The Amanita Toxins in Mushrooms

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Three emperors, a pope, and a multitude of lesser beings are reported to have succumbed to the poisons found in some wild mushrooms. Despite experience and education, the death toll still mounts. Unfortunately, superstition and folklore remain the guides for many mushroom collectors in distinguishing the poisonous species. The authors have reported a simple chemical procedure for identifying the most deadly poison in mushrooms, the amanita toxins. This paper contains experimental evidence on the reliability of that method. In 46 species of wild mushrooms, all the specimens that were found lethal to mice gave a positive test for the amanita toxins. In only one case did a species which failed to manifest toxicity give a test indicating the presence of these toxins.

THE AMANITA TOXINS IN MUSHROOMS may be identified by a method described in a recent paper (*3*). These toxins, found in the closely related *Amanita phalloides*, *Amanita verna*, and *Amanita virosa*, are responsible for at least 90% of the deaths caused by mushroom poisoning (*23*). Despite man's long and unhappy experience with wild mushrooms, there are still about 50 deaths and many more cases of poisoning annually in the United States alone (*21*). Unlike muscarine, the toxin of *Amanita muscaria*, the amanita toxins produce no toxic symptoms for 10 to 15 hours after ingestion, a time that allows the toxins to be thoroughly absorbed by the body (*11*). Although there is no specific antidote for the amanita toxins such as atropine for muscarine, the Pasteur Institute in Paris has developed and stocks an antiserum made by immunizing horses. Because this serum is not generally available, standard treatment consists of glucose and amino acids given intravenously to combat the symptoms (*17*).

Historical

The first reliable work on the amanita poison was done by Kobert (*13*, *15*), who demonstrated in *Amanita phalloides* the presence of a heat-labile, hemolytic principle which he named "phallin," and a heat-stable, alcohol-soluble toxin which Ford later termed the "amanita toxin" (*8*). Kobert postulated, but offered no evidence of a third poison, like thujone, pulegone, and similar substances occurring in poisonous higher plants (*14*). Ford (*8*) confirmed the presence of the amanita hemolysin and amanita toxins in *Amanita phalloides*. Owing to the instability of the hemolysin in the stomach and to cooking, he attributed the toxic nature of the mushroom to the amanita toxin. The hemolysin was found to be a glucoside with the elementary composition carbon 48.93, hydrogen 6.08, nitrogen 10.83, sulfur 1.94, and oxygen 32.22 (*1*). This gives an empirical formula of $C_{66}H_{99}O_{33}N_{13}S$. The amanita toxin was shown not to be a protein, glucoside, or alkaloid, but to yield indole and pyrrole on fusion with potassium hydroxide (*24*).

In a series of papers (*16*, *25–29*), the Wielands reported their extensive investigations of the chemistry of the amanita toxin of *A. phalloides*. The alcohol-soluble toxin was found to be made up of three peptides which were called "phalloidine," "α-amanitine," and "β-amanitine." Phalloidine, melting point 255-258° C., with the formula $C_{35}H_{46}O_{10}N_8S.6H_2O$ is a heptapeptide with the following molecules of amino acids in a cyclic structure: 2 alanine, 1 oxytryptophan, 1 cysteine, 1 allohydroxyproline, 1 threonine, and an unidentified unsaturated α-amino acid. The hydrolysis of α-amanitine, $C_{39}H_{52}O_{14}N_{10}S$, yields aspartic acid, glycine, hydroxyproline, cysteine, and an unidentified amino acid. It is believed that α-amanitine is the amide of β-amanitine. A 30-gram specimen of *A. phalloides* was found to contain about 1 to 2 mg. of the amanitines and 0 to 5 mg. of phalloidine. α-Amanitine, β-amanitine, and phalloidine required 2.5, 8–10, and 40 to 50 γ, respectively, to kill a 25-gram mouse. Phalloidine may be of less importance in mushroom poisoning than the amanitines, not only because of its lower toxicity, but also because of its lesser stability to heat. Wieland and others (*28*) described a chromatographic test for phalloidine and the amanitines. The authors have evaluated this test and developed it into a procedure for identifying the amanita toxins in mushrooms.

Chromatographic Procedure and Results

The work reported here describes the authors' investigations in establishing the validity of the procedure for identifying the amanita toxins in mushrooms. This required association of the chromatographic spots obtained by the procedure with toxicological symptoms produced by the amanita toxins, and evidence to demonstrate that all specimens containing these toxins would produce the identifying criteria and those not containing the toxins would not do so.

Over 50 mushrooms, representing 46 species, including 13 amanitas, were surveyed by the chromatographic procedure for identification of the amanita toxins. The extract of each mushroom was also tested biologically by injection into mice. Only two species, *Amanita verna* (the Destroying Angel) and *Amanita tenuifolia* (*19*), were found to be lethal. Considerable work was done with these species.

In their work with *Amanita phalloides*, Wieland and others (*28*) found three spots on the chromatogram associated with the toxins. The first two, α- and β-amanitine, when developed with cinnamaldehyde, gave violet-colored spots with R_f values of 0.41 and 0.24, respectively. The third, phalloidine, gave a bright blue spot with an R_f value of 0.50. In the present work, employing *Amanita verna*, three spots were obtained on the chromatogram developed with cinnamaldehyde: two violet spots with R_f 0.43 and 0.18 and an orange spot with R_f 0.07. No spot corresponding in color or R_f value to phalloidine was found on analysis of numerous specimens of *A. verna*. To be sure that the bright blue color and violet color were not confused, another color test was run: a chromatogram employing diazotized sulfanilic

acid (Pauley's reagent) which gives a cherry red color with the amanitines and a yellow color with phalloidine (*28*). The cherry-red colored spots were obtained with the *A. verna* extract, but no yellow color was observed. Thus it may be concluded that unlike *A. phalloides*, *A. verna* contains the two amanitines but no phalloidine.

Table I. Chromatographic Column Run of *Amanita verna* Extract

Fraction	Spot Color	R_f
0– 52	None	0.00
53	V	0.38
54	V	0.44
55	V	0.44
56	V	0.44
57	V	0.36
58	V	0.36
59	V	0.36
60	V	0.37
61	V	0.39
62	V	0.39
63	V	0.32
64	None	0.00
65	V	0.16
66	V	0.17
67	V	0.17
68	None	0.00
69	None	0.00
70	V, Y	0.17, 0.32
71	V	0.17
72	V	0.15
73	V	0.15
74	V	0.15
75	None	0.00
76	None	0.00
77	V	0.15
78	None	0.00
79	None	0.00
80	Or, Y, V	0.05, 0.15 0.44
81– 89	None	0.00
90	Y	0.05
91	Y	0.05
92	Y	0.05
93	None	0.00
94	None	0.00
95	None	0.00
96	Y	0.05
97	Y	0.05
98	Y, V	0.05, 0.20
99–104	Y, V	0.05, 0.17
105–107	Or, V	0.05, 0.22
108–110	Or, V	0.05, 0.21

Y. Yellow
V. Violet
Or. Orange

To confirm the identity of the components of the *A. verna* extracts and their R_f values, the extract was purified by running it through a 300 × 10 mm. chromatographic column with a sintered-glass base, charged with 5 grams of Whatman No. 1 standard grade powdered cellulose. The sample used was 0.3 ml. of a concentrated methanol extract. The powdered cellulose was mixed with acetone and poured into the column. After the column was packed, it was washed free of acetone, using a special solvent made up of 20, 6, 5, and 1 parts, respectively, of methyl ethyl ketone, acetone, water, and butanol. The sample was adsorbed on powdered cellulose, diluted with methanol, poured into the column, and packed down. The special solvent was run through the column, while a constant liquid head of solvent was maintained on top of the cellulose throughout the run. The effluent was collected in test tubes, fractions of approximately 0.5 ml. being taken every minute. All of the 110 fractions were concentrated by evaporation of the solvent and then each was run individually on a strip chromatogram. The color and R_f values of the spots of the fractions are given in Table I.

Although the results show evidence of some channeling and holdup in the column, this was not serious and the components were well separated, as demonstrated by the individual spots in the chromatograms of most of the fractions. Two extracts of other specimens of *A. verna* were similarly purified in the cellulose column with the same results.

Table II. Combined Selected Fractions of Powdered Cellulose Column Run on *A. verna* Extract

Fraction No.	Spot Color	R_f Value
0– 53	None	..
53– 63	Violet	0.43
66– 74 (except 70)	Light violet	0.17
102–110	Orange	0.07

Three definite spots were found as originally observed on the strip chromatogram of the whole extract. Those fractions of Table I, most of which represented chromatographically pure components, were combined as shown in Table II. The fraction with R_f 0.43 undoubtedly is α-amanitine. That with R_f 0.17 is probably β-amanitine, despite the lower R_f than Wieland's 0.24 which was consistently obtained. The orange spot which is not readily moved by the solvent is found in the chromatograms of many other mushrooms (Table III) and may represent fungus tissue peptides.

Chromatograms were made of extracts of specimens of *A. tenuifolia*, the only other highly toxic species found in these studies. As given in Table III, this species produced a chromatogram with one violet spot, corresponding in R_f value to β-amanitine, and an orange spot near the origin.

Toxicity of Mushroom Extracts

In order to determine whether or not the components indicated by the violet spots represented toxins, as assumed, the combined fractions given in Table II were injected intravenously into mice. Each mouse (strain A, female), approximately 20 grams, received 0.05 ml. of an aqueous solution (obtained by evaporating off the organic solvent and taking up the residue in water) in the tail vein. The mice receiving the fractions assumed to be α- and β-amanitines died in 32 to 48 hours. There were no toxic manifestations in less than 12 hours following injection. Earlier signs of toxicity and a heavier spot on the chromatograms indicated that the α-amanitine in *A. verna* was present in higher concentration than the β-amanitine. The mice receiving the fractions indicated by no color and the orange spot color showed no ill effects upon injection, even though the injected doses were subsequently multiplied severalfold.

Further tests of toxicity were performed with the whole extracts of specimens of *A. verna* and *A. tenuifolia*. An *A. verna* sporophore was extracted by the aqueous procedure of Ford (*9*). Forty grams of fresh tissues were boiled with 150 ml. of water for 30 minutes and refrigerated. The tissue was removed by filtration and the filtrate concentrated to 5 ml. by boiling. This solution was injected into two mice, one receiving 0.2 ml., the other 0.1 ml. The mouse receiving 0.2 ml. died in convulsions in 4.5 hours. The other mouse died in 31 hours. Post-mortem autopsy showed the livers to be darkened with hyperemia and the heart and kidneys similarly damaged. These were the typical symptoms found in all cases of poisoning produced by specimens of *A. verna* and *A. tenuifolia*.

Graduated doses of the above extract were tested in order to determine the minimum lethal dose. Four mice received 0.15, 0.075, 0.025, and 0.005 ml. of the extract intravenously. The mouse receiving 0.15 ml. died in 7.5 hours and those receiving 0.075 and 0.025 ml. died almost simultaneously 6.75 hours after injection. The mouse receiving 0.005 ml. became greatly affected after 18 hours, being unable to stand, with head and legs withdrawn, appearing very sick.

Figure 1. *Amanita verna*, the "destroying angel"

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After 72 hours, however, the animal had fully recovered.

Because all this work had been done employing intravenous injection, it was of interest to determine the effect of subcutaneous injection of this extract. When 0.1 ml. was injected subcutaneously, death occurred in 10 hours, giving evidence of the potency of the toxins and their ability to be translocated through the system. Other specimens of *A. verna* and *A. tenuifolia*, whether extracted with water or methanol, gave essentially the same toxic results described.

Table IV presents what is believed to be the first comparative compilation of doses for the amanita toxins. In addition to the data assembled from the literature, this table contains the authors' computation of the doses of the amanita toxins as amanitine. For the authors' entry in this table it was assumed that the minimum lethal dose was twice the smallest dose obtained in the series referred to above and that death would ensue in 48 hours or less, as has occurred in all their tests. In calculating the doses of the amanita toxins from the doses of mushroom tissue given in the literature, it was assumed that in all reported specimens of *A. phalloides* and *A. verna* the toxins were present in the same concentration as reported (*28*) for the amanitines in *A. phalloides*. In certain cases the investigators did not state whether the specimens tested were weighed fresh or dried. In these cases it was presumed that fresh tissue was employed. In many of the tests in the literature only one or two animals were reported on; therefore, the lethal dose that was computed may not necessarily be the minimum lethal dose. Despite these considerations, however, Table IV shows how highly poisonous these toxins are to several warm-blooded animals. The dose to the guinea pig and rabbit approximates that to man, which was estimated from poison case studies. The dog and mouse appear to be considerably more resistant.

All of the mushrooms tested were collected locally by the authors and among them were several specimens classified as *A. verna*, but which produced no toxic symptoms in mice. In the chromatographic test, these specimens yielded orange and yellow spots but not the violet spots associated with the amanita toxins. Whether these were closely related but different species such as *Amanita vernella* (*18*), which was tested and found to be nontoxic, or whether they were physiological mutants is not known. It was most surprising to find the local specimens of the brown *A. phalloides* nontoxic. Concentrated extracts from both the methanol and the aqueous extractions of different specimens on several mice showed no signs of affecting the animals. The chromatogram (Table III) gave no indication of the presence of the amanita toxins. In correspondence with Cook (*4*) it was learned that she had tested the local (Gainesville, Fla.) *A. phalloides* against the guinea pig without finding toxic symptoms. In a published paper (*5*), Cook reported *A. verna*, *A. verniformis*, and *A. virosiformis* highly toxic to guinea pigs but *A. cothurnata*, *A. flavivolva*, *A. roanokensis*, and four other Amanitas nontoxic. Of the latter three species, the authors obtained no toxicity with *A. cothurnata* and *A. roanokensis* but a few minutes after injection of the extract of *A. flavivolva* (*20*), a species closely related to *A. cothurnata*, the mouse showed signs of discomfort and irritation and had jerky movements which lasted for 3 hours. After that, the animal completely recovered. The rapid symptoms were similar to those of muscarine and the chromatogram was negative for the amanita toxins. *A. cothurnata* has been reported to be the causative agent in cases of human poisoning (*7*). Another species known to be poisonous but not deadly to humans, *Lepiota morgani*, had no effect on mice.

Of all the species given in Table III, only three, *A. verna*, *A. tenuifolia*, and *Lepiota cretacea*, gave evidence of the amanita toxins when tested by the chromatographic procedure (*3*). All of the species in Table III were tested in mice and only *A. verna*, *A. tenuifolia*, and *A. flavivolva* showed any signs of toxicity. The specimen of *Lepiota cretacea* tested was very small and it is possible that this species, which gave a light violet color with an R_f value close to that of α-amanitine in the chromatogram, may contain a small quantity of this toxin, but that the quantity present was insufficient to produce toxic symptoms in the animal. The α-amanitine spots on chromatograms of *A. verna* were much more intense.

Table III. Chromatograms of Poisonous and Nonpoisonous Mushrooms

Mushroom	Spot Colors	R_f Values
Agaricus campestris	Orange-pink	0.30
Agaricus pocillator	Light yellow	0.66
Agaricus rhoadsii	Orange, yellow	0.27, 0.38
Amanita flavivolva	Yellow	..
Amanita flavoconia	Light yellow	0.14
Amanita margarita	Yellow-orange	0.08
Amanita phalloides[a]	Orange	0.20
Amanita praelongispora	Yellow	0.15
Amanita rubescens	Yellow	0.15
Amanita solitariformis	Light orange	0.16
Amanita subsolitaria	Orange, yellow	0.07, 0.22
Amanita tenuifolia	Orange, violet	0.07, 0.25
Amanita verna	Orange, violet, violet	0.07, 0.18, 0.43
Amanita vernella	Yellow-orange	..
Amanitopsis pubescens	Brown streak	12 cm. long
Amanitopsis vaginata	Orange	0.16
Boletus alboater	Intense orange,	0.064
	Very light pink	0.56
Boletus bicolor	Orange	0.64
Boletus floridanus	Yellow	0.57
Boletus fumosiceps	Deep orange, pink	0.057, 0.11
	Light pink, orange	0.28, 0.48
Boletus luteus	Yellow	0.07, 0.59
Boletus pisciodorus	Light yellow	0.44
Boletus preanisatus	Orange, light yellow	0.07, 0.15
Boletus roseialbus	Pink, light yellow	0.19, 0.36
Boletus scaber	Pink	0.14
Boletus scaber var. Badinos	Orange, pink, light yellow	0.07, 0.26, 0.42
Boletus subvelutipes	Yellow	0.07, 0.73
Clitocybe adirondackensis	Pink	0.17
Collybia subluxurians	Orange, yellow	0.03, 0.21
Geaster sp.	Light orange	0.22
Hygrophorus subruber	Light orange	0.20
Hypholoma fasciculare	Bright orange, light orange	0.05, 0.20
Lactarius lactifluus	Orange	At origin
Lactarius pergamenus	Rose	0.05
Lepiota brunescens	Orange, yellow	0.08, 0.84
Lepiota cretacea	Light orange, pink,	0.21, 0.30
	Yellow, light violet	0.40, 0.46
Limacella illinita	Pink, light yellow	0.31, 0.60
Polyporus sp.	Light yellow streak	5 cm. from origin
Russula mariae	Orange, light yellow	0.07, 0.24
Russula radiata	Pink	0.22
Russula subalbidola	Orange, orange streak	0.05
Russula sp. (white)	Pink	0.19
Russula sp.	Yellow	0.22
Strobilomyces strobilaceus	Intense orange, light orange	0.052, 0.24
	Light orange	0.56

[a] The brown *A. phalloides*, not the greenish colored mushroom found in Europe.

Identification with Chromatography

The chromatographic prints may have further use in the identification and classification of the higher fungi. In identifying bacteria, workers, of necessity, rely heavily upon physiological

Table IV. Lethal Doses of the Amanita Toxin to Animals and Man

Animal	Species	Workers	Year	Reference	Dose	Material Tested	Calculated Lethal Dose (as Amanitine[a]), Mg./Kg.	Time for Death, Days	Remarks
Man	*A. phalloides*	Ford and Clark	1914	(*12*)	1–2 mushrooms	Fresh tissue	0.03	8–10	Estimate of M.L.D. from case histories
Guinea pig	*A. phalloides*	Ford	1909	(*9*)	0.4 mg./animal	Purified toxin	1.0	1	Not minimum dose Wt. of animal est.
Guinea pig	*A. phalloides*	Ford	1909	(*9*)	0.3 g./animal	Fresh(?) tissue	0.015	25	Not minimum dose Wt. of animal est.
Guinea pig	*A. phalloides*	Ford	1911	(*10*)	0.5 g./420 g.	Fresh(?) tissue	0.063	0.75	
Guinea pig	*A. phalloides*	Raab and Renz	1933	(*22*)	350 mg./kg.	Fresh tissue	0.0175	...	
Guinea pig	*A. verna*	Raab and Renz	1933	(*22*)	250 mg./kg.	Fresh tissue	0.0125	...	
Guinea pig	*A. verna*	Cook	1954	(*5*)	122 mg./kg.	Dry tissue	0.06	2	Not minimum dose
Rabbit	*A. phalloides*	Ford	1911	(*10*)	0.5 g./1035 g.	Fresh(?) tissue	0.025	0.75	
Dog	*A. phalloides*	Binet and others	1944	(*2*)	0.168–0.194 mg./kg.	Purified amanitine	0.168–0.194	...	
Mouse	*A. phalloides*	Wieland and others	1949	(*29*)	2.5γ/25 g. 8–10γ/25 g. 40–50γ/25 g.	α-Amanitine β-Amanitine phalloidine	0.1 0.3–0.4 1.6–2.0	5 3 2–3	
Mouse	*A. verna*	Block and others	1955	This paper	0.08 g./20 g.	Fresh tissue	0.2	2	

[a] In all cases (except data of Wieland and others) lethal dose was calculated as amanitine, assuming 0.005% amanitine in fresh specimens.

tests. The higher fungi, however, are identified by morphological descriptions, although Davidson and others (*6*) have employed a physiological test to differentiate the mycelia of white rot fungi from brown rotters. The chromatographic patterns in Table III, with different species producing spots with different colors and R_f values, suggest such a procedure as an aid in classification, just as odor and color are used to identify the sporophores. Considerably more investigation would be necessary to ascertain the dependability of such a method.

Summary and Conclusions

Over 50 mushrooms, representing 46 species of fungi, which included 13 species of Amanitas, were tested chromatographically for the amanita toxin and simultaneously for evidence of poisonous characteristics by intravenous injection into mice.

The only fungi producing death were *Amanita verna* and *Amanita tenuifolia*, both of which gave a positive chromatographic test for the amanita toxins. *A. verna* gave evidence of containing α- and β-amanitine, whereas *A. tenuifolia* showed only the chromatographic spot for β-amanitine. Neither contained phalloidine. By running it through a powdered cellulose column, the *A. verna* extract was separated into chromatographically pure components. Those components, chemically identified with the amanitines, killed mice in 32 to 48 hours after injection, whereas the other fractions were harmless.

Lepoita cretacea showed a weak chromatographic spot suggesting that it may contain a small quantity of α-amanitine, but the small dose employed had no harmful effect on the mouse. None of the other specimens gave a positive test for the amanita toxins and none produced any ill effects on mice, except *Amanita flavivolva*, which caused rapid but fleeting symptoms characteristic of muscarine.

Amanita phalloides, the brown variety, collected locally, and some specimens of *A. verna* were found to be nontoxic. These gave no chemical evidence of containing the amanita toxins. *Amanita cothurnata* and *Lepiota morgani*, both of which are toxic to man, were harmless to mice.

A table was prepared from data in the literature and computations were made to give the lethal doses of the amanita toxins to animals. Man, guinea pig, and rabbit are shown to be much more sensitive to this poison than dogs and mice.

The individual patterns and spot colors produced by the chromatographic procedure employed may be an aid in the identification and classification of the higher fungi.

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