On the Relationship of Glutathione Reductase to Mushroom Poisoning (34267)

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Numerous studies have shown that individuals having genetic defects related directly or indirectly to glutathione metabolism are more vulnerable than normal persons to a variety of agents, such as drugs, alcohol, or radiation. Recent reports (1, 2) from Germany have described such a situation involving pathological changes in the erythrocytes after the consumption of poisonous mushrooms of the *Amanita* variety. In 10 cases of mushroom poisoning (1) there was a characteristic decrease in the GSH stability (Beutler test) of erythrocytes incubated with acetylphenylhydrazine, and increased Heinz body formation, both effects indicating an impaired ability to maintain glutathione in a reduced state. With recovery from the poisoning the above tests returned to normal. Eight of the 10 individuals had normal levels of erythrocyte glutathione reductase but the other two apparently were carriers of a glutathione reductase deficiency and had only half the normal level of enzyme (2). These two showed Heinz body formation and decreased GSH stability in the erythrocytes to a much greater degree than was observed for the others, and all out of proportion to their otherwise mild symptoms. These facts strongly suggest that there is a relationship between glutathione reductase, the transitory erythrocyte changes, and mushroom poisoning.

Waller *et al.* (2) recognized the above possibility but found no evidence for reduced activities of glutathione reductase or of other enzymes concerned with glutathione reduction in erythrocytes of patients suffering from mushroom poisoning. They believed the decline in GSH was an indirect result of acidosis; if the pH were much below the optimum for hexokinase (about 7.4 or 7.5), less glucose-6-phosphate would be formed, less TPNH would then be generated by glucose-6-phosphate dehydrogenase, and reduction of GSSG by TPNH and glutathione reductase would be impaired. This mechanism may be questioned because not all patients with metabolic acidosis showed glutathione instability, and the most marked instability occurred in a glutathione-reductase deficient individual who showed no signs of acidosis. The marked erythrocyte changes seen in glutathione-reductase deficient persons relative to normal persons might be better explained as the loss of an already marginal level of this enzyme under the influence of the mushroom poisons. Since the *Amanita* poisons are cyclic sulfur-containing hepta- or octa-peptides (3), and thus have a certain similarity to the disulfide hexapeptide substrate, GSSG, we looked for a direct inhibition of glutathione reductase by two of the poisons.

Materials and Methods. The mushroom poisons α-amanitin and phalloidin, which are the most abundant lethal peptides in *Amanita*, were generously provided by Dr. T. Wieland, Institute for Organic Chemistry, Frankfurt, Germany. The compounds were homogeneous upon thin-layer chromatography (4) on silica gel in methanol:2-butanone (1:1) and developed the typical colors with the standard detection reagents (3, 4). Intraperitoneal injection of α-amanitin and phalloidin at 1 and at 20 μg/g of body weight, respectively, caused 100% mortality in mice

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TABLE I. Effect of Mushroom Poisons on GSH Stability (Beutler test) in Normal Erythrocytes.

Expt.	Sample	Poison (M)	GSH after incubation (mg/ml of rbc)	GSH stability (% of unincubated control)
1	control	—	0.52	91
	α-amanitin	1×10^{-7}	0.54	95
		1×10^{-5}	0.53	93
		5×10^{-4}	0.52	91
2	control	—	0.57	92
	phalloidin	1×10^{-7}	0.55	89
		1×10^{-5}	0.57	92
		5×10^{-4}	0.50	81

(strains C57BL/6, BALB/c, DBA/2, and SJL). Mortality was low in a preliminary test of α-amanitin at 0.2 μg/g, a lethal dose for some strains of mice (5).

Five-ml samples of blood were drawn from normal persons and mixed with 6 mg of EDTA to prevent clotting. Total blood GSH by the nitroprusside method and GSH stability in the presence of acetylphenylhydrazine were determined by the methods of Beutler (6). For the stability test, 1-ml portions of blood were preincubated with or without the mushroom poisons for 1.5 hr at 37°, then 5 mg of acetylphenylhydrazine was added in solid form to each tube and the incubation continued for 2 hr; the contents were then analyzed for GSH.

Crystalline yeast glutathione reductase was obtained from the Sigma Chemical Company, St. Louis, Missouri, and assayed under the conditions of Massey and Williams (7). After preincubating the enzyme with the poisons for 15 min at 25°, oxidized glutathione was added to start the reaction.

Results and Discussion. Typical results with the Beutler test for GSH stability are shown in Table I. A decline in GSH under the acetylphenylhydrazine stress was observed but was not appreciably altered by the presence of the poisons, although a somewhat greater loss of GSH occurred with the highest level of phalloidin. On the basis of these results a direct inhibition by these particular poisons of any erythrocyte enzyme concerned with glutathione reduction seems unlikely at levels of poisons that are likely to be attained *in vivo*.

The above conclusion is supported by additional studies of these compounds with pure glutathione reductase, where possible complications such as permeability barriers are not a factor. For convenience, the crystalline yeast enzyme was used. The pure enzymes from yeast and human erythrocytes have many similarities, including molecular weight, flavin moiety, high specificity for the disulfide substrate, and sensitivity to sulfhydryl inhibitors (8). As shown in Table II, phalloidin had no effect on glutathione reductase, and only a slight inhibition was observed with the highest level of α-amanitin.

These results indicate that a direct inhibition of glutathione reductase by the mushroom poisons, α-amanitin and phalloidin, cannot explain the marked erythrocyte changes seen in mushroom poisoning of persons genetically deficient in this enzyme or of normal individuals showing no signs of a metabolic acidosis. The less direct studies of enzyme activities in erythrocytes of patients poisoned with mushrooms (2), or in tissues from animals poisoned with phalloidin (5),

TABLE II. Effect of Mushroom Poisons on Glutathione Reductase Activity.

(M) Poison[a]	GR activity (% of control)	
	α-Amanitin	Phalloidin
1×10^{-6}	98	100
1×10^{-5}	100	98
1×10^{-4}	100	97
3×10^{-4}	85	99

[a] Molarity of enzyme-bound flavin was approximately 1×10^{-9}.

also showed no inhibition of glutathione reductase or ancillary enzymes. The latter studies are complicated, however, by the possible effects on measured activity when tissue extracts containing the enzyme and a dissociable inhibitor are diluted and assayed in the presence of optimal amounts of substrate. Because of the circumstantial evidence that glutathione metabolism and the effects of poisonous mushrooms on erythrocytes are related in some fashion, we would leave open the possibility that whole mushrooms contain compounds other than these two peptides that strongly inhibit glutathione reductase, and thus produce erythrocyte changes in susceptible individuals out of proportion to overall toxicity. Alternatively, the compounds known to be present in mushrooms might act synergistically with other compounds or might undergo metabolism after their ingestion to form inhibitors of glutathione reductase.

Summary. The effects of two purified mushroom poisons, α-amanitin and phalloidin, on glutathione-reducing enzyme systems were investigated to determine if the decrease stability of glutathione and the pathological changes reported for erythrocytes of persons poisoned with mushrooms might result from inhibition of glutathione reductase by these cyclic sulfur-containing peptides. The poisons at levels up to 5×10^{-4} *M* had little or no effect on the activity of pure glutathione reductase from yeast and did not appreciably alter the level of reduced glutathione in human erythrocytes stressed with acetylphenylhydrazine. We conclude that a direct inhibition by these particular compounds of any erythrocyte enzyme concerned with glutathione reduction is unlikely, and that the reported relationship between glutathione reductase and mushroom poisoning must involve other compounds or other mechanisms.

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