Physical Properties and Function of Phallolysin†

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ABSTRACT: Phallolysin, a mixture of two to three cytolytic proteins (all of M_r 34 000), has been isolated from *Amanita phalloides* mushrooms and purified to homogeneity (specific activity 24 000 hemolytic units/mg of protein). After separation by isoelectric focusing, the amino acid composition of two of these proteins has been determined. They are rich in water-soluble amino acids and contain one tryptophan residue each, but no cysteine or methionine. M_r was determined to be 34 000 in the native form as well as under denaturing conditions, indicating that the native proteins exist as monomers. Many of the physical properties of phallolysin are strikingly similar to those of staphylococcal α-toxin, e.g., molecular weight, existence of multiple forms, p*I* values, amino acid composition, and thermolability (60 °C). Pure phallolysin allowed us to prepare a radioactively labeled toxin. Labeling was achieved by reaction with formaldehyde, followed by reduction with sodium [^{3}H]borohydride. With the labeled toxin (specific activity 7–14 Ci/mmol, ca. 60% biological activity), we investigated its binding to human A_2 erythrocytes. We determined the number of receptors on these cells (2×10^4 per cell) as well as their affinity to the toxin ($K_D = 4 \times 10^{-9}$ M). In studies on the mechanism of cytolytic activity, we were able to distinguish at least three sequential events: binding of the toxin to human erythrocytes, K^+ release, and membrane rupture (hemoglobulin release). These steps could be characterized by different kinetics as well as by different temperature dependencies. Again, the kinetic data for phallolysin are very closely related to those obtained for staphylococcal α-toxin. The similarity in the mechanism of the two toxins is discussed in detail. On the basis of the observation that both toxins also affect liposomes, a mechanism of cytolysis is proposed which may be valid for both toxins.

Phallolysin is a component of the mushroom *Amanita phalloides* and causes cytolysis in a great number of mammalian cells. Conspicuous through its hemolytic activity, it was the first toxin detected in this plant (Kobert, 1891). There is agreement today that it is the most potent toxin of this mushroom, although it does not contribute to human poisoning because it is labile in acidic media or on heating to temperatures >60 °C. LD_{50} values were determined in the rabbit (40 μg/kg; Faulstich & Weckauf-Bloching, 1974) and in the rat (50 μg/kg; Odenthal et al., 1975).

All preparations of phallolysin described so far (Faulstich & Weckauf-Bloching, 1974; Seeger, 1975) were not pure enough for exact studies of the physical and chemical properties of the protein. We have therefore modified the purification procedure and have obtained a homogeneous protein, allowing us to study in more detail the physical properties of the toxin as well as the mechanism of cytolysis.

Materials and Methods

Purification of Phallolysin. Frozen *A. phalloides* mushrooms (550 g) were thawed, minced in a Star mixer together with 0.9% NaCl (up to a final volume of 1200 mL), and stirred for 3 h at room temperature. After centrifugation at 20000*g*, solid ammonium sulfate was added (ca. 130 g/L) to the supernatant until the conductivity (Radiometer, Copenhagen) was 120 mS. The mixture was gently stirred for another 3 h at room temperature to achieve complete precipitation and centrifuged at 4 °C for 30 min at 20000*g*. The precipitate was discarded, and again solid ammonium sulfate (ca. 100 g/L) was added to the supernatant to 4 °C until the conductivity had reached 155 mS. After centrifugation (30 min at 4 °C and 20000*g*), the precipitate was dissolved in 50 mL of water and extensively dialyzed against 0.9% NaCl for 18 h at 4 °C. After some turbidity was removed by centrifugation, the dialyzed solution was started on a DEAE-Sephadex A-25 (Pharmacia, Upsala) column (2 cm i.d. × 100 cm) equilibrated with tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] buffer (0.02 M, pH 8.2). The eluate was monitored by optical density (280 nm, Uvicord II, LKB, Sweden) and tested for hemolytic activity. The fractions containing the hemolytic activity were combined (ca. 100 mL).

The toxin fraction was directly applied to another column (2 cm i.d. × 100 cm) of CM-Sephadex C-25 (Pharmacia, Upsala) equilibrated with 0.02 M sodium phosphate, pH 8.0. The fractions containing the hemolytic activity were detected as described above, combined, and lyophilized.

A column (2 cm i.d. × 60 cm) with Bio-Gel P100 (Bio-Rad, München) was prepared after removal of fine, floating particles from the sedimenting gel and equilibrated in 0.02% sodium azide solution. The lyophilized protein was dissolved in 5 mL of water, centrifuged at 20000*g* for 5 min, and applied to the column. Detection of the protein was as described above.

The fraction containing the hemolytic activity was directly applied to a column (0.5 cm i.d. × 20 cm) containing *N*-acetylglucosamine attached to polyacrylamide (Selectin 1, Pierce, Eurochem. Rotterdam) equilibrated with 0.02% sodium azide solution. Again, the toxin fraction was detected by its optical density (at 280 nm) and hemolytic activity and lyophilized. The gel was recovered by washing with 0.45 M *N*-acetylglucosamine, followed by 0.02% sodium azide solution.

Protein concentration was determined according to Bradford (1976).

Hemolytic activity was measured as described by Faulstich & Weckauf-Bloching (1974); however, the erythrocytes (A_2, Behring Werke, Marburg/Lahn) were washed 3 times with 0.154 M NaCl before use. Erythrocytes of various mammals were isolated from fresh blood by three washings with 0.154

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[1] Abbreviations: HU, hemolytic unit(s) (based on washed human standard erythrocytes; former assays used unwashed human standard erythrocytes); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hb, hemoglobin.

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M NaCl and centrifugation (ca. 2000*g*). The hematocrit was adjusted to 5%.

Isolation of the Multiple Forms of Phallolysin by Isoelectric Focusing on a Preparative Scale. Swollen Sephadex G-200 Superfine (308 mL, Pharmacia, Upsala) was mixed with 10 mL of a 40% (w/v) ampholyte solution (Servalyt, pH 5–9, Serva, Heidelberg) and poured into a gel chamber (20 cm × 25.6 cm × 0.6 cm, Desaga, Heidelberg) at 4 °C. Supernatant solution was withdrawn with a strip of filter paper, and a furrow was formed with a spatula in the middle of the gel; 20 mg of the lyophilized phallolysin, as obtained from the affinity column described above, was dissolved in 2 mL of a 1% ampholyte solution and applied to the furrow which was then refilled with the removed gel material. Two strips of thick filter paper, soaked with 1 M NaOH and 1 M H_3PO_4, respectively, were used as electrodes. The chamber was put into a Desaphor electrophoresis apparatus with cooling (Desaga, Heidelberg) and run at 200 V for 3 h, followed by 600 V for 12 h. A 3-cm strip of filter paper as usually used for chromatography (Whatman, no. 3) was put on the wet gel for a copy. The strip was dried at 110 °C for 10 min, and the proteins were detected by incubation with Coomassie Blue and destaining in ethanol/water/acetic acid (750:1950:240). The areas of the gel corresponding to the colored bands on the paper strip were removed, suspended in the same volume of 0.5 M NaCl, and applied to a column (2 cm i.d. × 25 cm) of Sephadex G 25 equilibrated with 0.5 M NaCl. The protein material was eluted in the first peak and separated from the ampholytes. After removal of the salts by dialysis, the protein material was lyophilized.

Amino Acid Analysis. About 1 mg of each of phallolysins A and B, isolated from the preparative isoelectric focusing procedure, was dissolved in 3 mL of 6 N HCl, containing 1% phenol (Sanger & Thompson, 1963), and hydrolyzed for 22 h at 110 °C in sealed ampules. The amino acid composition was determined on an automatic analyzer (Biotronik, Frankfurt).

Assay for Cysteine. One milligram of phallolysin was carboxymethylated with iodo[^{14}C]acetic acid and analyzed by dialysis for the incorporation of radioactivity into the protein material (Heil et al., 1974).

Assay for Tryptophan. Four milligrams of phallolysin was reacted with *N*-bromosuccinimide according to Spande & Witkop (1967). The decrease of the UV spectrum at 280 nm, caused by oxidative degradation of tryptophan, was measured.

Assay for Saccharides. One milligram of phallolysin was hydrolyzed in 3 mL of 2 N HCl at 110 °C for 6 h. The hydrolysate was evaporated to dryness, and the dry residue was silylated as reported by Wulff (1965). Dissolved in 100 μL of benzene, an aliquot of the solution was applied to an OV 101 column (200 cm, 3%, 10^{-11} g/s intensity, 80–220 °C program, 4 °C/min rise in temperature, Fractovap 2150, Carlo Erba Instrumentazione, Hofheim/Taunus).

SDS Gel Electrophoresis. Gels and samples were performed according to the method of King & Laemmli (1971).

Analytical Electrofocusing. Isoelectric focusing was carried out on ampholine PAG plates (LKB, Sweden) comprising a pH range from 3.5 to 9.5 essentially according to Wellner & Hayes (1973), modified as described by Bühring (1981).

Polyacrylamide Gradient Gel Electrophoresis. The molecular size of the native toxin was determined as described by Bühring (1981).

Preparation of ^{3}H-Labeled Phallolysin. Three milligrams of phallolysin was dissolved in 750 μL of borate buffer (0.1 M, pH 9.0) and centrifuged at 20000*g*. Two aliquots of the supernatant were taken for a protein assay and for the determination of hemolytic activity. The aliquot of 2 mg of protein (60 nmol) was reacted with 9 μL of a 1% solution of formaldehyde (300 nmol) for 10 min under stirring. Sodium [^{3}H]borohydride (600 nmol, dissolved in 20 μL of borate buffer, specific activity 7.1 Ci/mmol, Amersham-Buchler, Braunschweig) was added, and stirring was continued for 15 min; 0.1 N HCl was added cautiously to adjust the pH to 4.0 (at pH 3.5, the hemolytic activity would be irreversibly lost), and the solution was stirred for 2 h. After extensive dialysis against 0.154 M NaCl, the reaction mixture was applied to a Bio-Gel P-10 column (0.5 cm i.d. × 20 cm) equilibrated with 0.02% NaN_3 solution. The fraction containing phallolysin as detected by its hemolytic activity was filtered through a sterile filter tip on a 10-mL syringe (pores 450-nm Millex, Millipore, Neu-Isenburg, West Germany) and used directly. In an aliquot of the solution, the specific radioactivity (curies per 34000 mg of protein) and the specific hemolytic activity (HU per mg of protein) were determined by using the method of Bradford (1976) for measuring the protein mass.

Determination of the Equilibrium Dissociation Constant (K_D) of Phallolysin. Portions (1 mL) of human erythrocyte suspensions (1.3%) were incubated with varying concentrations (5×10^{-9}–10^{-7} M) of [^{3}H]methylphallolysin (specific activity 3×10^8 dpm/mg of protein) for 30 min at room temperature. Incubation was stopped by addition of 4 mL of 0.154 M NaCl and filtered through a microfilter (Schleicher & Schuell, BA 85, 0.45-μm pores). In controls, the corresponding amount of [^{3}H]methylphallolysin was filtered, but without cells. After three washings (10 mL of 0.154 M NaCl), the filters were transferred to a counting vessel filled with Instagel (Packard). In a Scatchard plot, B' (nanograms of toxin bound) was plotted against B/F, where B = cpm bound and F = cpm free = total cpm − cpm bound.

Calculation of the Number of Binding Sites for [^{3}H]-Methylphallolysin on the Surface of Human Erythrocytes. The number of binding sites (n) per human erythrocyte was calculated according to

$$n = \frac{ML}{m}$$

where M is the maximum number of moles of [^{3}H]methylphallolysin bound under assay conditions, as taken from the point of intersection of the ordinate in the Scatchard plot, L is Lohschmitt's number, and m is the number of human erythrocytes in the assay (ca. 1.3×10^8 cells).

Measurement of K^+ Release. The K^+ concentration present in the supernatant of erythrocytes incubated with phallolysin was measured in an atomic absorption spectrometer (Unicam SP90) at 760 nm. For calibration, a KCl solution of known concentration was used. The K^+ concentration found after osmotic shock of erythrocytes was taken as the 100% value.

Kinetic Measurements. Portions (1 mL) of human erythrocyte suspensions (1.3%) were incubated with [^{3}H]methylphallolysin (specific activity in one experiment 7 Ci/mmol, in another 14 Ci/mmol) at a concentration causing 50% hemolysis after 20 min. Aliquots were taken after defined time intervals. One sample was subjected to the binding assay as described above; another was used for the determination of K^+ ion and hemoglobin release.

Results

Definition of Hemolytic Units. One hemolytic unit (HU) is defined as the amount of phallolysin causing 50% hemolysis in a 1.3% suspension of human erythrocytes (A_2) within 45 min at 22 °C (Faulstich & Weckauf-Bloching 1974). Since

Table I: Efficiency and Yield of the Purification Procedures for Phallolysin

purification step	hemolytic activity (HU)	protein mass (mg)	sp act. (HU/mg)	relative concn (extract = 1)	yield (%)
extraction	2110000	3600	586	1	100
ammonium sulfate precipitation	1600000	240	6660	11.2	76.2
DEAE-Sephadex	1406250	128	10986	18.9	67.0
CM-Sephadex	1276500	99	12894	22.2	60.8
Bio-Gel P100	625000	30	20833	35.8	29.8
N-acetylglucosamine–polyacrylamide	600000	25	24000	41.0	28.5

FIGURE 1: Disc PAGE (12%) of purified phallolysin in the presence of SDS (0.1%). The marker protein next to phallolysin is pepsin (M_r 34700).

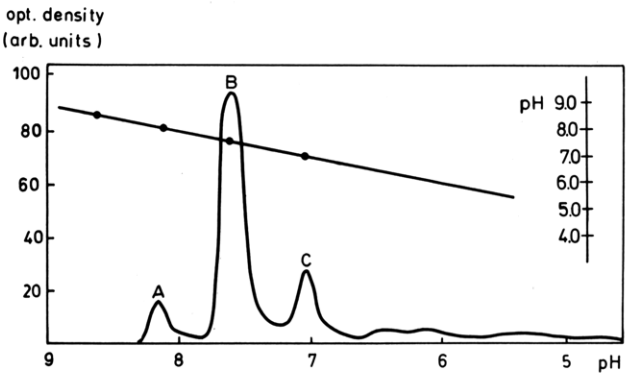

FIGURE 2: Densitogram of an electrofocusing gel showing the amount of the three multiple proteins (A, B, and C) in a phallolysin preparation.

standard erythrocytes increased in sensitivity after several washings in 0.9% NaCl, we concluded that the cells were suspended in solutions containing unknown additives for stabilization. Therefore, in the present study, we used such erythrocytes only after three washing steps, after which their susceptibility to phallolysin had become constant. The washings increase the sensitivity of the erythrocytes by a factor of 3–6. Accordingly, the numbers for the specific activity (HU per milligram of protein) presented in this paper may differ from previous numbers by a factor of 3–6.

Sensitivity of Erythrocytes of Various Animal Species. The use of nonstabilized erythrocytes allows the direct comparison of human and mammalian red blood cells. Their different resistances to phallolysin, expressed as relative concentrations required for 50% hemolysis, are the following: man (A_2), 1; dog, 1.7; pig, 8; goat, 170; ox, 640; sheep, >1000. When the hemolytic activity was assayed at 37 °C, instead of 22 °C, human erythrocytes became more resistant (2), while the bovine cells became more sensitive (256).

Purification of Phallolysin. The purification of phallolysin as reported here resulted in a 41-fold increase in the specific hemolytic activity. Table I shows the efficiency of the different purification steps. Phallolysin was obtained with >95% purity as proved by a single band in SDS–PAGE (Figure 1).

Multiple Forms of Phallolysin. Previous studies with phallolysin have reported multiple forms of the protein, either two (Faulstich and Weckauf-Bloching, 1974) or three (Seeger, 1975). They were designated A, B, and C, in order of decreasing p*I* values. A and B were present in all our preparations (n = 8) while C was found in only two of them. The p*I* values were determined from analytical isoelectric focusing gels to be 8.1 for A, 7.6 for B, and 7.0 for C.

Form B predominated in all preparations (>50%); form A was between 10 and 50%, and form C was 10–20%, if present at all. The densitogram as obtained from isoelectric focusing of one of the preparations containing protein C is shown in Figure 2. In this batch, the contribution of the three proteins to the total protein mass was the following: A, 11%; B, 70%; and C, 19%. The three forms were separated by isoelectric focusing. All of the forms were capable of destroying human erythrocytes in the assay described above. However, their specific activity was found decreased to about 3000 HU/mg for each, i.e., ~10% of the original hemolytic activity. Evidently, the greater part of the proteins was denatured by the electrofocusing procedure or the following separation step from the ampholine material. In any case, the protein material was homogeneous, and for two of them (A and B), the amount was sufficient for determining the amino acid composition (Table II).

Table II: Amino Acid Composition of Phallolysin Forms A and B and, for Comparison, Staphylococcal α-Toxin Forms A and B (Six & Harshman, 1973) and the Mixture of A and B (Kato & Watanabe, 1980)

amino acid	A	B	α_A	α_B	α
Asx	25	24	40	43	43
Thr	21	34	22	23	24
Ser	36	22	19	19	21
Glx	27	30	19	20	20
Pro	18	24	8	9	10
Gly	50	30	20	24	36
Ala	17	16	11	11	17
Val	21	21	13	14	16
Leu	15	19	13	14	16
Tyr	7	7	9	10	7
Phe	8	6	8	8	9
Lys	22	21	21	23	31
His	7	12	4	4	9
Arg	8	8	8	8	8
Trp	1	1	4	4	not reported
Met	0	0	6	6	8
Cys	0	0	0	0	0

Amino Acid Composition. While the number of arginine, tyrosine, and valine residues is identical for both forms, amounts of other amino acids vary widely, e.g., proline, glycine, serine, threonine, isoleucine, and histidine. Provided M_r = 34000 is correct, both phallolysins A and B would contain 294 amino acid residues composed as shown in Table II. Both forms are free of cysteine and methionine and contain one tryptophan residue each. This was proved by the UV ab-

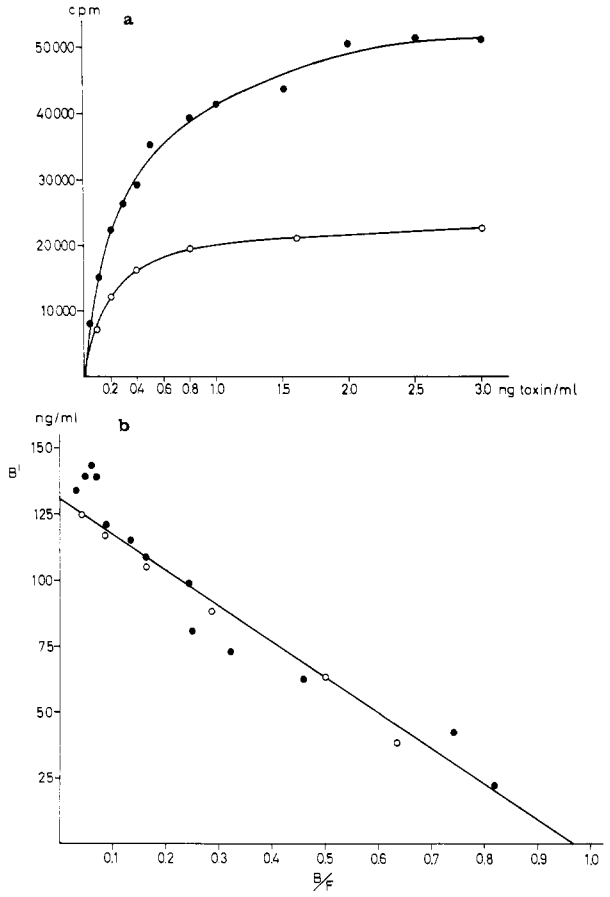

FIGURE 3: (a) Binding of [^{3}H]methylphallolysin to human erythrocytes (A_2, 1.3 × 10^8 cells/mL) as determined by a filter assay. (○) Experiment with [^{3}H]methylphallolysin of specific activity 7 Ci/mmol. (●) Second experiment with [^{3}H]methylphallolysin of specific activity of 14 Ci/mmol. (b) The same data plotted according to Scatchard: B', nanograms of toxin bound per milliliter; B, cpm bound; F, cpm free.

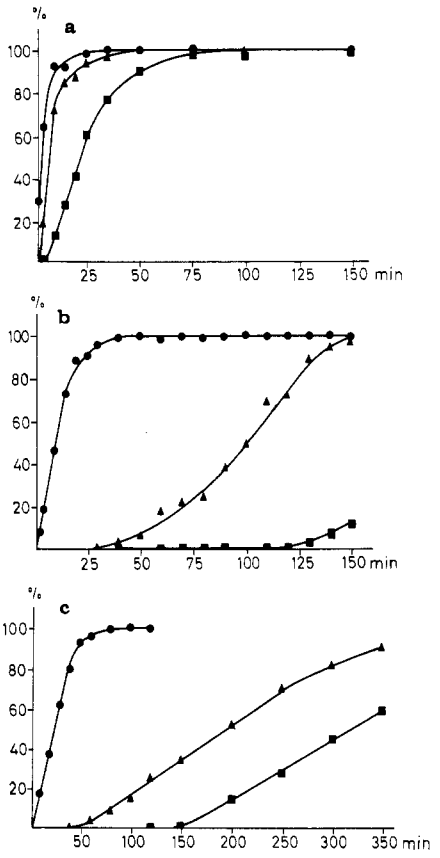

FIGURE 4: Kinetics of binding of [^{3}H]methylphallolysin (●) and K^+ efflux (▲) and Hb release (■) from human erythrocytes at 22 °C (a), human erythrocytes at 4 °C (b), and bovine erythrocytes at 2 °C (c).

sorption spectrum possessing the typical shoulder at 290 nm (not shown) and quantitated by the decrease in UV absorption at 280 nm after oxidative degradation of the protein with *N*-bromosuccinimide (Spande & Witkop, 1967). We determined 1.09 tryptophan residues per M_r 34000.

Radioactive Labeling. Methylation of ε-amino groups in phallolysin can be achieved by reaction with formaldehyde, followed by reduction of the Schiff base formed with tritiated borohydride. The reaction needs exact dosing: too large an excess of formaldehyde or borohydride, or lowering the pH to values slightly below 4.0, even for a short time, will destroy the biological activity. The specific activity of the methylated phallolysin (7 Ci/mmol), as achieved with a batch of sodium borohydride of 7.1 Ci/mmol, suggests that 4 lysine residues out of 21 have been substituted. This was proved by amino acid analysis, which separated the peaks of lysine and methyllysine, indicating a ratio of 4:1. The modification reduced the specific hemolytic activity to 58%.

A second labeling experiment (specific activity of sodium [^{3}H]borohydride 10.7 Ci/mmol) yielded the protein with a specific radioactivity of 14 Ci/mmol, corresponding to 5.2 methylated lysine residues. In this experiment, the hemolytic activity had been reduced to 66%.

Affinity of [^{3}H]Methylphallolysin for Human Erythrocytes. Specificity of the Binding and Number of Binding Sites per Cell. When the concentration of toxin was increased under constant assay conditions (1.3 × 10^8 human erythrocytes/mL), the amount of toxin bound reached a saturation value. The results of two experiments using the [^{3}H]methylphallolysin preparations (see above) are shown in Figure 3a.

The data were also plotted according to Scatchard (B' vs. B/F, Figure 3b). From the slope of the line, we calculate the equilibrium dissociation constant K_D = 4.0 × 10^{-9} M. The maximum number of phallolysin molecules bound to a defined number of erythrocytes allows the calculation of the number of receptors per cell (n). This value for human erythrocytes was n = 2 × 10^4/cell. Specificity of binding was demonstrated by competition of unlabeled phallolysin with [^{3}H]methylphallolysin for the receptors on human erythrocytes: dilution of [^{3}H]methylphallolysin by 1 equiv of native phallolysin reduced the radioactivity bound to 50% (47% ± 15%). This value indicates that the labeled and unlabeled toxin compete for the same binding site and that the K_D values of the labeled and unlabeled toxins are comparable.

Kinetics of Binding of [^{3}H]Methylphallolysin and Release of K^+ and Hemoglobin. Kinetic studies were performed to show the correlation of toxin binding with K^+ efflux and with release of hemoglobin. The experiments were performed by using human erythrocytes at 22 (Figure 4a) and 4 °C (Figure 4b) as well as bovine erythrocytes at 22 °C (Figure 4c).

With human erythrocytes at 22 °C (Figure 4a), half-saturation is reached after 3 min. In contrast, the release of K^+ and hemoglobin occur later, namely, after 6 and 20 min, respectively (50% values). This indicates that K^+ efflux and hemoglobin release (rupture) are events separate from each other as well as from binding.

This becomes even more evident at 4 °C, where binding is only slightly retarded (half-saturation after 8 min), but K^+ release takes 100 min and rupture would take several hours if the experiments were carried on for more than 2.5 h. After

Table III: Physical Properties of Phallolysin and Staphylococcal α-Toxin

property	phallolysin	staphylococcal α-toxin	ref for data on staphylococcal α-toxin
M_r	34000	36000	Kato & Watanabe (1980)
multiple forms	3	2-4	
p*I*	7.0, 7.6, 8.1	7.2, 8.4	Six & Harshman (1973)
		6.3, 7.4, 8.6, 9.2	McNiven et al. (1971)
biological activity			McCartney & Arbuthnott (1978)
is destroyed at temp (°C) higher than	65	60	
affinity for receptor, K_D (M)	4×10^{-9}	6×10^{-9}	Cassidy & Harshman (1976)
LD_{50} (μg/kg of rabbit)	40	5-7	Arbuthnott (1970)

2.5 h, the hemoglobin release was measured to be only 10%.

In a similar experiment, using bovine erythrocytes and a concentration of phallolysin 50-fold higher than with the human cells, the corresponding values for half-saturation, K^+ release, and rupture were 25, 200, and 320 min, respectively. In comparison to human erythrocytes, which bound 3.8 pmol of [^{3}H]methylphallolysin per 1.3×10^8 cells, the toxin amount bound on bovine erythrocytes was 25 times less (0.15 pmol/1.3×10^8 cells, not shown).

Discussion

Purification Procedure. The purification procedure described above yielded phallolysin with the highest specific hemolytic activity obtained so far (24000 HU/mg of protein). Some of the purification steps applied were reported previously but were either improved or optimized while other steps were added. For example, in the ammonium sulfate precipitation, the extremely high water solubility of phallolysin allows the separation of a number of other less soluble proteins in the first fraction obtained at 22% salt saturation. Further, ion-exchange chromatography on DEAE-Sephadex was superior to that on DEAE-cellulose. The ion-exchange procedure was performed at pH values near the isoelectric point of phallolysin (around 8.0), where the toxin will pass through the column with minimal interaction with the gel matrix while other proteins with p*I* < 8 are held back. This concept was also extended to proteins with p*I* > 8 associated with the toxin, which were easily removed by another passage through a column of CM-Sephadex, again at pH 8. An attempt to concentrate the toxin by immobilization on an affinity column with *N*-acetylglucosamine attached to polyacrylamide failed but accidently removed some minor contaminants. After the five purification procedures listed in Table I, phallolysin can be regarded as >95% pure. This was shown by disc PAGE in SDS (Figure 1) as well as by isoelectric focusing experiments in which besides the band of the three hemolytically active isoproteins only traces of other protein material were detected (Figure 2).

Amino Acid Composition of Phallolysins A and B. Both forms of the protein are rich in amino acid residues contributing to high water solubility (Ser, Thr, Asx, Glx, Pro, Gly). Obviously, the high content of these amino acids implies that phallolysin will not be precipitated on the addition of trichloroacetic acid, either in aqueous solution or in electrophoresis gels. In neither form were sulfur-containing amino acids found, indicating that phallolysin cannot belong to that class of cytolytic agents which act through thiol groups, such as alveolysin (Geoffroy et al., 1981) or streptolysin O (Halbert, 1970).

Comparison of the amino acid composition of the two forms shows only little variation in the basic amino acids, which are believed to be involved in membrane interaction (lysine, arginine) (A. C. Vaisius et al., unpublished results). The small difference (only one lysine residue) cannot account for the different p*I* values of forms A and B. Therefore, some differences must exist also in the asparagine/aspartic acid and glutamine/glutamic acid ratios, which have not been determined so far. Large variation (up to 40%) was found for serine, threonine, glycine, isoleucine, and histidine, indicating that the isoforms of phallolysin represent different gene products.

Radioactive Labeling. Iodination of the tyrosine residues in phallolysin was accompanied by complete loss of biological activity. Evidently, an essential amino acid residue was affected by this reaction. This result is similar to that obtained with staphylococcal α-toxin (Cassidy & Harshman, 1976) which lost ca. 90% of its biological activity during iodination.

We therefore tried methylation of the ε-amino groups of lysine, a reaction which introduces tritium in a hydrogenation step. Four or even five methyl groups were introduced with more than 60% recovery of biological activity. Moreover, the affinity of the toxin to erythrocyte receptors seems not be diminished by the modification because addition of 1 equiv of unlabeled toxin substituted ca. 50% of the labeled toxin, indicating that the affinities of the native toxin and the toxin derivative are comparable.

The decrease in hemolytic activity after modification did not parallel the degree of methylation of lysine residues. We therefore assume that the loss of cytolytic activity was not caused by the methylation itself but rather by the exposure of the reaction mixture to acidic conditions during the preparation procedure.

Physical Properties of Phallolysin and Staphylococcal α-Toxin. For comparison, the physical properties of phallolysin and staphylococcal α-toxin are compiled in Table III.

Evidently, both toxins are slightly basic proteins (M_r ca. 35000) and exist in multiple forms. They are unstable on heating, have comparable high affinity for receptors on erythrocyte membranes, and are extremely toxic when injected into animals. Also, the amino acid compositions (Table II) seem related, particularly the high content of hydroxylated amino acids (serine, threonine), the constant portion of aromatic amino acids (phenylalanine, tyrosine), and the absence of cysteine. Only the values of methionine and tryptophan are completely different.

Functional Analogies of Phallolysin and Staphylococcal α-Toxin. Our present knowledge on the toxic activities of phallolysin and α-toxin suggests that with both toxins cytolysis is the final event of a multistep process. Moreover, the two toxins seem to correspond even in the details of their prelytic phases. By kinetic analysis, a sequence of at least three events was characterized: binding of the toxins, efflux of K^+ ions, and membrane rupture as indicated by hemoglobin release.

At concentrations giving comparable cytolytic activity (release of 50% of the hemoglobin after 20 min of incubation from human erythrocytes with phallolysin and from rabbit erythrocytes with α-toxin), the kinetics of the events preceding cytolysis, namely binding and K^+ release, are nearly identical for the two toxins [see Figure 4a of this paper and Figure 2 in Cassidy & Harshman (1976)]. Thus, half-saturation of the

binding sites was reached with both toxins after 1–3 min, while it took 4–6 min to release 50% of the K^+ ions. We conclude that binding and K^+ release are two distinct events, which occur in a similar way with both toxins. This can be demonstrated even more clearly if the experiments are run at 4 °C instead of 22 °C (Figure 4b). Under these conditions, both effects are retarded, but K^+ release is retarded much more so than binding. Binding is complete after 30 min, when K^+ release is just starting. On the other hand, K^+ release is complete after 2 h, when cytolysis begins. Again, the corresponding experiments with α-toxin at low temperature, as reported by Cassidy & Harshman (1976), showed that binding, ^{86}Rb release, and hemoglobin leakage from rabbit erythrocytes occurred in a way strikingly similar to phallolysin.

The time course of ion (K^+, $^{86}Rb^+$) release, as well as of hemoglobin leakage (cytolysis), as caused by either phallolysin or α-toxin, shows characteristic lag times. The duration of the lag time depends, for both toxins, inversely on the toxin concentration. After the lag time, the respective release of ions and hemoglobin proceeds linearly. As a result, sigmoidal time courses are observed which represent another feature common to the two toxins. By this kinetic behavior, the two toxins differ distinctly from other cytolytic agents like melittin or the toxin of sea anemone (*Stoichactis helianthus*), which both lyse cells instantly after binding (Seitz et al., 1981).

For understanding the events occurring during the lag time, the experiments at low temperature were particularly important. They showed not only that the event determining the time course of cell rupture is a prelytic step, occurring even prior to K^+ release, but also (from the strong retardation at low temperature) that this unknown event is highly temperature dependent.

A hypothetical explanation would be that the lag time represents the formation of pores or channels, a prerequisite for the dissipation of K^+ ions. Such pores may form from toxin units alone or from membrane components in cooperation with the toxin. The formation of such pores would then be the rate-limiting step for the whole chain of events occurring after binding of the toxin. That this step was found to be slowed down at low temperature is understandable since the formation of such pores would be expected to depend largely on membrane fluidity and thus on temperature.

Proposed Mechanism for the Toxic Activity of Phallolysin and Staphylococcal α-Toxin. Phallolysin damages not only cells but also liposomes (Seeger & Wachter, 1980; Bühring et al., 1983). Thus, multilamellar liposomes, as well as unilamellar liposomes, release incorporated [^{14}C]glucose when treated with the toxin. The concentration used for disrupting multilamellar liposomes was ca. μM. In comparison, the most sensitive mammalian cells (Faulstich et al., 1974) were affected at concentrations of 10^{-8} M. In these experiments, the composition of the liposomes was either phosphatidylcholine, cholesterol, and dicetyl phosphate or phosphatidylserine. There was no [^{14}C]glucose release from liposomes when dicetyl phosphate or phosphatidylserine was substituted by neutral or cationic lipid components like stearylamine. Obviously, the negative net charge of liposomes is a prerequisite for the activity of phallolysin (Bühring et al., 1983). Again, similar results have been reported for α-toxin: the bacterial toxin is also able to damage liposomes, i.e., protein-free artificial membranes, preferentially those containing lipid components with a negative net charge like dicetyl phosphate or phosphatidylserine (Weissmann et al., 1966; Freer et al., 1968).

On the other hand, receptor proteins in the membrane of, e.g., erythrocytes seem to determine whether a cell species is more or less sensitive to phallolysin (Faulstich & Weckauf, 1975). For example, bovine erythrocytes were shown to bind 25 times less of the labeled toxin. This probably corresponds to a much decreased number of receptor molecules and possibly even a decreased affinity to the toxin. As a result, the toxin concentration required for the lysis of bovine erythrocytes is 640-fold higher than that for human cells and comparable to the concentration used for the disruption of liposomes.

Very different sensitivities have also been reported for the various species of erythrocyte against staphylococcal α-toxin. Here some erythrocyte species were shown to be devoid of receptor molecules (rat, guinea pig) but nevertheless susceptible to lysis (Barei & Fackrell, 1979). Obviously, also here the presence and number of receptor molecules determine the lytic activity of the toxin. Notably, the receptor for the staphylococcal toxin has a specificity distinctly different from that of phallolysin receptor. This must be concluded from the fact that, e.g., bovine erythrocytes are nearly insensitive to phallolysin but highly sensitivity to staphylococcal α-toxin.

Considering all the data, we suggest that the basic mechanism of action of phallolysin, and probably also of α-toxin, is on phospholipids, causing a leakage of membranes by the formation of pores.

Organization of these pores is relatively slow and seems to require a critical concentration of toxin. At high concentrations of the toxins in the medium, this critical concentration can be achieved without receptors, by nonspecific interaction with the membrane, provided the membrane has a net negative charge. This may be the case for the above-mentioned liposomes as well as for "insensitive" or "resistant" erythrocytes (ovine or bovine erythrocytes for phallolysin; rat or guinea pig erythrocytes for α-toxin). In contrast, in cells containing specific receptor molecules on the surface, the critical concentration in the membranes can be reached at much lower concentrations of the toxins in the medium. Receptors with high affinity will bind the toxin and accumulate it in or on the membrane.

We assume that when the critical concentration is reached, toxin molecules or membrane components, or both, form pores, causing leakage of small particles, e.g., K^+ ions. Balancing of the K^+ gradients in cells, however, is associated with an influx of Na^+ ions, as shown for phallolysin (Seitz et al., 1981). Concomitant with the Na^+ ions, water will penetrate, inducing so-called colloid osmotic swelling, as originally pointed out by Wilbrandt (1941). Such swelling induced in human erythrocytes by phallolysin has been visualized by microscopy; it seems indistinguishable from the swelling caused by hypotonic medium. Swelling beyond the critical volume of the cell will finally end with cell rupture and hemoglobin release (Seitz et al., 1981).

The receptors assumed to specifically bind phallolysin or α-toxin are present in only low concentrations (for phallolysin, 20 000 per human erythrocyte; 5000 for α-toxin on the erythrocytes of rabbits; Harshman, 1979). They must be species specific, because otherwise the large differences in the susceptibility of erythrocytes of various animal species cannot be explained: 1:1000 for phallolysin (human:ox) and 1:100 for α-toxin (rabbit:human; McCartney & Arbuthnott, 1978; Barei & Fackrell, 1979). It is therefore unlikely that such receptors are among the well-characterized, major components of the erythrocyte membrane.

The formation of pores suggested here is supported by structural changes as recently reported for the membranes of human erythrocytes treated with phallolysin (Seitz et al., 1981). In freeze-etching pictures, an aggregation of particles

was found in the P face, together with numerous troughs which were not detectable in controls.

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Correlation of Enzymatic Properties and Conformation of Smooth Muscle Myosin†

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ABSTRACT: In the presence of adenosine 5′-triphosphate (ATP) and 1–10 mM $MgCl_2$, the relative viscosity (η_{rel}) of dephosphorylated gizzard myosin is reduced markedly over a range of KCl from 0.35 to 0.15 M. Sedimentation patterns show that the decrease in η_{rel} is due to the conversion of the 6S to 10S forms of myosin. Under similar conditions, η_{rel} of phosphorylated myosin is not altered, and at 0.2 M KCl, the 10S form is not observed. In 1 and 2 mM $MgCl_2$ and <0.2 M KCl, 10S can be formed from both phosphorylated myosin plus ATP and dephosphorylated myosin minus ATP. In the presence of ethylenediaminetetraacetic acid (EDTA), the decrease of η_{rel} and corresponding change in sedimentation pattern are independent of ATP and show only a dependence on KCl. Therefore, ATP and dephosphorylation are not obligatory for the 6S to 10S transition. In all instances, the 6S–10S transition of monomeric myosin is paralleled by an alteration of adenosine-5′-triphosphatase (ATPase) activity; i.e., the KCl dependence of the two processes is the same. Transition from 6S to 10S causes a decrease in Mg^{2+}- and Ca^{2+}-ATPase activity of myosin and an increase in K^+-EDTA-ATPase activity. The relationship between myosin shape and the ATP dependence of Mg^{2+}-ATPase activity also is consistent with this generalization. The phosphorylation dependence of the viscosity transition from 6S to 10S is not linear, and phosphorylation of both heads is required for the complete transition. In contrast, the ATP dependence of the transition is linear, and the binding of 2 mol of ATP/myosin is required for maximum effect. The idea is developed that the enzymatic properties of myosin are determined by its conformation, and thus, analogous changes in filamentous myosin may be important in the regulation of the activity of smooth muscle actomyosin.

The most popular theory to account for the regulation of the contractile apparatus of smooth muscle involves phosphorylation of the two M_r 20000 light chains of the myosin molecule. Several facets of this scheme have been analyzed, and considerable evidence in favor of the phosphorylation hypothesis has been accumulated [for reviews, see Adelstein & Eisenberg (1980) and Walsh & Hartshorne (1982)]. However, the role of phosphorylation in activating actomyosin adenosine-5′-

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