei of the liver parenchyma of mice poisoned with α-amanitin decreases continuously[14]. A strong inhibition of RNA-synthesis, also in isolated liver cell nuclei, was soon found to be the reason for this. Specifically, it was an inhibition of the DNA-dependent RNA-polymerases II (or B). More details can be found in literature references[9,36]. RNA-polymerases II are contained in the nuclei of all eukaryotic cells, where they are responsible for the transcription of DNA to give hnRNA, called messenger-RNAs after processing. In contrast to type II RNA-polymerases, type III transcription enzymes, which are responsible for the synthesis of transfer-RNAs and low molecular weight RNAs, are 10^3 to 10^4 times less sensitive to amatoxins, while type I RNA-polymerases, which provide for the transcription of ribosomal RNAs, are not inhibited at all, even by high concentrations of amanitin. RNA-polymerases of prokaryotic (lacking a nucleus) organisms, such as bacteria as well as RNA-polymerases from mitochondria, chloroplasts, and viruses are also completely unresponsive to amanitin.

Table 2. Inhibition constants K_i of α-amanitin for RNA-polymerases II and III from different eukaryotes

Species	RNA-polymerase II	III
	M	M
Various mammals	3×10^{-9}	$1-4\times10^{-5}$
HeLa cells	3×10^{-9}–10^{-8}	$1-4\times10^{-5}$
Amphibian (*Xenopus laevis*)	5×10^{-8}	2×10^{-5}
Fruitfly (*Drosophila melanogaster*)	3×10^{-8}	
Slimy mushroom	3×10^{-8}	
Plants (corn, soja, wheat)	5×10^{-8}	
Yeast (*Saccharomyces cerevisiae*)	10^{-6}	
Champignon Common meadow mushroom (*Agaricus bisbosporus*)	7×10^{-6}	
Deadly Agaric Death cup (*Amanita phalloides*)	2×10^{-4}	

A detailed investigation of the individual steps of transcription revealed that amanitin inhibits the elongation step. This was concluded from the fact that the enzyme in the presence of amanitin can still form dinucleotides, but can no longer form trinucleotides[32]. Evidently, binding of amanitin to RNA-polymerase blocks the movement of the protein along the 'single-stranded' DNA.

The RNA-polymerases II in the cell nuclei of various species differ in their susceptibility to amatoxins within wide limits. Also, many cell mutants resistant to α-amanitin could be cultivated, from which RNA-polymerases II were isolated having up to a thousand-fold less sensitivity to α-amanitin. In table 2 the K_i-values for RNA-polymerases II from various eukaryotes are compared; for some the (up to 10^4 times) higher values for RNA-polymerases III are also listed.

As can be seen, RNA-polymerases II from mushrooms are very insensitive to α-amanitin. While the enzyme from edible mushrooms is $> 10^3$ times less sensitive than that from mammalian cells, a $> 10^5$ times higher concentration of α-amanitin is needed for the inhibition of RNA-polymerases II of species like *A. suballiacea* which accumulate α-amanitin. With the aid of amatoxins marked with radioactive or fluorescent labels, at least one further protein besides RNA-polymerase was discovered that binds amanitin, albeit with little affinity. So far, the question of the possible biological function of this protein could not be answered.

Tool of biological research

Since the specific inhibition of RNA-polymerases of type II from eukaryotic cells occurs even in the nanomolar range, the amatoxins are an ideal tool for biological research on the molecular and cellular level. Utilizing the response to amanitin, even extremely complex biological processes can be investigated to see whether their completion is dependent on a step of transcription, namely de

novo synthesis of m-RNA. It is this attribute which today makes α-amanitin as indispensable and specific a tool for the inhibition of transcription, as cycloheximide is for the inhibition of translation.

An important area of application of amatoxins is exploration of hormones. These frequently display their effect by stimulating the transcription of certain gene segments. As a consequence, certain hormones lose their effect in the presence of amanitin. A classical example is an early observation made in 1970 by Constantin Sekeris and co-workers, who found that the formation of tyrosine transaminase in the rat liver, induced by cortisol, can be suppressed by α-amanitin [29]. In the following 20 years, it was shown that not only many hormones but also several vitamins, second messengers, growth factors and drugs act through induction of transcription. Substrates even became known which are able to induce the transcription of RNA coding for metabolizing enzymes. For instance, the induction of acetyl coenzyme-A-carboxylase by glucose can be inhibited by α-amanitin in cultivated rat hepatocytes [18]. Likewise, the de novo formation of proteases does not occur in female malaria mosquitos treated with amanitin when the animals are fed with blood after a hunger phase [2]. An enumeration of all reports published in this field would go far beyond the scope of this article; however, a review up to the year 1980 can be found in ref. 9.

Numerous studies in the last ten years have dealt with plant RNA-polymerases. As enzymes of eukaryotic cells, they also are inhibited by α-amanitin, but only at higher toxin concentrations (for a comparison see table 2 and ref. 40). For instance, after treatment of the epicotyl tissue of the pea with auxins, a transcription was demonstrated, the value of which was 50- to 100-fold greater than that of the control. Transcription already started after 15 min, and could be completely inhibited by α-amanitin. Therefore, the most likely conclusion is that the 'primary response' to these growth hormones consists of de novo synthesis of mRNA [31]. With the aid of α-amanitin, it could be demonstrated that in algae the smaller subunit of ribulose-1,5 diphosphate-carboxylase is coded in the nucleus (the transcription of this protein is light-activated and can be fully inhibited by α-amanitin), while the genetic information of the large subunit is located in the chloroplast. As a consequence the transcription of the large subunit is barely influenced by α-amanitin [27].

Quite soon after the discovery of its specific inhibitory action, α-amanitin was employed in virus research. When the replication of a virus is inhibited by amanitin, it is an indication that the development of the virus is dependent on the transcription apparatus of the host cell. Generally, the rule holds true that viruses which multiply in the cytoplasm are resistant to α-amanitin, while those that multiply in the nucleus are inhibited by amanitin. In both groups of viruses, the RNA-viruses and the DNA-viruses, species are known which are sensitive to and others that are resistant to amanitin. Viruses and viroids of plant origin were also investigated, such as the 'hop stunt virus' [42] and the striped mosaic viruses of wheat and barley [33]. The replication of both is inhibited by α-amanitin, as is that of viroids like PSTV (potato spindle tuber viroid) [3, 30] and CEV (citrus exocortis viroid) [16].

Many laboratories have also employed amanitin as a tool in developmental biology, prompted by the observation that first divisions of an egg-cell frequently can occur in the presence of α-amanitin. Evidently, the transcription products of partition sequences whose synthesis cannot be inhibited by α-amanitin must have been coded by maternal mRNA, that is, by RNA from the ovular cell. As a rule, no response to amanitin can be observed up to the eight cell stage. On the other hand cases are known, for example in the mouse, where products of the embryonic genome could be proven to exist already in the two cell stage [15]. From this field of research also, only two arbitrarily selected studies will be mentioned. In mouse embryos the synthesis of hypoxanthine-phosphoribosyltransferase is dependent on maternal RNA before implantation, since the formation of this enzyme can be inhibited by cycloheximide, but not by α-amanitin [20]. Similarily, sea urchin eggs do not respond to amanitin until the blastula phase [24], in which m-RNA even for later phases of development is synthesized.

In the mid-sixties Fiume and Laschi studied by electron microscopy the cell nuclei of mice poisoned with amanitin. They found that the nucleolus was degraded into fragments and that chromatin had condensed [13]. Therefore, it is not surprising that even today α-amanitin represents an important tool for morphological studies of the cell nucleus, such as the exploration of nucleolus-organizing regions, NOR [25], or, using tritiated amanitin, the identification of gene locations in the cell nucleus during the interphase [19]. Amanitin was also employed to explore the individual steps of transcription, and to investigate the complex sequence of events during RNA processing, by which the initially formed hnRNA is finished to give mRNA. As has been shown by the example of the gene for β-globulin, the exact transcription of genes demands that flanking and structural gene sequences also be transcribed. The transcription of these flanking genes is not inhibited by α-amanitin [5, 6]. One aim of the exploration of RNA processing is the elucidation of the structure and function of the ribonucleoprotein complexes [21]. An example of this type of research is the realization that the maturation of hnRNAs is dependent on the small snRNA U6, and that U6 is transcribed by RNA-polymerase III. But the synthesis of other snRNAs, such as U1, can be inhibited by α-amanitin and therefore, obviously, is a product of RNA-polymerase II [23].

Potential applications in medicine

Already in 1969 Cessi and Fiume established that amanitin increases in toxicity by an order of magnitude when

bound to proteins[7]. Experimental animals died when treated with protein-bound amanitin in doses of one tenth, in many cases even one hundredth of the LD_{50} of the free toxin. The reason for the high toxicity of amanitin conjugates was soon recognized. Although amanitin bound to proteins can no longer penetrate its natural target cells, hepatocytes, it is taken up via endocytosis by the sinuosoidal cells of the liver (an overview of amanitin protein conjugates is found in ref. 10). Disregarding the unfavorable highly toxic side effects, this principle presented a tool for introducing amanitin into cells other than the natural target cells.

Receptor-mediated endocytosis is a variant of endocytosis by which certain cells can be addressed specifically. The macromolecule, the polymer-bound amanitin, is provided with a ligand which binds to a receptor of the cell surface, and together with it, is internalized by the cell. When a ligand, such as the epidermal growth factor (EGF), possesses a high affinity for its receptor, an EGF-labeled toxin vehicle can be taken up at such a low toxin concentration that poisoning of the sinusoidal cells by unspecific phagocytosis becomes improbable. Since there are tumor cells which carry an extremely high number of EGF-receptors on their surfaces (10–200 times more than a normal cell), a tumor therapy could perhaps be based on an EGF-mediated endocytosis of amanitins bound to macromolecules.

Ute Bermbach[1] succeeded in loading the many side chains of poly-L-ornithine (32 kD) with β-amanitin in high amounts, and in attaching 1–2 molecules of EGF as recognizing molecules. Indeed, it was shown that this 'hormonotoxin', already at a concentration of 28 nM of amanitin (IC_{50}), was able to inhibit the growth of A431 cells, a human tumor cell line with 2×10^6 EGF receptors per cell (as compared to 10^4 EGF receptors on normal cells.) With unbound amanitin the same effect could be obtained with an only 80-times higher concentration. At present, experiments are under way which will show whether, with the aid of this 'hormonotoxin', the growth of solid human A431 tumors can be suppressed in vivo, for example in immuno-incompetent (nude) mice.

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Research Articles

Periodicity in fish otolith Sr, Na, and K corresponds with visual banding

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Abstract. Examination of a section of an otolith from a teleost fish by fast atom bombardment-secondary ion mass spectrometry (FAB-SIMS) revealed seasonal periodicity in Sr, Na, and K concentrations that corresponded with visually observed annual banding. Strontium maxima also corresponded with Na and K minima and vice versa. In addition there was a general, apparently age-related, trend in Sr levels, with concentrations at the edge of the otolith being higher than in the core.
Key words. Fast atom bombardment (FAB); secondary ion mass spectrometry (SIMS); fish otolith; calcareous tissues, elemental analysis.

Otoliths are hard, stone-like, non-bony structures which form part of the organs, located in the inner ear, which sense position and motion in vertebrates[1]. In teleost fish, otoliths are made of calcium carbonate in the aragonite crystal form, deposited in a protein matrix[1,2]. Three pairs are present in the labyrinths of each fish of which the sagittae, or sagittal otoliths, are the largest[2]. Two types of information can be obtained from the sagittal otoliths of teleost fish. Firstly, banding evident to the eye or by low-powered microscopy can often provide a method of determining the age of individual fish. The nucleus of an otolith is present at the beginning of larval life, and grows daily by the addition of layers to the outer surface. In sections through an otolith there is usually visible evidence of periodicity in the formation of concentric layers on daily, annual and sometimes intermediate time scales[3,4]. Age-determination of fish is an important part of the study of population dynamics and calculation of sustainable fishery yields. Various hard parts of the fish which show periodic banding, including scales, bones and fin-rays, have been used for this purpose[4]. Otoliths possess the advantage that they continue to thicken by the addition of layers to the medial surface even when the fish has ceased growing. In some fish species the process