Staphylococcal $\beta$-Hemolysin

II. Phospholipase C Activity of Purified $\beta$-Hemolysin

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Sheep erythrocyte ghosts released water-soluble organic phosphorus when treated with purified $\beta$-hemolysin. Phospholipid analysis demonstrated that sphingomyelin accounted for 53% of the phospholipids present in sheep erythrocytes. Purified $\beta$-hemolysin showed phospholipase C activity when purified ox brain or sheep erythrocyte sphingomyelin was used as substrate. Such studies have also revealed that the disappearance of sphingomyelin from the reaction mixture was accompanied by a comparable increase in the concentration of phosphoryl choline. Thin-layer chromatography of phospholipids, extracted from sheep erythrocytes which had been exposed to $\beta$-hemolysin, demonstrated that sphingomyelin was rapidly degraded. Activators of $\beta$-hemolysin, such as Mg$^{2+}$, enhanced the release of organic phosphorus from erythrocyte ghosts and from sphingomyelin. Inhibitors of $\beta$-hemolysin, such as ethylenediaminetetraacetic acid, $p$-chloromercuribenzoate, and iodoacetamide, inhibited the release of organic phosphorus from erythrocyte ghosts and from sphingomyelin. These studies strongly suggested that $\beta$-hemolysin enzymatically degraded the sphingomyelin of the erythrocyte membrane. Such degradation probably resulted in the eventual lysis of the erythrocyte.

Doery et al. (9) reported that the extracellular products from an $\alpha$-$\beta$ strain of Staphylococcus aureus (B.S.M. 24) contained two phospholipases. One of these phospholipases hydrolyzed phosphatidyl inositol and lysophosphatidyl inositol, while the other hydrolyzed sphingomyelin. By use of column electrophoresis, they demonstrated that the distribution of the phospholipase which hydrolyzed sphingomyelin corresponded closely with the distribution of $\beta$-hemolysin.

The same workers (10), using $\beta$-hemolysin of undefined purity, suggested that $\beta$-hemolysin, sphingomyelinase, and lysophospholipase are activities of one protein.

In a preliminary communication, Maheswaran and Lindorfer (Bacteriol. Proc., p. 44, 1966) reported that $\beta$-hemolysin of demonstrated purity degraded sphingomyelin, and that it released water-soluble organic phosphorus from sheep erythrocyte stroma. The organic phosphorus-containing compound was demonstrated to be phosphoryl choline.

Wiseman and Caird (18) used partially purified $\beta$-hemolysin to confirm the above findings.

In the work reported here, purified $\beta$-hemolysin was used to study the kinetics of phosphorus release from sheep erythrocyte ghosts and sphingomyelin. These studies also revealed that sheep erythrocyte sphingomyelin served as substrate for $\beta$-hemolysin.

**MATERIALS AND METHODS**

The methods used for the production and purification of $\beta$-hemolysin, evidences for purity, measurement of hemolytic units, and measurement of protein were identical to those previously reported (13).

Preparation of ghosts. Sheep blood was collected in acid-citrate-dextrose (15 ml/100 ml of blood). The blood was centrifuged at 1,600 × $g$ for 10 min. The supernatant fluid was discarded, and the packed red cells were washed five times with 0.145 M sodium chloride. Theuffy coat was removed by aspiration during each step of the washing procedure. The erythrocytes were then hemolyzed by mixing with 10 volumes of distilled water which was saturated with carbon dioxide. The mixture was refrigerated for 14 hr at 4°C in a closed container. The supernatant fluid was discarded, and the sedimented ghosts were centrifuged at 1,600 × $g$ for 5 min. The sediment was then washed 10 times in cold carbonated distilled water to remove the hemoglobin. Ghosts which were to be used for the extraction of lipids were lyophilized. Ghosts which were to be used for kinetic studies were dialyzed for 48 hr against distilled water to remove all detectable free phosphorus, and then were lyophilized.

Determination of phosphorus. The organic phosphorus present in various samples was quantitatively determined by the method of Bartlett (3).
Kinetics of release of water-soluble phosphorus from erythrocyte ghosts treated with \( \beta \)-hemolysin. The \( \beta \)-hemolysin used in these studies was dialyzed for 48 hr against distilled water to remove all detectable free phosphorus. All kinetic studies were performed with the same batch of erythrocyte ghosts and the same batch of \( \beta \)-hemolysin [128 hemolytic units (HU)]. In all studies, 10 mg of dried ghosts was suspended in 1 ml of 0.025 M tris(hydroxymethyl)aminomethane chloride buffer, pH 7.2, with or without Mg\(^{2+}\). Two ml of \( \beta \)-hemolysin was added and mixed with a Vortex mixer for 1 min. The mixture was incubated in a shaker water bath at the stated temperature for 30 min. The erythrocyte ghosts were removed by centrifugation at 71,000 \( \times \) g for 20 min, and the supernatant fluid extracted were subjected to extraction by the method of Doery et al. (10) with modifications. A mixture was prepared with 1 ml of \( \beta \)-hemolysin (156 HU), 0.5 ml of phingomycin (5 mg/ml), 0.5 ml of 0.3 M ammonium acetate buffer (pH 7.2), and 0.5 ml of distilled water (or different volumes of 0.1 M magnesium sulfate). This mixture was shaken in a water bath at 41 C for 3 hr. After incubation, 0.3 ml of albumin (5\%, w/v; Bovine Albumin Fraction V, Calbiochem, Los Angeles, Calif.) and 0.7 ml of trichloroacetic acid (20\%, w/v) were added. The resultant precipitate was removed by centrifugation at 71,000 \( \times \) g for 30 min at 4 C. The supernatant fluid was filtered through a 10-µm filter (Millipore Corp.). The organic phosphorus content in 1 ml of the filtrate was determined. Phingomycin and \( \beta \)-hemolysin controls were also subjected to phosphorus analysis and used as blanks.

Isolation and identification of phospholipid composition. Phospholipid composition of erythrocyte ghosts was determined by thin-layer chromatography (TLC) using Silica Gel G (E. Merck, AG, Darmstadt, Germany). The developing solvent was chloroform-methanol-glacial acetic acid-water (25:15:4:2, v/v) as described by Skipski et al. (17). Phospholipids were detected by means of the reagents described by Skidmore and Entenman (16) and were identified by comparing their \( R_f \) values with those of pure reference compounds. For quantitation, phospholipids were extracted by the method of Abramson and Blecher (1), and the phosphorus content of each phospholipid was determined by calculating the mean of seven identical analyses. The quantity of each phospholipid was then expressed as the percentage of total phosphorus as described by Parker and Peterson (15).

**Phingomycin (bovine brain).** The phingomycin was prepared through a Biochemicals, Chagrin Falls, Ohio. When examined by TLC, it moved as a single component and stained with Dragendorff reagent, but not with ninhydrin. For phospholipase C assay, 5 mg/ml of aqueous phingomycin was sonically treated in a MSE (Measuring & Scientific Equipment, Ltd., London, England) sonic disintegrator (20 kc, 1.5 amp, for 10 min).

Phospholipids extracted from sheep erythrocyte ghosts were subject to TLC. The phingomycin was extracted from the chromatogram by the method of Abramson and Blecher (1) and dried under reduced pressure in the presence of nitrogen. For phospholipase C assay an aqueous sonically treated suspension was used.

Estimation of phospholipase C activity with ox brain phingomycin used as substrate. This was done by the method of Doery et al. (10) with modifications. A mixture was prepared with 1 ml of \( \beta \)-hemolysin (156 HU), 0.5 ml of phingomycin (5 mg/ml), 0.5 ml of 0.3 M ammonium acetate buffer (pH 7.2), and 0.5 ml of distilled water (or different volumes of 0.1 M magnesium sulfate). This mixture was shaken in a water bath at 41 C for 3 hr. After incubation, 0.3 ml of albumin (5\%, w/v; Bovine Albumin Fraction V, Calbiochem, Los Angeles, Calif.) and 0.7 ml of trichloroacetic acid (20\%, w/v) were added. The resultant precipitate was removed by centrifugation at 71,000 \( \times \) g for 30 min at 4 C. The supernatant fluid was filtered through a 10-µm filter (Millipore Corp.). The organic phosphorus content in 1 ml of the filtrate was determined. Phingomycin and \( \beta \)-hemolysin controls were also subjected to phosphorus analysis and used as blanks.
TLC was also used to isolate the phosphoryl choline from the acidic phase of the 22-hr reaction mixture. Developed chromatograms were air-dried for 2 hr and exposed to iodine vapor. The pale yellow areas were quickly outlined with a sharp instrument, and the plates were exposed to air until the color disappeared. The silica gel containing the phosphoryl choline was scraped off and quantitatively transferred to a centrifuge tube. Phosphoryl choline was extracted by suspension in four successive 2-ml portions of distilled water; the extract was separated at each stage by centrifugation at 1,600 × g for 2 min. The same extraction procedure was done on silica gel from a blank portion of each chromatoplate. The pooled extracts were lyophilized, resuspended in 3 ml of 1 N HCl, and heated for 90 min at 100 C. One ml of the above product was used for choline determination by the method of Appleton et al. (2), and 1 ml was subjected to phosphorus analysis by the method of Bartlett (3).

Phospholipid analysis of sheep erythrocytes after treatment with β-hemolysin. β-Hemolysin (312 HU) and sheep erythrocytes were allowed to react at 41 C for 1 hr. The lipids were extracted from the reaction mixture by using 10 volumes of chloroform-methanol solvent (2:1, v/v). The water-soluble contaminants in the extract were removed by extraction with 0.2 volume of 0.05 M KCl. The phospholipids were separated from the neutral lipids by using silicic acid column chromatography (4). The extracted phospholipids were then spotted on a TLC plate (17).

Proteolytic activity of β-hemolysin. β-Hemolysin was tested for proteolytic activity by the method of Burke and Pattee (5) with casein and Azocoll (Calbiochem) as substrates. Pronase Grade B (Calbiochem) was used as a control against the same substrates.

RESULTS

Kinetics of release of water-soluble organic phosphorus from β-hemolysin treated erythrocyte ghosts. These studies were done at five different temperatures (33, 37, 41, 43, and 45 C) in the presence of 0.01 M Mg++. Figure 1 demonstrates that a direct linear relationship existed between the temperature of incubation and the amount of phosphorus released. Studies done at 43 C revealed that there was also a direct linear relationship between the time of incubation (30 to 180 min) and the amount of phosphorus released.

The studies depicted in Table 1 showed that the optimal concentration of Mg++ for phosphorus release was 0.01 M. β-Hemolysin inactivated with ethylenediaminetetraacetic acid (0.004 M) did not release phosphorus from sheep erythrocyte ghosts. However, when such hemolysin was reactivated with Mg++ (0.01 M), phosphorus was released. In addition, it was demonstrated that erythrocyte ghosts treated with electrodialyzed (12) β-hemolysin did not release phosphorus.

Figure 2 shows the results of a study of the effects of β-hemolysin concentration on the amount of phosphorus released from erythrocyte ghosts. It will be noted that a direct straight-line relationship existed between the amount of phosphorus released and concentration of hemolysin.

TABLE 1. Effect of Mg++ concentration on the release of phosphorus from sheep erythrocyte ghosts treated with purified β-hemolysin

<table>
<thead>
<tr>
<th>Mg++</th>
<th>Amt of phosphorus (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>0.0001</td>
<td>3.0</td>
</tr>
<tr>
<td>0.001</td>
<td>5.7</td>
</tr>
<tr>
<td>0.01</td>
<td>12.9</td>
</tr>
<tr>
<td>0.035</td>
<td>10.8</td>
</tr>
</tbody>
</table>

β-Hemolysin = 128 HU.

FIG. 1. Effects of temperature on the release of phosphorus from erythrocyte ghosts by β-hemolysin. Conditions: Mg++ concentration = 0.01 M; β-hemolysin = 128 HU.

FIG. 2. Effect of β-hemolysin concentration on phosphorus release from erythrocyte ghosts. Conditions: Mg++ concentration, 0.01 M; temperature, 43 C.
Phospholipid composition of sheep erythrocyte ghosts. The phospholipid extracted from erythrocyte ghosts was dissolved in chloroform, spotted on a TLC plate, and developed with the solvent. Five iodine-positive spots were observed between the origin and the solvent front. The ninhydrin-positive spots close to the solvent front were identified as phosphatidyl ethanolamine and phosphatidyl serine, whereas three Dragendorff positive spots were identified as lecithin, sphingomyelin, and lysolecithin (Fig. 3). These five phospholipids were then extracted and quantitated. These studies revealed that 95.4% of phospholipid phosphorus was recovered after TLC. Sphingomyelin accounted for 53.15% of the total phospholipid, phosphatidyl ethanolamine for 39.3%, phosphatidyl serine for 2.05%, lecithin for 0.90%, and lysolecithin was found in trace amounts.

Phospholipase C activity of β-hemolysin with purified ox-brain sphingomyelin used as substrate. Studies showed that β-hemolysin reacted more efficiently (more phosphorus released) with sonically treated than with emulsified sphingomyelin. Therefore, sonically treated sphingomyelin was used for all kinetic studies.

A direct linear relationship was found between time of incubation at 37 C (30 min to 4 hr) and amount of phosphorus released.

Figure 4 demonstrates that a direct linear relationship existed between the temperature of incubation (37 to 41 C) and the amount of phosphorus released. Note, at temperatures above 41 C, there was a decrease in the amount of phosphorus released.

These studies also revealed that the optimal concentration of Mg++ for phosphorus release from sphingomyelin was 0.01 M. β-Hemolysin inactivated with ethylenediaminetetraacetic acid (0.004 M) did not release phosphorus from sphingomyelin.

The results presented in Fig. 5 show the relationship between the amount of phosphorus released and the concentration of hemolysin. Note that the relationship was linear over the ranges used for this experiment.

β-Hemolysin inactivated by iodoacetamide, p-chloromercuribenzoate, pronase, trypsin, and chymotrypsin (S. K. Maheswaran, Ph.D. Thesis.)
University of Minnesota, 1967) did not release a detectable amount of phosphorus from sphingomyelin.

Isolation and identification of phosphoryl choline. The acidic extracts from the 3-, 6-, and 22-hr reaction mixtures, subjected to TLC, showed one spot which was stained by molybdic acid (phosphorus) and Dragendorff (choline) spray reagents (Fig. 6). This phosphorus- and choline-containing compound had the same RF value as the phosphoryl choline which was used as standard. When this spot was extracted from the chromatograms and hydrolyzed, it was found to contain both phosphorus and choline in equimolar concentration.

The acidic extract (phosphoryl choline) and ethanolic extract (sphingomyelin) from the 0-, 3-, 6-, and 22-hr reaction mixture were analyzed for phosphorus. Results showed that at zero time all the phosphorus from the reaction mixture was found in the ethanolic extract. At 3 hr, about 25% of the total phosphorus was found in the acidic extract. At 6 hr, the total phosphorus in the acidic extract rose to 53.2%. Finally, after 22 hr of incubation, about 85% of the total phosphorus was found in the acidic extract.

TLC studies with these acidic and ethanolic extracts showed that, as the time of incubation increased, there was a quantitative decrease in sphingomyelin. This decrease in sphingomyelin was associated with a comparable increase in the concentration of phosphoryl choline.

Phospholipid analysis of sheep erythrocytes after treatment with β-hemolysin. This study was conducted because the rate of degradation of purified ox-brain and sheep erythrocyte sphingomyelin was slow (85% degradation in 22 hr at 41°C), and because other authors (6, 7) have suggested that native phospholipids are more susceptible to phospholipase than are purified phospholipids. The study revealed that of the five phospholipids present in sheep erythrocytes (phosphatidylin ethanolamine, phosphatidyl serine, sphingomyelin, and lysolecithin) only sphingomyelin was degraded. Under the conditions of this study, the sheep erythrocyte sphingomyelin was completely degraded within 1 hr or less.

Purified β-hemolysin was not proteolytic even after 22 hr of incubation with casein or Azocoll.

**Discussion**

This work demonstrated that sheep erythrocyte ghosts released water-soluble organic phosphorus when treated with purified β-hemolysin. Studies concerning the effects of time, temperature, Mg++ concentration, and hemolysin concentration on the release of phosphorus from erythrocyte ghosts all suggested that the reaction was enzymatic.

The effects of purified β-hemolysin on purified ox-brain sphingomyelin were also investigated. These kinetic studies supported the contention that β-hemolysin possessed phospholipase C (sphingomyelylase) activity.

In addition, studies with ox-brain sphingomyelin revealed that activators of β-hemolysin, such as Mg++, also activated the degradation of sphingomyelin. Further, inhibitors of β-hemolysin, such as ethylenediaminetetraacetic acid,
p-chloromercuribenzoate, and iodoacetamide (S. K. Maheswaran, Ph.D. Thesis, University of Minnesota, 1967), also inhibited the release of organic phosphorus from sphingomyelin.

More exact information on the phospholipase C activity of β-hemolysin was obtained by studies which isolated and identified the products of the reaction. These studies used purified sheep erythrocyte sphingomyelin as well as purified ox brain sphingomyelin as substrate. The results demonstrated that the organic phosphorus disappeared from the ethanolic phase at about the same rate that it appeared in the acidic phase. Further, TLC studies verified these results (Fig. 6). In addition, it was demonstrated that the organic phosphorus which appeared in the acidic phase had the same $R_f$ value as phosphoryl choline. Finally, extraction of the spot identified as phosphoryl choline yielded a product which contained equimolar quantities of phosphorus and choline.

The fact that the degradation of purified sphingomyelin by β-hemolysin was slow seemed to discount the importance of this reaction as an explanation of hemolysis. However, several other facts seemed to support the contention that sphingomyelinase played an important part in β-hemolysis. Wiseman (18) has recently reported that a partially purified β-hemolysin preparation degraded native sheep erythrocyte sphingomyelin more rapidly than purified ox brain sphingomyelin. The independent work reported herein employed β-hemolysin of demonstrated purity. It showed that the rate of degradation of native sheep erythrocyte sphingomyelin was more rapid than the rate of degradation of purified sheep erythrocyte sphingomyelin. Furthermore, Condrea et al. (6) have made similar observations with other phospholipases. They found that these phospholipases were more active on native substrates than they were on purified phospholipids. Dawson and Bangham (7) reported on the importance of the electrokinetic potential in some phospholipase-substrate interactions. They suggested that native phospholipids possessed favorable electrokinetic properties and thus were more susceptible to phospholipases than purified phospholipids.

De Gier and Van Deenen (8) and the work reported here have demonstrated that 53 to 54% of the phospholipid present in sheep erythrocyte ghosts was sphingomyelin. This suggested that there was an adequate amount of substrate (sphingomyelin) present in sheep erythrocytes to account for damage sufficient to cause hemolysis. Furthermore, it may not be necessary to degrade all of the sphingomyelin of the erythrocyte membrane to produce sufficient damage to allow the eventual release of hemoglobin from the erythrocyte.

Activators and inhibitors of β-hemolysis (S. K. Maheswaran, Ph.D. Thesis, University of Minnesota, 1967) also exerted the same effect on the release of phosphoryl choline from sphingomyelin. This indicated that sphingomyelinase does play a role in β-hemolysis.

In conclusion, these studies showed that purified β-hemolysin had a phospholipase C (coded EC 3.1.4.3 in the report of the Commission on Enzymes of the International Union of Biochemistry, Oxford, Pergamon Press, 1961) activity against sphingomyelin from ox brain and sheep erythrocytes.

Acknowledgments

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Literature Cited

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