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Curative Potencies against α-Amanitin Poisoning by Cytochrome c

Abstract. *The fatalities occurring after ingestion of the poisonous mushroom* Amanita phalloides *are thought to be due to α-amanitin. Cytochrome c provided antidotal effects against a lethal dose of this toxin in mice. Significant survival was obtained even when the treatment was withheld for 8 hours. The enzyme was superior to a previously explored therapeutic regimen consisting of penicillin and its combination with chloramphenicol and sulfamethoxazole.*

Clinical intoxications resulting from ingestion of the poisonous mushroom *Amanita phalloides* (death cap) are not frequent. In view of the lethality (about 30 percent) (*1*), such cases are dramatic events. Wieland and his collaborators (*2*) have elucidated the chemistry of the poisonous principles. Study of their biological activity (*2, 3*) has revealed that the symptomatology is associated with two main categories of toxins. The rapidly acting phallotoxins, which experimentally cause death in mice within a few hours, are responsible for the gastrointestinal phase of the poisoning. This phase sets in after a latency period of approximately 8 hours and is characterized by diarrhea, nausea, and vomiting. The principal phallotoxin is phalloidin. The amatoxins, which have a 10- to 20-fold greater toxicity, kill mice at lethal doses only after a few days and are thought to cause the late and often fatal hepatorenal phase of the intoxication that sets in after 3 to 5 days. As α-amanitin is quantitatively the most important of the highly toxic amatoxins, it can be assumed to be mainly responsible for the clinical mortality.

No specific treatment of *Amanita* poisoning has been available so far. Several compounds have been recommended on the basis of their alleged clinical effectiveness, but, because of the lack of controlled investigations, the effectiveness could never be substantiated. Also, when tested under controlled conditions, some allegedly useful agents such as thioctic acid, glucocorticosteroids, and coenzyme A did not counteract lethal poisoning with α-amanitin (*4, 5*).

The availability of the pure components of *Amanita phalloides* afforded the opportunity for studying their mode of action. As for α-amanitin, either the liver or the kidney has been shown to be the target organ, depending on the dose (*6*). The poison early induces ultrastructural changes in the nuclei with fragmentation of the nucleoli and segregation of their granular and fibrillar components appearing within only 15 to 30 minutes. Quantities in the range of the minimal lethal dose cause death occurring between 3 and 5 days in mice, with cytoplasmic changes and necrosis of the epithelial cells in the proximal tubules of the kidneys, where the reabsorption of α-amanitin leads to a high concentration of the toxin. With more elevated doses of α-amanitin, the identical damage in the hepatocytes becomes irreversible and causes death at about 2 days. α-Amanitin acts by binding to RNA polymerase in eukaryotic cells and inhibiting this enzyme (*7*). Because of this property, the toxin has been adopted as a tool in molecular biochemical research.

Various agents displaying a protective effect against phalloidin were uncovered, including hepatotoxic compounds such as carbon tetrachloride, thioacetamide, and sodium-cinchophen (*8, 9*). Phenylbutazone and rifampicin also protect against phalloidin (*4*). The first four substances are thought to prevent the transformation of phalloidin to a toxic metabolite by interfering with the function of hepatic microsomal drug-metabolizing enzymes. Carbon tetrachloride and 6-aminonicotinamide have some protective effects against α-amanitin (*9*). Moreover, antamanide, a structural analog of the toxic cyclopeptides, is present in minute quantities in the mushroom and counteracts phalloidin at a suitable dosage (*10*). However, all of the mentioned agents were only effective if given before or at least simultaneously with the toxins. Therefore, the search for antidotes capable of reversing the effects of the toxins at a later stage was pursued but carried out exclusively with α-amanitin.

In a first set of experiments, a series of agents including penicillin, phenylbutazone, chloramphenicol, and sulfamethoxazole were found to counteract lethality if applied after the α-amanitin (*11*). With application as late as 8 hours after the poisoning, a high dose of penicillin or a lower dose of it combined with chloramphenicol and sulfamethoxazole led to survival rates of 30 to 40 percent against the LD_{95} (lethal dose, 95 percent effective) of α-amanitin (0.6 mg/kg). These substances may enhance the excretion of

Table 1. Survival of mice poisoned with α-amanitin and treated with an intraperitoneal injection of cytochrome c. Female white mice (*19*) weighing 18 to 20 g were treated 30 minutes or 8 hours after the intraperitoneal injection of α-amanitin (0.8 mg/kg). The α-amanitin and cytochrome c were dissolved in isotonic saline and administered in a volume of 0.1 ml per 10 g of body weight. The controls received saline (0.1 ml/10 g) only. Penicillin G (sodium benzylpenicillinate) was applied intraperitoneally, chloramphenicol (sodium succinate) subcutaneously, and sulfamethoxazole in water by gavage. Survival of the mice was assessed at 1 week after the administration of α-amanitin. No more fatalities occurred after this time.

Treatment	Dose (mg/kg)	Time after α-amanitin (hours)	No. of mice per group	Survival (%)
Controls			50	0
Cytochrome c	50	½	25	44*
Cytochrome c	50	8	38	34*
Cytochrome c	150	½	19	53*
Cytochrome c	150	8	20	30*
Penicillin-G	1000	8	26	4
Penicillin-G + cytochrome c	1000 150	8	8	62*
Penicillin-G Chloramphenicol Sulfamethoxazole	500 125 500	8	26	8
Penicillin-G Chloramphenicol Sulfamethoxazole + cytochrome c	500 125 500 150	8	8	37*

* The chi-square test, $P < .01$

α-amanitin by displacing it from binding sites on the plasma albumin molecule (*4*, *11*).

The most effective antidote to α-amanitin, however, is cytochrome c (Table 1). It was tested against α-amanitin at a dose of 0.8 mg/kg, which invariably killed the control mice between 50 and 100 hours later. At this dose, the activity of penicillin and of the combined treatment with chloramphenicol and sulfamethoxazole were barely discernable, whereas cytochrome c, at 50 or 150 mg/kg, provided a survival rate of about 30 percent if applied 8 hours after the α-amanitin. If given only after 30 minutes, approximately 50 percent of the mice survived. The cytochrome was not consistently effective at either a lower dose (15 mg/kg), or a higher dose (450 mg/kg). The highest survival rate (62 percent) observed with any treatment following a dose of 0.8 mg/kg by 8 hours occurred if cytochrome c was combined with penicillin. Whether the combined regimen provides statistically significant advantages is unknown.

The cytochrome c preparation (*12*) was extracted from horse heart (*13*) and purified chromatographically (*14*).

The toxicity of malachite green in dogs and rats is antagonized by cytochrome c (*15*), but I have not found conclusive reports on its efficacy in other experimental intoxications. Clinical applications of the enzyme, mostly in the therapy of anoxic states, have been generally abandoned after a temporary surge of positive reports (*16*). The divergencies may be related to the variable activity displayed by different enzyme preparations. Moreover, cytochrome c has been reported to penetrate only into cells that have previously suffered toxic damage (*17*). This may also explain both the inconsistency of the clinical results and the effectiveness of the enzyme in α-amanitin poisoning. My finding also suggests a search for biochemical targets of α-amanitin other than RNA polymerase. The myocardial damage encountered in death cap poisoning and the reversibility by cytochrome c of electrocardiographic changes caused by malachite green (*15*) may represent a hint. Phalloidin has been reported to interfere in rat liver mitochondria with oxidative phosphorylation at the cytochrome c stage of the respiratory chain (*18*). Whether this applies for α-amanitin too has not yet been studied.

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Dibutyryl Cyclic Adenosine Monophosphate Stimulation of Colcemid-Inhibited Axonal Elongation

Abstract. *Adenosine 3′,5′-monophosphate (cyclic AMP) and its dibutyryl derivative induce a variety of morphological changes, including those associated with in vitro axonal maturation. Established sensory ganglia treated with dibutyryl cyclic AMP show significant increases in average axonal length and number in comparison with controls; those treated with maintenance doses of Colcemid show no increases in either parameter; simultaneous treatment with both agents results in growth statistically similar to that produced by dibutyryl cyclic AMP alone. The data are consistent with our hypothesis that cyclic AMP promotes axonal elongation by stimulating microtubule assembly from a preexisting subunit pool.*

Adenosine 3′,5′-monophosphate (cyclic AMP) and its dibutyryl derivative have recently been reported to induce a variety of effects on neoplastic tissue, including morphological transformation of various tumor cell lines to a more normal appearance (*1*) as well as induction of differentiation in neuroblastomal cells (*2*). These morphological alterations suggest the possibility that cyclic AMP and dibutyryl cyclic AMP act specifically on some component of the cytoskeletal system. Recently we reported that both nucleotides are capable of significantly enhancing neurite maturation in a nonneoplastic neuronal system (*3*). The elongating axonal processes in this system contain two prominent cytoskeletal elements, microfilaments and microtubules. Treatment of these neurites with Colcemid or colchicine inhibits further elongation and at higher concentrations induces retraction (*4*). These effects can reasonably be attributed to the specific disruptive action of these agents on the microtubular components of the axonal cytoskeletal system (*5*).

One possible mechanism of action for cyclic AMP and dibutyryl cyclic AMP's promotion of neurite maturation might be stimulation of microtubule assembly from a preexisting subunit pool. In order to investigate this possibility, dibutyryl cyclic AMP was applied to established cultures in the presence of concentrations of Colcemid sufficient to prevent neurite elongation but not high enough to initiate a significant retraction (maintenance dose). Under these conditions, dibutyryl cyclic AMP significantly reversed Colcemid's inhibitory effects on axonal development.

Dorsal root ganglia from 8½-day-old chick embryos were explanted onto collagen-coated cover slips and cultured (*6*). In order to insure sufficient neuronal growth all cultures were treated for 48 hours beforehand in standard medium (basal medium 199 supplemented with 10 percent heat-inactivated, fetal calf serum) containing 5 m*M* dibutyryl cyclic AMP. The effective removal of the nucleotide from all cultures was accomplished by three 5-minute washes with standard medium before the experimental treatment. That this was an effective measure can be seen by comparison of those cultures subsequently incubated for 24 hours in standard medium with the other test groups (Fig. 1). The various test agents were incorporated into the standard