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# STUDIES ON EXTRACELLULAR PROTEINS FROM *STAPHYLOCOCCUS AUREUS*

## VII. STUDIES ON β-HAEMOLYSIN

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SUMMARY

Some properties of an extensively purified β-haemolysin were investigated[4]. The purified β-lysin was confirmed to release *N*-acylsphingosine from sphingomyelin, thus being a sphingomyelinase C. The haemolytic spectrum for different red blood cells was investigated and sheep erythrocytes were found to be most sensitive. No synergism or antagonism was noticed on the combined action of staphylococcal α-, β-, and δ-haemolysin on sheep, rabbit, or human red blood cells. Purified β-haemolysin was lethal for rabbits, guinea pigs, mice (10–100 μg), and chicken embryos (0.25–10 μg). Both effects were inhibited by an anti-β-serum. Human thrombocytes and their α-granules were also rapidly lysed, while human leucocytes and bacterial protoplasts were resistant for a comparative dose. These observations on the susceptibility of different cells are compatible with the different phospholipid composition of these cell membranes. No β-haemolytic activity was found in the staphylococcal cell fractions. Thus, β-haemolysin is probably a true extracellular protein, actively excreted during growth.

## INTRODUCTION

Recent investigations suggested that β-haemolysin of *Staphylococcus aureus* is an enzyme which hydrolyses sphingomyelin[1–3] and liberates *N*-acylsphingosine and phosphorylcholine. β-Haemolysin from strain R 1 was purified in a large scale and by acrylamide electrophoresis and other methods[4] the preparation was shown to be highly pure. It was also found to be devoid of all well established extracellular toxins and enzymes produced by strains of *S. aureus*. In spite of the fact that highly purified preparations of β-haemolysin have recently been obtained, there are still contradictory results in the literature with regard to the physical properties and toxicity of this haemolysin[5–8].

This investigation was carried out in order to study some properties of the

Abbreviations: p*I*, isoelectric point; HU, haemolytic unit(s); PCMB, *p*-chloromercuribenzoate.

β-haemolysin with special regard to its cytotoxic effects on mammalian cells and lethal activity in mice and rabbits. The toxin has been shown to be a true extracellular protein (exoprotein), actively excreted by growing cells of *S. aureus*.

## MATERIALS AND METHODS

The methods for the production and purification of β-haemolysin, measurement of protein and evidences for purity were identical with those previously reported[4]. Unless otherwise stated, purified β-haemolysin (Stage 3, Table I, ref. 4) with a specific activity of $10^{11}$ haemolytic units (HU)/mg was used for the procedures described below.

### *Measurement of haemolytic activity*

Measurement of haemolytic activity was performed as previously described[4]. To investigate the haemolytic spectrum, red blood cells of several species were used (see Table I) and the assays were performed in the ordinary test system. The end points of 50% haemolysis were read after incubation at 37° for 1 h and at 4° for 2 h. In order to investigate the possible synergistic and antagonistic effects of purified α-, β-, and δ-haemolysin, assays were performed on cells of rabbit, sheep, and human red blood cells. Purified α-haemolysin ($\alpha_{Ia}$, p*I* 8.6; specific activity, 8000 HU/mg[9]), β-haemolysin (p*I* 9.4; specific activity $10^{11}$ HU/mg[4]), and δ-haemolysin (p*I* 9.6; specific activity 100 HU/mg[10]) were diluted in phosphate buffered saline containing $MgSO_4$ and bovine serum albumin[4] and assays were performed in this buffer system.

### *Sphingomyelinase activity. Qualitative determination*

The incubation mixture containing β-haemolysin $10^9$ HU (Stage 3 haemolysin, specific activity $10^{11}$ HU/mg) sphingomyelin, Tris–HCl buffer, and $MgCl_2$ was the same as in the quantitative assay described in ref. 4. The chloroform phase after extraction of the phospholipids was concentrated by perevaporation and subjected to thin layer chromatography on glass plates covered by silica gel (DC Fertigplatten, 0.25-mm-thick layer, Merck, Darmstadt, Germany) with the solvent chloroform–methanol–water (65:25:4, v/v/v) according to WAGNER *et al.*[11]. A control was also made for every test, in which the enzyme was added to the incubation system after addition of chloroform and methanol. Samples of 20 μl containing approx. 100 μg of phospholipid and a control containing 100 μg *N*-acylsphingosine (ceramide) were applied on the chromatograms. $H_2SO_4$ (10%, v/v) was used as a spray reagent.

### *Measurement of lethal and dermonecrotic activity*

*Mice.* The lethal activity of the purified β-haemolysin was observed in Swiss white mice weighing about 20 g. Samples of β-lysin were filtered through a Millipore filter (pore size 0.45 μm, Millipore, Bedford, Mass., U.S.A.) and tested for sterility. 0.1 ml of lysin diluted in sterile phosphate buffer saline was injected into the tail vein, or 0.5 ml was injected intraperitoneally. Deaths occurring within 24 h were recorded.

*Rabbits.* The lethal effect of purified β-haemolysin for rabbits was also investigated. The skin necrotising property of crude and purified β-haemolysin was also demonstrated in mature rabbits about 2.5 kg in weight. Rabbits were handled as

previously reported[7]. Skin reactions were read and recorded every 24 h for 7 days.

*Guinea pigs.* Grown up animals were injected intraperitoneally with Stage 3 $\beta$-lysin.

*Chicken embryos.* $\beta$-Haemolysin (0.1-ml samples) was injected intravenously into 10-days-old embryos as described by SMITH AND THOMAS[12]. The same doses of $\beta$-lysin were in other experiments also applied on the chorio-allantoic membrane[12,13]. Deaths were recorded after 2 h and overnight.

*Thrombocytes*

Human blood was obtained by veni-punction and 350 ml was collected in eight siliconized glass cylinders of 50 ml volume each containing 5 ml of 0.1 M sodium citrate. Platelet rich plasma was obtained after 45 min at 20° by suction of the 2–4 cm buffy coat on top of the sedimenting erythrocytes followed by centrifugation (190 $\times g$, 15 min, 20°). The thrombocytes were resuspended in Hank's balanced salt solution. When stored at 4°, the platelet rich plasma could be used for as long as 4–8 h. Phase contrast microscopy revealed a contamination of a few erythrocytes and leucocytes per field (400 and 950 $\times$ magnification). This preparation contained $0.2 \cdot 10^6$–$3 \cdot 10^6$ platelets/mm$^3$ and $10^3$–$5 \cdot 10^3$ each of erythrocytes and leucocytes/mm$^3$. Incubation of one drop of platelet rich plasma on a siliconized glass slide in a moisten chamber (20 min, 20°) permitted removal of these red blood cells by spraying the surface with Hank's balanced salt solution[14]. Incubation of thrombocytes together with $\alpha$-, $\beta$-, and $\delta$-haemolysin in suitable dilutions was also performed, followed by a study of the morphological changes in the microscope. The assay conditions for determination of platelet aggregation and lysis were based on the method described by O'BRIEN[15]. The decrease in absorbance at 540 nm ($A_{540\,nm}$) was automatically recorded for 10 min in a Beckman DB-G instrument. ADP (Sigma) was dissolved in saline (1 mg/ml) and used as a positive control for platelet aggregation[15]. The appearance of exo-$\beta$-*N*-acetylglucosaminidase (EC 3.2.1.29)[16] and lactate dehydrogenase (EC 1.1.1.27)[17] in the soluble fraction was estimated after removal of the intact thrombocytes by centrifugation (500 $\times g$, 10 min).

*Leucocytes*

Parafilm slide preparations of human polymorphonuclear leucocytes were prepared from capillary blood as previously described[14,18]. The cells were observed for morphological changes in the phase contrast microscope (400 and 950 $\times$ magnification).

*Tissue cultures*

The following tissue cultures were used: (1) HeLa-cells[19]; (2) primary cultures of cynomolgus monkey kidney cells; (3) serially propagated cell cultures of African green monkey kidney; (4) two strains of fetal human diploid fibroblasts derived from skin and muscle were used at the 8–10th passage[20]. All cells were outgrown in different media and maintained in EAGLE's[21] medium containing Earle's buffer supplemented with penicillin (100 $\mu$g/ml) and streptomycin (100 $\mu$g/ml). Maintaining media for Tissue culture 1 and 4 also contained bovine fetal serum (2%, v/v). After the medium was exchanged and 0.1 ml of purified $\beta$-haemolysin in appropriate dilutions was added, the tubes were incubated stationary at 37° once more. The cells were examined

for cytotoxic effects under the microscope after 2, 6, 24, and 72 h of incubation. Two tubes of each culture were incubated with β-haemolysin from each dilution. Negative controls were incubated with β-haemolysin neutralized by antiserum "EX 1480" as determined in the ordinary haemolytic assay system. Positive controls of β-haemolysin were treated with the same amount of normal human serum or a polyvalent staphylococcal antiserum "M18" without anti-β-haemolytic activity.

*Protoplasts*

*Bacillus megaterium* KM, was grown in liquid medium[22] and protoplasts were prepared in principle as previously described[23]. *S. aureus*, strain R 1 was grown in casein hydrolysate–yeast extract medium[4] and protoplasts were prepared by enzymatic digestion with lysostaphin[24]. A drop of the suspended whole protoplasts was also examined with a phase-contrast microscope (magnification × 950) after the incubation was stopped (37°, 2 h). Ten independent fields did not reveal a single intact organism.

A sample of each protoplast preparation was also centrifuged, suspended, and lysed in 0.1 M phosphate buffer (pH 7.0). The isolated membranes were centrifuged twice (20 000 × *g*, 20 min, 4°) and resuspended in the same buffer. No *N*-acetylaminosugar or aminosugar was detected in this preparation, suggesting that no cell wall material was present.

Assay procedure: protoplasts were resuspended in 0.1 M phosphate buffer (pH 7.0) containing 0.6% (w/v) $MgSO_4$ and 0.4 M sucrose. Lysis was followed by continuous recording of the absorbance at 640 nm ($A_{640}$) in a Beckman DB-G spectrophotometer with the reaction mixture at 37°.

*Experiments on whole cells of strain R 1*

*Disintegration and release experiments.* Suspensions of whole cells were washed twice with 0.01 M phosphate buffer (pH 7.0) at 4°. The standard procedure for disintegration of washed whole cells of *S. aureus*, strain R 1 (20 g wet wt.) was a freeze-press technique (X-press, Biotec Labtjänst, Stockholm, Sweden) according to EDEBO[25]. Cells were also lysed by lysostaphin[24] and/or staphylococcal endo-β-*N*-acetylglucosaminidase (1000 U[26]). Cell suspensions of strain R 1 ($A_{640\ nm} = 2.0$) were cleared during 20 min at 37°.

The disintegrated material was centrifuged (4000 × *g*, 10 min) and the pellet was discarded. The supernatant was centrifuged once more (15 000 × *g*, 30 min), β-haemolytic activity was assayed on the supernatant, "the cytoplasmic fraction", and the pellet was suspended in Hank's balanced salt solution, "the cell wall fraction".

In order to investigate the possibility to release β-haemolysin from whole cells, cells from the early, middle and late part of the exponential growth phase were washed as just described and suspended in the same buffer supplemented with sodium phosphate, sodium citrate, RNA (from yeast, commercial grade, Sigma), and 1 M KCl[27–29]. Selective release by suddenly cooling of the cells was also tried[30].

*Chemical and analytical methods.* (1) *N*-Acetylaminosugar (Morgan Elson reaction, 30-min heating time) according to GHUYSEN *et al.*[31]; (2) aminosugar according to BELCHER *et al.*[32].

*Chemicals* were of analytical grade unless otherwise stated and were purchased as previously reported[4]. Lysostaphin (5619-7A) was kindly donated by Dr. P. A. Tavormina and coworkers at Mead Johnson, Evansville, Ind. (U.S.A.). Tissue

cultures were kindly supplied by Dr. S. Jeansson and Dr. G. Carlström, Dept. Clinical Virology and human blood from healthy donors (Blood Transfusion Center) at Karolinska sjukhuset, Stockholm.

*Antisera.* An anti-β-serum, "EX 1480", was generously supplied by Dr.P. Knight, Wellcome Res. Labs, Beckenham (England). A polyvalent staphylococcal antiserum, devoid of anti-β-haemolysin, was obtained by immunizing rabbits with crude exoproteins of *S. aureus*, strain M18 (T. WADSTRÖM, unpublished data). It was used as a control serum in the tests for the different biological activities of purified β-haemolysin.

## RESULTS

Contradictory results on the stability of purified β-haemolysin were reported[4,8,33]. The purified β-haemolysin used in this investigation was found to be unstable upon storage both at 4° and at −20°[4]. Glycerol and carrier ampholytes were found to partially stabilize this protein. On storage of purified β-haemolysin (Stages 1–3)[4] at 4°, the β-haemolytic and sphingomyelinase activities and the cytotoxic, dermonecrotic, and lethal activities were inactivated in parallel. A preparation, containing $10^9$ HU/ml of Stage 3 β-haemolysin, was completely inactivated after 5 days of storage at 4° and was then used as a control in the biological tests.

Purified β-haemolysin was found to be extremely unstable upon storage in a diluted state[4]. Addition of serum albumin (1 mg/ml), glycine (1%, w/v), 0.1 mM dithiotreitol, 1 mM *p*-chloromercuribenzoate (PMCB), and several other compounds did not stabilize the activity. Lyophilization has up to now been found to be the only method for a safe long time storage of purified toxin[4].

### *Haemolytic spectrum*

The haemolytic spectrum of purified β-haemolysin, $10^9$ HU/ml (sheep red blood cells), was assayed on red blood cells on a variety of species (Table I). No cells from other species were found to be as sensitive as the sheep red blood cells. The "hot–

TABLE I

SENSITIVITY TO β-HAEMOLYSIN OF ERYTHROCYTES FROM DIFFERENT ANIMALS

| *Animal* | *HU/ml (37°, 1 h)* | *HU/ml (4°, 2 h)* |
|---|---|---|
| Sheep | 10 | $10^9$ |
| Ox | 10 | $10^8$ |
| Goat | 10 | $10^5$ |
| Rabbit | 10 | $10^2$ |
| Human | 10 | 10 |
| Fowl | 10 | 10 |
| Chicken | 10 | 10 |
| Pig | 10 | 10 |
| Cat | 10 | 10 |
| Guinea pig | <10 | <10 |
| Horse | <10 | <10 |

TABLE II

COMBINED EFFECT OF α-, β-, AND δ-HAEMOLYSIN ON RABBIT, SHEEP AND HUMAN RED BLOOD CELLS

| *Haemolysin* | *Rabbit (HU/ml)* | *Sheep (HU/ml)* | *Human (HU/ml)* |
|---|---|---|---|
| α- | $10^5$ | $10^2$ | 20 |
| β- | $10^2$ | $10^9$ | 20 |
| δ- | 40 | $<10$ | 160 |
| α- + β-* | $10^5$ | $10^9$ | 20 |
| α- + δ-* | $10^5$ | $10^2$ | 160 |
| β- + δ-* | 40 | $10^8$ | 160 |
| α- + β- + δ-* | $10^5$ | $10^8$ | 160 |

* $10^5$ HU of α-haemolysin (rabbit red blood cells), $10^9$ HU of β-haemolysin (sheep red blood cells) and 160 HU of δ-haemolysin (human red blood cells) were used in these experiments.

cold" phenomenon after incubation at 37° and 4° was observed for sheep and goat cells[5,34].

*Haemolytic action by combination of α-, β-, and δ-haemolysin*

Purified preparations of α-, β-, and δ-haemolysin were assayed singly and two together in the three possible combinations on rabbit, sheep, and human red blood cells (Table II). No obvious antagonistic or synergistic effects were noticed by any of these combinations on the three red cell systems.

*Phospholipase C. Identification of sphingomyelinase activity*

Purified oxbrain sphingomyelin (Sigma) was incubated with purified β-haemolysin at 37° for 30 min. The phospholipids were then extracted with chloroform and methanol and subjected to thin-layer chromatography. One spot was obtained after staining with the spray reagent, which had the same $R_F$ value as *N*-acyl-sphingosine. Thin-layer chromatography of the chloroform–methanol extracts showed that, as the time of incubation increased, there was a quantitative decrease in sphingomyelin, which was associated with a comparable increase in the concentration of *N*-acyl-sphingosine.

*The lethal activity*

Purified staphylococcal β-haemolysin was shown to be lethal for mice which died within 10–20 sec from 10–100-μg quantities. Upon intravenous injection in rabbits of the same dose, spasms were recorded within 5–20 min and the animals then died after some minutes. After intraperitoneal administration death of rabbits came 2 to 12 h later and for guinea pigs within 2 h. The instability of purified β-haemolysin in a diluted state has prevented determination of the $L.D._{50}$ dose. A specific anti-β-serum ("EX 1480") inhibited the haemolytic and lethal activity in the same dilution while a polyvalent antistaphylococcal serum ("M18") did not.

A dose of 0.25 μg was lethal for chicken embryos. These died within 2 h after intravenous injection. The chorio-allantoic membrane technique was found to be somewhat less sensitive, since about 10 μg was required to kill the embryos. The dead embryos were changed in morphology, the membranes and other macroscopical

structures were partly dissolved into an amorphous mass, while the control embryos killed by storage over night at 4° were unchanged in morphology.

*Dermonecrotic effect*

β-Haemolysin in 1–10 μg quantities was injected intracutaneously in rabbits. An area of dermonecrosis was surrounded by a zone of erythema and visible already 12 h after the injection and it increased both in diameter and depth during the whole observation period. These effects were very similar to the results obtained upon injection of purified α-toxin (0.1 mg) into another two animals.

*Thrombocytes*

Human platelets lyse rapidly and completely when in contact with an appropriate amount of β-haemolysin in 0.25 ml. No clumping similar to the aggregation obtained by ADP was visible in the microscope when 1 μg was added. However, a sample of unconcentrated crude β-haemolysin diluted 1/10 yielded aggregation and a slow process of lysis. No membrane ghosts were visible after the action of β-lysin, as found after the action of purified α- and δ-haemolysin. That the decrease in $A_{540\ nm}$

TABLE III

CYTOTOXIC ACTIVITY OF β-HAEMOLYSIN

Cells were cultured and the cytotoxic activity was examined as described in MATERIALS AND METHODS. Symbols: −, no change in the cell culture when compared to the control; +, some cells are damaged; ++, most cells are morphologically changed but the cell layer is not distorted; +++, the cell layer is broken up; ++++, only scattered damaged cells are seen and no cells adhere to the glass surface.

| *Amount of β-haemolysin (μg)* | *6 h* | *24 h* | *72 h* |
|---|---|---|---|
| 10 | ++ | ++++ | No cells |
| 1 | + | ++ | ++++ |
| 0.1 | − | − | ++(+) |
| *Human embryonic fibroblasts* | | | |
| 10 | +(+) | +++ | No cells |
| 1 | + | ++ | ++++ |
| 0.1 | − | − | − |

was mainly caused by lysis of thrombocytes was confirmed by estimation of the α-granule specific exo-β-*N*-acetylglucosaminidase and the cytoplasmic lactate dehydrogenase. Staphylococcal anti-β-serum ("EX 1480") neutralized the platelet lysing activity at a concentration close to that necessary to neutralize the haemolytic activity on sheep red blood cells, while M18 antiserum and normal rabbit and human serum did not influence the activity.

*Leucocytes*

Human granulocytes adhering to siliconized slides and covered by a drop of Hanks balanced salt solution were incubated with 1 μg of β-haemolysin at 20° for 3 to 30 min. No morphological changes were visible in any of these experiments.

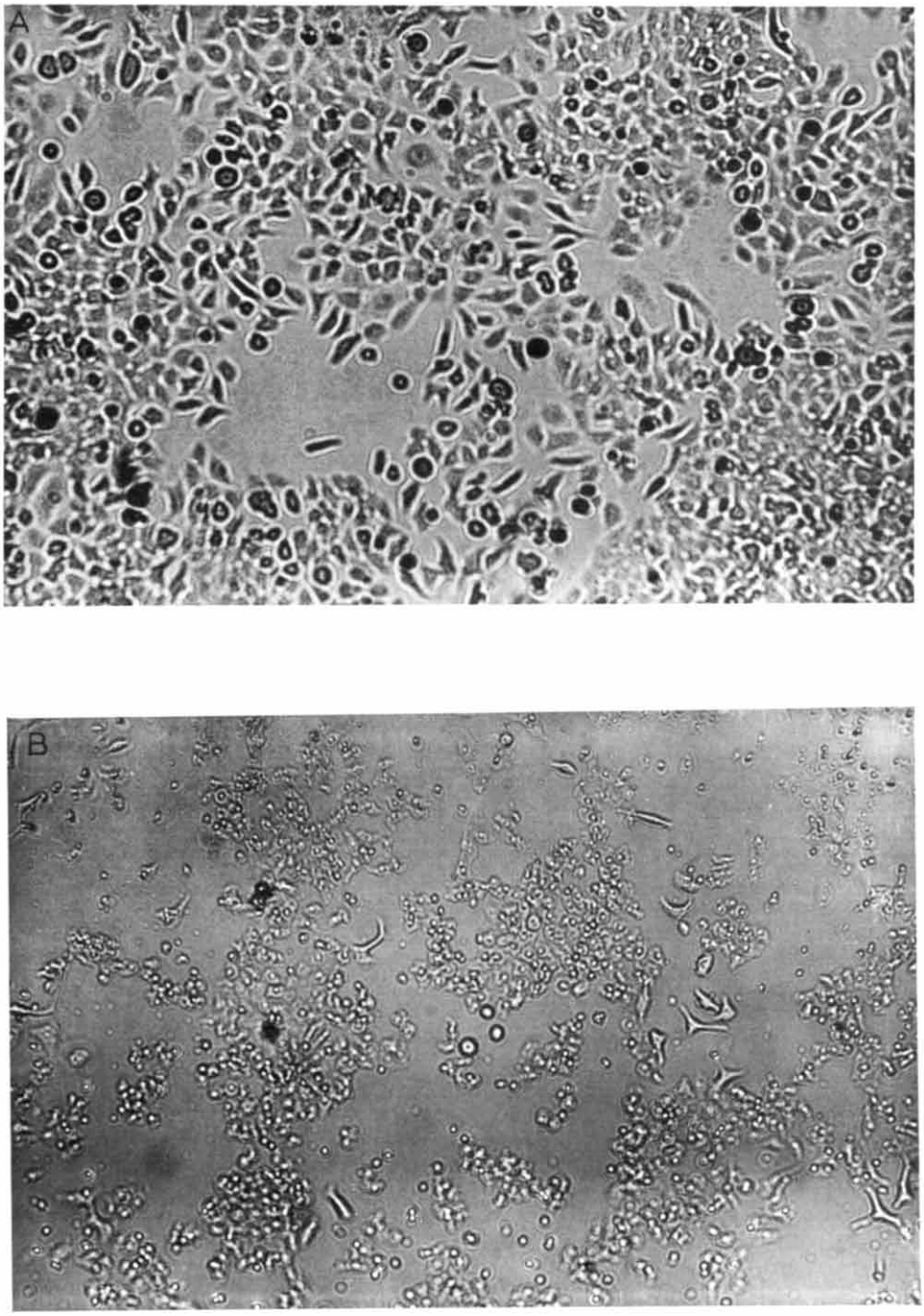


Fig. 1. Cytotoxic effect of 2 μg of purified β-haemolysin on a monolayer of HeLa cells. A. Before inoculation with β-haemolysin. B. 24 h after inoculation. According to Table III this cytopathic change is scored as +++.

*Cytotoxic effect in tissue cultures*

HeLa-cells, monkey kidney cells, primary and serially passed and diploid human fetal fibroblasts were used for the possible cytotoxic action of β-haemolysin. When 0.1–10 μg of purified β-lysin was added to the cultures a cytotoxic effect was noticed already after 2 h of incubation at 37° (Fig. 1). The cytopathic effect was considerably delayed in cultures incubated with a smaller dose of β-haemolysin (Table II). These results clearly show that 0.1–10 μg of purified β-haemolysin is cytotoxic for a variety of different cells. Anti-β-serum ("EX 1480") inhibited the cytopathic effect, while a polyvalent antiserum ("M18") did not.

*Protoplasts*

Protoplasts of *B. megaterium* and *S. aureus*, strain R 1, were suspended to a turbidity $A_{640\ nm}$ of 0.8. No decrease in this value was noticed upon addition of 1 μg of purified β-haemolysin.

*Proof for the extracellular nature of β-haemolysin*

Washed whole cells of strain R 1 were disintegrated in the X-press. Cells were also lysed by treatment with lysostaphin and endo-β-*N*-acetylglucosaminidase. No β-haemolytic activity was found either in the crude lysates or in the cytoplasmic or cell wall fractions obtained after high speed centrifugation. Digestion of whole cells by lysostaphin in 1.2 M sucrose yielded protoplasts which were lysed by suspending the protoplasts in distilled water. No β-haemolytic activity was detected in the cytoplasmic or the protoplast membrane fractions after centrifugation (30 000 × *g*, 30 min).

Experiments performed to actively release β-haemolysin from washed whole cells of strain R 1, showed that less than 1/1000 of the amount of haemolysin found extracellularly in 100-ml supernatant could be recovered from the total amount of cells from the same volume. A similar amount was recovered from the cytoplasmic fraction of the lysates, while the cell walls were totally negative. Thus, β-haemolysin seems to be a true extracellular protein, and no significant cellbound fraction could be detected.

## DISCUSSION

In addition to the *in vitro* hot-cold lysis of sheep red blood cells a number of other toxic manifestations have also been ascribed to β-haemolysin. Contradictory results on the *in vivo* effect of β-haemolysin upon intradermal administration were reported[5–8,34,35]. The discrepancy in these reports on the possible dermonecrotic and lethal effect of this protein might be explained by the difference of the dose administrated and the degree of contamination by other toxic factors, *e.g.* α-toxin.

CHRISTIE AND NORTH[36] reported that β-haemolysin was able to augment the activity of α-toxin on human erythrocytes. In this investigation neither a significant synergistic nor antagonistic effect between purified α-, β-, and δ-lysin could be demonstrated on rabbit, sheep, or human red blood cells. However, β-lysin was previously reported to act synergistically with δ-lysin[37] and to stabilize sheep red cells against the action of α-lysin at 37°, but was in a small amount shown to act synergistically with α-toxin[38]. It is probable that the individual doses employed and the degree of

purity of the haemolytic preparations will influence the results of such an investigation.

The action of β-lysin in membrane lysis is most probably caused by dissolution of the sphingomyelin, while the modes of action of α- and δ-haemolysin are unknown. No synergism or antagonism was noticed on the combination of any two of these haemolysins. Thus it seems at least probable that the action of α- and δ-lysin is not dependent on intact membrane phospholipids. Similar studies with *Clostridium welchii* lecithinase and other phospholipases are in progress (R. MÖLLBY AND T. WADSTRÖM, unpublished data). The mode of action of δ-lysin is probably like a surfactant[39] and thus very unspecific, while both the α- and β-lysins, according to this and previous studies, show a high specificity for certain membranes (Table I)[38–40].

The lethal effect of purified β-haemolysin was shown on both rabbits, mice, and chicken embryos. Since the purified lysin was very susceptible to dilution, the appropriate $L.D._{50}$ dose could not be calculated. The lethal dose was found to be 10–100 μg for rabbits and mice, which seems to be in good agreement with a recent report[8]. This means that β-haemolysin on the basis of weight is about as potent lethal toxin as α-haemolysin[41] while the $L.D._{50}$ dose on rabbits for δ-lysin was very recently shown to be about 125 mg[37]. This result is surprising, considering the previous conflicting reports which disagree completely with regard to toxicity. These three haemolysins are probably up to now the only purified lethal factors from *S. aureus*[42].

In view of these findings, the toxicity for a variety of tissue culture cells, human platelets, human leucocytes, and bacterial protoplasts was investigated. All cultures were highly susceptible to a μg amount of this toxin. Depending on the concentration of the lysin, cytopathic changes developed from within some hours to 1 or 2 days, while the effect of an appropriate amount of α-toxin and δ-lysin appeared within half an hour. While δ-lysin instantly lysed the cells forming membrane ghosts the cytopathic change produced by α-toxin was an increased granularity of the cytoplasm followed by lysis and ghost formation. On the other hand, β-lysin seemed to detach islands of cells from the surface and disorganize the monolayer (Fig. 1). A similar phenomenon was noticed when thrombocytes were incubated with the three staphylococcal toxins. Ghosts were visible after the action of α- and δ-lysin, while all platelets drastically disappeared without the formation of ghosts upon the action of β-haemolysin. These results illustrate that the action of β-lysin was not only a process of detachment but also of plasma membrane lysis. This was proved by determining the liberation of an enzyme located in the α-granules of thrombocytes, exo-β-*N*-acetylglucosaminidase and a cytoplasmic enzyme, lactate dehydrogenase[43]. HOVIG[44] studied the effect of various enzymes on rabbit platelets, and found that a phospholipase A caused complete destruction of the cells, while trypsine and a lipase and some other enzymes did not induce any morphological changes. The lipid composition of membranes, granules, and the soluble fraction of human platelets has been analyzed[45]. The phospholipid composition of these subcellular fractions was very similar, which could explain that both plasma membranes and α-granule membranes were dissolved by β-haemolysin.

KORBECKI AND JELJASZEWICZ[46] and JELJASZEWICZ *et al.*[47] showed that there was a disturbance in the lipid metabolism in KB and monkey kidney cells upon the action of β-lysin. A cytopathic action on platelets of a partially purified β-haemolysin

was also described by BERNHEIMER AND SCHWARTZ[48] and by JELJASZEWICZ *et al.*[49]. A partially purified preparation was found to be leucocidal for guinea pig macrophages[6], while GLADSTONE AND VAN HEYNINGEN[14] found human leucocytes to be resistant to the action of β-lysin which is in accordance with results reported in this paper. Bacterial protoplasts of *B. megaterium* and *S. aureus* were not lysed by β-haemolysin either. These results agree with the observation that all bacterial membranes investigated were found to lack phospholipids[50,51] containing sphingosine with the exception for one species of Bacteroides[52]. It is thus obvious that purified β-lysin is not protoplast lytic, while staphylococcal δ-lysin and probably α-toxin[53] lyse protoplasts and spheroplasts. Since no β-lysin was released upon disintegration of whole cells it is very probable that this protein is a true extracellular enzyme, actively excreted as described by POLLOCK[54], and that this phospholipase is not involved in the bacterial cell metabolism. This is also obvious from the fact that sphingomyelin is not found in staphylococcal cells[55].

The variation in susceptibility of several cultures of fibroblasts to this toxin might be due to several factors: (1) the dose of toxin applied, (2) the status of the cells of each culture, *e.g.* the age of the individual cells in terms of the number of cell divisions after isolation of the primary cells, (3) the content of sphingomyelin and its accessibility to β-lysin in the cell membranes of the different cell types. Thus, a difference in the sensitivity of *e.g.* the primary monkey kidney and the human foetal dermal fibroblasts to the action of β-lysin might be caused by a combination of these three possibilities. Recently, WISEMAN AND CAIRD[2] found a good correlation between the susceptibility of the red blood cells from several species and the amount of sphingomyelin in the cell membrane. In Table I a similar correlation is found, as β-haemolysin is more active against the species sheep, ox and goat, which all have a significantly higher percentage of sphingomyelin in their red cell membranes than other species[56,57]. The difference in sensivity between these species might be due to the accessibility of the sphingomyelin to the lysin because of different steric configurations of the other phospholipids and the membrane proteins. Since probably no systematic analysis of the phospholipid composition of the different types of other mammalian cells except erythrocytes yet exists, it is not possible to show if a similar correlation between the cytopathic change and the sphingomyelin content exists for the cells investigated. However, recently a comparison of the phospholipid spectrum in human erythrocytes, thrombocytes, and leucocytes[58] showed a lower content of sphingomyelin in the leucocyte membrane, which would favour the hypothesis that this correlation is not only typical for different species of red blood cells. A comparison of the lipid composition of different mammalian cell membranes from several reports has recently been summarized[59]. The amount of phosphilipid in the whole cell of rat liver cell and the mouse L fibroblast was significantly higher than in the membranes, while the sphingomyelin content was much higher in the membrane. It is reasonable to believe that a sphingomyelinase, such as β-haemolysin in lower concentrations, might disturb the cell metabolism by interference with the surface membrane without inducing a morphological change. Preparations of phospholipase C were recently also found to enhance the glucose uptake of adipose cells[60] and influence the metabolism in thyroid slices[61]. A disturbance in the cell metabolism without a morphological change of leucocytes subjected to the action of β-haemolysin was described by WISEMAN[62].

As far as the authors know no investigation concerning the toxicity or action on cell membranes of other sphingomyelinases has been reported in the literature, and since probably only two enzymes of this category, one from rat liver[63] and one from *Cl. welchii*[64], have been purified before, further characterization of the staphylococcal enzyme is in progress.

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