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Toxic Effects of Phallolysin from Amanita phalloides

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Summary. Phallolysin, a protein from Amanita phalloides with cytolytic effects *in vitro*, was highly toxic when given intravenously to rats, mice, rabbits and guinea pigs: i.v. LD_{50} in rats was 85 Haemolytic Units (HU)/kg, corresponding to 0.05 mg protein/kg b.w. Death ensued from intravascular haemolysis. In rats large doses (600 HU/kg b.w.) caused cardiac death within a few minutes due to liberation of potassium from lysed cells. The serum contained lethal concentrations of potassium. There was also histological evidence of severe renal damage as a result of the haemolysis. In addition, phallolysin directly damaged the isolated guinea pig heart and the isolated rat liver, probably by its action on membranes.

Given by mouth, phallolysin was not poisonous to rats.

Key words: Phallolysin — Hemolysins — Amanita phalloides — Mushrooms — Toxicology.

Phallolysin is a toxic protein derived from the death cap fungus Amanita phalloides (Vaill. ex Fr.) Secr. It haemolyses erythrocytes from man and various other species *in vitro* (Seeger, 1972; Seeger *et al.*, 1973). Its isolation (Seeger, 1975a) and physicochemical properties (Seeger, 1975b) have been reported in previous communications. Toxicity studies were originally restricted to intraperitoneal administration to mice (Seeger, 1972). The following investigations, which have already been published in part (Odenthal and Seeger, 1974), were intended to elucidate certain organotoxic effects of phallolysin and to ascertain the cause of death in animals poisoned with it. Faulstich *et al.* (1974a, b) recently reported the isolation and toxic properties of two cytolytic glycoproteins from Amanita phalloides. They adopted the term phallolysin for these substances. Their findings will be discussed below.

Materials and Methods

Phallolysin was obtained in concentrated form from aqueous extracts of Amanita phalloides by precipitation with 40% ammonium sulphate, ion exchange chromatography on DEAE-cellulose and gel chromatography on Biogel P-30, and

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standardized in haemolytic units (HU) as previously reported (Seeger, 1975a). The preparation employed in the present study was free from low molecular weight toxins such as phalloidin and amanitins. It was obtained as eluate in 0.1 M sodium phosphate buffer pH 5.5 + 0.2 M sodium chloride + 10^{-3} M sodium azide and had an activity of 2500 HU/ml, 1 HU corresponding to 1.1 μg of protein and 0.06 μg of sugar. It was diluted with 0.9% saline for intravenous or oral administration.

The following substances were obtained from commercial sources: bradykinin (Sandoz, Nürnberg); eosin, formaldehyde solution (DAB 7), glucose (analytical grade), various salts (analytical grade) for Tyrode solution (see below), haematoxylin, creatinine (Merck, Darmstadt); mannitol (Boehringer, Ingelheim); p-aminohippuric acid (Casella-Riedel, Frankfurt); sodium pentobarbital, bovine serum albumin (lyophilized, pure, Serva, Heidelberg), polyvinylpyrrolidone (BASF, Ludwigshafen).

Tyrode Solution for the Langendorff Preparation. NaCl 6.96; KCl 0.45; $MgSO_4 \times 7H_2O$ 0.25; $CaCl_2 \times 2H_2O$ 0.41; $NaHCO_3$ 2.1; $NaH_2PO_4 \times 2H_2O$ 0.02; Na_2HPO_4 0.06; glucose 1.0; distilled water to 1000.0.

Modified Tyrode Solution for Liver Perfusion. NaCl 8.0; KCl 0.2; $MgCl_2 \times 6H_2O$ 0.21; $CaCl_2 \times 2H_2O$ 0.26; $NaHCO_3$ 1.0; $NaH_2PO_4 \times 2H_2O$ 0.58; glucose 1.0; bovine serum albumin 13.0; distilled water to 1000.0.

Animals. Female NMRI mice (19—25 g; Mus Rattus KG, Brunnthal); male and female rats, Wistar strain SPF (150—250 g; Mus Rattus KG, Bunnthal); female rabbits, White Vienna strain of own breeding (2.5—4.0 kg); male and female guinea pigs, Pirbright strain (300—400 g; Bäumler, Wolfratshausen). All animals had free access to drinking water and were fed on standard food (Altromin®), appropriate to each species. Food was withdrawn 24 hrs before the experiments.

General Toxicity

Mice were given test doses of phallolysin dissolved in a volume of 0.01 ml/g b.w. by injection into the tail vein over a period of 60 sec. 8—16 mice were used for each dose. For rats the volume was 1 ml/200 g b.w. and there were 10 animals in each group. Statistical calculations were carried out with the aid of probit analysis, fitting the regression line according to R. A. Fisher's method of maximum-likelihood solution, fiducial limits corresponding to t-values (Weber, 1972).

Ten female rats were each given 100 HU phallolysin/kg b.w. intravenously. Two hrs later the rats were killed, their haematocrit values determined and the Na^+ and K^+ serum concentrations measured by flame photometry (Eppendorf flame photometer, Hamburg). Their kidneys were fixed in 4% formalin, and 5 μm sections were cut and stained with haematoxylin-eosin[1].

For tests of oral toxicity groups of 5 rats of either sex were given 400 or 800 HU/kg b.w. by oesophageal tube.

Cardiovascular Effects

Wistar rats were anaesthetized with urethane (1.25 g/kg b.w.), fixed in a supine position on a heated operating table and tracheotomized. A polyethylene catheter was tied into the jugular vein for injections and another into the carotid artery for monitoring the blood pressure with a Statham transducer type P 37 and an amplifier unit (ifd Mescher, Mülheim/Ruhr). The ECG (standard lead II) was

[1] We are indebted to Dr. H. Uebel, Department of Toxicology, Dr. Madaus & Co., for his help.

recorded by subcutaneous needle electrodes, amplified and plotted together with the blood pressure on a Hellige 8 channel thermorecorder (Hellige, Freiburg).

Phallolysin was given either by continuous infusion in a dose of 6.7 HU/0.05 ml × min (7 rats) until death or by a single injection of 600 HU/kg b.w. in 1 ml within 60 sec (7 rats).

The serum potassium concentration was measured by flame photometry immediately after death in 5 rats which had received 600 HU/kg b.w. by i.v. injection. The results were compared with the potassium concentrations found in an untreated control group (19 rats) and with those found in 6 rats which had been given an infusion of 20 mg KCl/kg b.w. × min until death.

Phallolysin infusions were also given under the same experimental conditions to pairs of guinea pigs and rabbits anaesthetized with pentobarbital (40 mg/kg b.w., i.p.) unitil death occurred.

In order to study the effects of phallolysin on renal haemodynamics 10 male rats were anaesthetized with pentobarbital (40 mg/kg b.w., i.p.). Glomerular filtration rate was assessed by creatinine clearance, and renal plasma flow by PAH clearance and Na^+ clearance. For this purpose the rats were given a combined infusion of mannitol, creatinine and PAH by the technique of Vogel *et al.*, (1966) together with phallolysin 0.5 HU/min for a period of 1 hr. Creatinine was measured as total chromogens by the method of Popper *et al.* (1937), and PAH by the method of Bratton and Marshall (1937). Na^+ was determined by flame photometry. The results were compared with those obtained from a control group (10 rats). In 3 other rats the blood pressure measured directly by a cathcter in the carotid artery was recorded throughout the entire duration of the clearance test as described.

In rats anaesthetized with pentobarbital (30 mg/kg b.w., i.p.) thoracic duct lymph was collected by the technique of Vogel *et al.* (1967) for a period of 4 hrs. The so-called PVP filtration ratio was calculated from the plasma/lymph concentrations of infused polyvinylpyrrolidone (PVP) of known molecular weight. Injections of 15 (8 rats) or 25 (10 rats) HU phallolysin/kg b.w. were given 1 hr after starting the collection of lymph in order to study the effects of phallolysin on the plasma/lymph barrier. In another experiment (10 rats) an additional injection of 100 µg bradykinin/kg b.w. was given 60 min later to study the combined effects of phallolysin and bradykinin.

Studies on Isolated Organs

Guinea pig hearts ($n = 7$) prepared according to Langendorff's (1895) technique were perfused with Tyrode solution of the composition described above. A mechanogram was recorded with strech sensor and flexible rod type W (H. Sachs, Elektronik, Hugstetten/Freiburg), amplifier (ifd Mescher) and plotted on a Hellige thermorecorder. By this means the contraction rate (rate meter FR 2, ifd Mescher) was determined. Coronary perfusion rate was measured by a drop counter constructed as an optical sensor and recorded simultaneously.

Isolated rat livers were perfused without erythrocytes with modified Tyrode solution (see above) under a constant pressure of 15 mm Hg with a total perfusion volume of 150 ml (Schimassek, 1963). The K^+ concentration of the perfusate was determined in the liver outflow by flame photometry at hourly intervals. Bile was collected by a polyethylene catheter tied into the common duct and weighed at hourly intervals throughout the perfusion period. In addition, the increase of liver weight was measured after 3 hrs of perfusion. A control group of 10 livers was compared with a test group of 10 livers in which 75 HU of phallolysin had been added to the perfusion fluid 30 min after the start of the experiment.

Results

Acute Toxicity

The LD_{50} of phallolysin given intravenously to mice was 199.3 (22.2—1790; $P < 0.05$) HU/kg. The values were homogeneously distributed.

Doses below approx. 50 HU/kg caused merely sedation or short lasting convulsions. Higher doses—up to approx. 280 HU/kg—produced increasing haemoglobinuria, provided that the mice did not die immediately after the injection. Higher doses caused death within a few minutes, or in some instances even within 1 min. Death was preceded by marked excitation or convulsions.

For rats the LD_{50} by intravenous injection was 84.9 (50.4—142.9; $P < 0.05$) HU/kg. The values were also homogeneously distributed and the dose-response curve was steep.

Survival was dose-dependent. After a dose of 62.5 HU/kg it was 2—4 days, after 125 HU/kg 4 hrs to 3 days, and after 250 HU/kg only 55 min to 2 days. All these doses produced haemoglobinuria and pathological signs of severe haemolysis: copious effusions in the abdominal and thoracic cavities and in the pericardium, while the spleen, liver, heart and kidneys were soaked with blood-stained fluid. The bladder contained dark red urine. Reddish fluid dripped from the cut surfaces of the kidneys, cortex and medulla were no longer distinguishable. After doses exceeding 400 HU/kg most of the rats died within a few minutes with tonic-clonic convulsions. In such cases the signs of haemolysis were barely detectable at autopsy.

In rats killed 2 hrs after injection of 100 HU phallolysin/kg b.w. the haematocrit had fallen to $27.70 \pm 2.47\,\%$ [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 10$] as compared with $40.70 \pm 0.39\,\%$ in the controls ($n = 10$). Their serum was haemolytic and the serum potassium concentration was significantly increased to 4.58 ± 0.10 mEq/l [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 10$] as compared with a value of 3.63 ± 0.06 mEq/l in the controls. The serum sodium concentration did not differ significantly from that of the controls. Histological examination showed haemoglobin nephrosis with haemoglobin infiltration of glomeruli and interstitial tissue as well as haemoglobin casts in the tubules.

Oral doses of 400 or 800 HU/kg b.w. caused no obvious signs of toxicity in any of the rats.

Cardiovascular Effects

The effect of phallolysin depended on the rate at which it was infused into circulation.

Given by continuous infusion at a rate of 6.7 HU/min to urethane-anaesthetized rats it caused death within 25—50 min. As early as

5 min after starting the infusion the mean arterial pressure had fallen from 111.5 mm Hg to 83.5 mm Hg, and 15 min after starting the infusion to 50 mm Hg ($n = 7$); it then continued to fall until death occurred. Heart rate remained constant in all rats for the first 15 min after starting the phallolysin infusion. However, few minutes before death the heart rate dropped and the ECG showed a variety of abnormalities including widening of the QRS complex. AV block and ventricular fibrillation.

Spontaneous voiding of blood-stained urine was noted in a few cases. Autopsy showed a picture of massive haemolysis as described above for anaesthetized rats.

Phallolysin infusions administered under similar conditions to guinea pigs and rabbits caused death accompanied by the same toxic manifestations.

When anaesthetized rats were given an intravenous injection of 600 HU phallolysin/kg b.w. within 60 sec (approximately LD_{95}), they died within 5—35 min. The ECG showed a drop in heart rate at a very early stage at the end of the injection, accompanied by a fall in mean arterial blood pressure from 118.0 ($n = 7$) to 85.0 mm Hg ($n = 5$); (2 animals omitted because of early arrhythmia.) These changes were followed by delay in the conduction and spread of the excitation wave, and then by polymorphic heterotopic arrhythmias which finally ended in ventricular fibrillation or sudden cardiac arrest (Fig. 1). With the onset of arrhythmia the mean arterial blood pressure dropped to levels of 30—40 mm Hg.

Blood collected immediately after death was haemolytic. The serum contained potassium in a concentration several times higher than normal, namely 17.43 $\pm$ 7.25 mEq/l [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 5$] as compared with 3.63 $\pm$ 0.06 mEq/l in the untreated controls ($n = 19$).

Rats given a continuous infusion of potassium chloride (20 mg/kg $\times$ min) died of cardiac arrhythmia after 25.3 $\pm$ 4.1 min [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 6$]. Mean arterial blood pressure and heart rate dropped during the infusion: 10 min after starting the infusion mean arterial blood pressure had fallen from 117.1 to 96.3 ($n = 6$) and heart rate from 403 to 311 beats/min. At the time of death the mean serum potassium concentration was 17.78 $\pm$ 4.45 mEq/l [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 6$].

During the 1 hr clearance tests, infusion of phallolysin at a rate of 0.5 HU/min caused no change in arterial blood pressure ($n = 3$). However urine excretion decreased sharply to 0.32 $\pm$ 0.09 ml/min $\times$ kg [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 10$] as compared with 1.10 $\pm$ 0.06 ml/min $\times$ kg in the control group ($n = 10$). Na^+ clearance fell from 0.76 $\pm$ 0.05 to 0.12 $\pm$ 0.06 ml/min $\times$ kg, creatinine clearance from 8.88 $\pm$ 0.52 to

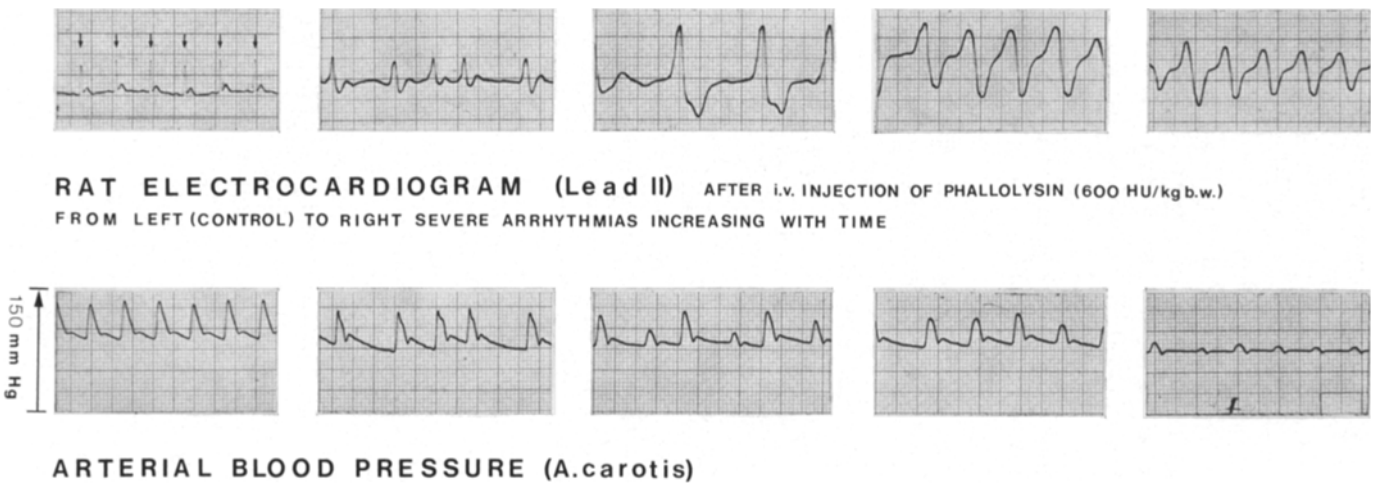

Fig. 1. Electrocardiogram and arterial blood pressure of a phallolysin-poisoned rat

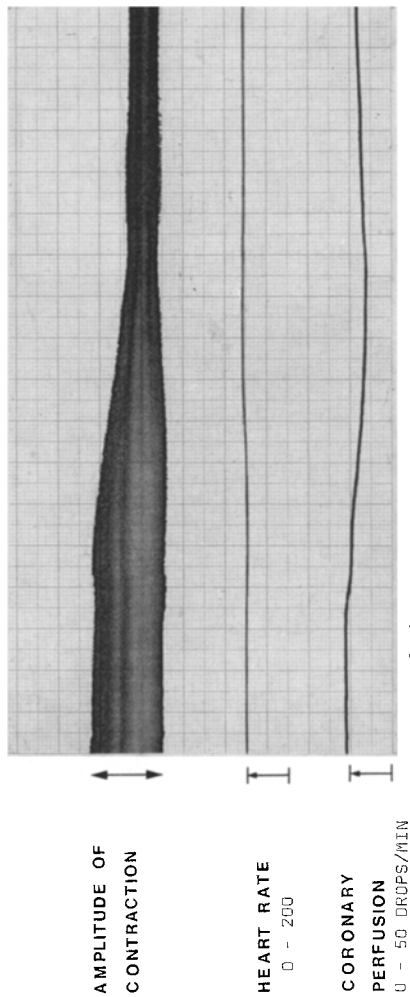

Fig. 2. Effect of phallolysin on isolated guinea pig heart (Langendorff preparation)

2.20 ± 0.54 ml/min × kg and PAH clearance from 30.72 ± 2.20 to 7.93 ± 2.10 ml/min × kg.

Administration of phallolysin in doses of 15 or 25 HU/kg b.w. to anaesthetized rats had no effect on lymph flow or PVP filtration ratios for up to 3 hrs after the injection. Supplementary injection of bradykinin under the described experimental conditions likewise caused no change as compared with the control animals.

Effects on Isolated Organs

In experiments on isolated perfused guinea-pig hearts, phallolysin (5 HU/heart) irreversibly decreased the contraction amplitude (Fig. 2).

Whereas the outflow of perfusate from the coronary vessels was greatly diminished and remained low as long as 60 min afterwards, heart rate was unaffected apart from a slight increase at the time of the maximum negative inotropic effect.

Higher doses of phallolysin (10 HU or more per heart) produced cardiac arrest within 3—5 min.

In experiments on the isolated rat liver there was a sharp rise in the outflow rate of potassium from the liver into the perfusion medium as early as 30 min after the addition of phallolysin (75 HU). The potassium concentration in the perfusate rose from 2.49 ± 0.17 mEq/l to 6.38 ± 1.05 mEq/l [$\bar{x} \pm (\text{S.E.})_{\bar{x}}$; $n = 10$]. The corresponding values in controls were 2.60 ± 0.13 and 3.40 ± 0.40 mEq/l ($n = 10$). Up to the end of the experiment potassium loss from the phallolysin-poisoned livers continued to rise, finally reaching 6.86 ± 0.98 mEq/l, the corresponding figure in the controls being 4.70 ± 1.11 mEq/l. Throughout the experiment these differences were highly significant.

In the phallolysin-poisoned livers the perfusion rate decreased by 41.3% over a period of 180 min with a steep decline after 90 min. Bile secretion ceased in 8 out of 10 livers within 90 min. In the controls, the decrease in perfusion rate was 14.9%, while bile secretion continued throughout the experiment.

The weight of the phallolysin-poisoned livers seemed to increase to a greater extent than that of the controls [weight gain 3.36 ± 1.07 g $(\bar{x} \pm (\text{S.E.}))_{\bar{x}}$ as compared with 1.75 ± 1.31 g in controls], but the difference was not significant.

Discussion

From earlier investigations it was known that phallolysin causes haemolysis *in vitro* and is highly toxic to mice (LD_{50} 1000 HU/kg b.w.) by intraperitoneal injection. The signs of poisoning were not characteristic and were obscured by a pain reaction (Seeger, 1972). Our present investigations have shown that phallolysin is five times more toxic to mice by intravenous than by intraperitoneal route, the LD_{50} being 199 HU/kg. Wistar rats are approximately 2.5 times more susceptible, the intravenous LD_{50} for rats is only 84.9 HU/kg. For reasons of economy, the phallolysin preparation was purified only to the extent described above and represented a 15-fold concentration compared to the protein content of the fungus extract. This appeared justified because the toxicity is correlated with the haemolytic activity and the impurities hence appear to be nontoxic. Further purification (Seeger, 1975a) with an average enrichment of 25-fold (at best 75-fold) yielded a preparation of phallolysin A in which 1 HU is equivalent to only 0.62 μg protein (or in

the most highly concentrated preparation 0.20 μg). The LD_{50} for mice calculated from these figures is 0.123 mg/kg (or possibly as low as 0.04 mg/kg), the LD_{50} for rats 0.05 mg/kg (or 0.017 mg/kg respectively). Hence, phallolysin is more toxic than α-amanitin, the LD_{50} in mice being 0.20 mg/kg (Wieland, 1967).

Doses which would certainly have been fatal if given intravenously were without effect when given to rats per os. Quite apart from the still unexplored problem of intestinal absorption, this can be explained by the known acid lability of phallolysin (Seeger, 1972; 1975b).

Our investigations also showed that phallolysin has not only haemolytic effects *in vitro*, but also causes intravascular haemolysis *in vivo* in mice, rats, rabbits and guinea pigs, Haemolysis was obviously the cause of death in rats which died from low doses; this conclusion was derived from autopsy findings and confirmed by the decrease in haematocrit.

However, as the dose was raised, cardiotoxic signs dominated. Rats died within few minutes and mice often in less than 1 min, too early indeed for autopsy signs of haemolysis to be demonstrable. Proof of cardiac arrest was obtained in rats.

Relevant in this respect are findings of Lin *et al.* (1973) who isolated a haemolysin from *Volvariella volvacea*, a fungus likewise belonging to the family Amanitaceae, but in view of its predominant effect they classified it as a cardiotoxin.

At first sight phallolysin seems to have two different lethal actions—a haemolytic action at one dose level and a cardiotoxic action at another. However, even in the rats which died of cardiac arrest within a few minutes, the blood was haemolytic and the serum potassium was raised to concentrations which were not compatible with life as shown by our comparative tests with KCl infusions. It seems clear that these animals likewise were affected by haemolysis, although the pathological signs had not fully developed in such a short time.

The release of potassium from erythrocytes in the quantities demonstrated in the serum after injection of phallolysin, especially after a dose of 600 HU/kg, is sufficient to explain the cardiotoxic effect of phallolysin. It could also explain the cardiac arrhythmias (Vanderark *et al.*, 1973; Ettlinger *et al.*, 1974; Surawics, 1974) and might in part be responsible for the decrease in blood pressure (diminution of peripherial resistance) through the dilator effects of potassium on vascular smooth muscle (Biamino and Wessel, 1973). The sudden drop in blood pressure after high doses of phallolysin is mainly due to cardiac arrhythmia and, therefore, of haemodynamic origin.

Our experiments on isolated guinea pig hearts show that phallolysin also possesses a direct cardiotoxic effect expressed in an irreversible decrease in contraction amplitude. Like the damage observed in the

isolated perfused rat liver and the irreversible lesions in the isolated guinea pig ileum (Mosthaf, unpublished) and in the neural and muscular components of the isolated rat diaphragm (Seeger, unpublished), this may also be the result of the membrane-damaging action of phallolysin. It may be assumed that this direct cardiotoxic action is involved in the cardiac arrest produced by high doses of phallolysin. In view of the fact that phallolysin has cytotoxic effects on all mammalian cells so far investigated (Fiume, 1967; Seeger and Lehmann, 1973; Faulstich *et al.*, 1974), the release of other intrinsic substances such as histamine or neurohumoral transmitters has also to be considered. This aspect has not yet been investigated.

The drasic reduction in urine output and in sodium, PAH and inulin clearance, raises the possibility that phallolysin may also possess nephrotoxic effects. In view of the histological changes seen after injection of 100 HU/kg b.w.—haemoglobin infiltration in the glomeruli and interstitium together with haemoglobin casts in the renal tubules—it can be assumed that the abnormalities of renal function are due to phallolysin-induced haemolysis. In our clearance experiments we were able to exclude any interference with renal haemodynamics.

Faulstich and Weckauf-Bloching (1974) observed haemolysis in only 20—30% of the rats which had died 20—22 hrs after injection of their phallolysin preparation (which was 10-fold concentrated as measured by the protein of the fungus extract). However, as they found haemorrhages in the intestine and retroperitoneal space in the other rats they postulated an angiotoxic effect of phallolysin as the cause of death. Our studies of the plasma-lymph barrier in rats yielded negative results with sublethal doses of phallolysin and hence indicate that phallolysin, if it attacks vessel membranes at all, has at least no preferential effect on them.

Our results lead to the conclusion that in mice, rats, rabbits and guinea pigs, phallolysin causes death by intravascular haemolysis. The cardiotoxic effect seen after high doses is due to release of potassium from lysed cells, though direct damage, as demonstrated in the isolated heart, may also be a factor.

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