Phospholipase Activity of the Delta Hemolysin of *Staphylococcus aureus** (33029)

GORDON M. WISEMAN AND JOHN D. CAIRD (Introduced by A. M. Wallbank)
Department of Medical Microbiology, University of Manitoba Medical School, Winnipeg, Canada

The alpha, beta, and delta hemolysins of *Staphylococcus aureus* are implicated in the pathogenicity of this microorganism for man and animals. However, only the mode of action of the beta hemolysin is known with certainty. This enzyme hydrolyzes sphingomyelin, a common constituent of cellular membranes, resulting in the liberation of water-soluble phosphorylcholine and water-insoluble *N*-acyl sphingosine (1,2). In the present experiments, we have shown that two highly purified preparations of the delta hemolysin liberate organic phosphorus from phospholipid extracts of mammalian erythrocytes. Of several phospholipids investigated as substrates, phosphatidylinositol was the most susceptible to degradation by the delta hemolysin.

Materials and Methods. Crude delta hemolysin from the Newman and E-delta strains of *S. aureus*[1] was prepared in stainless steel trays of Dolman–Wilson agar (3) overlain with sterile cellophane and covered with aluminum foil. The cellophane surface was inoculated with 2 ml of a saline suspension of a 24-hour agar slope culture of the organisms, which was spread with a glass rod. After 24-hours incubation at 37°C in a sealed plexiglass tank in an atmosphere of 25% CO_2 in air, 0.01 *M* phosphate buffered saline at pH 7.0 was added to the trays. The growth was taken up in the buffer, pooled, and centrifuged at 11,000*g* for 30 min. The supernatant fluid containing the hemolysin was stored at −20°C.

Crude delta hemolysin prepared in this manner was dialyzed against 0.05 *M* acetate buffer at pH 4.0 for 48 hours. The resulting precipitate which contains the active material was collected by centrifugation and dissolved in 0.05 *M* tris (hydroxymethyl) aminomethane (Tris) buffer at pH 9.0 to effect solution. Further dialysis of the preparation was then carried out against 0.1 *M* phosphate buffer at pH 7.0 for 48 hours. It was centrifuged and the supernatant fluid was added to hydroxylapatite (4) in the amount of 10 ml/0.2 gm of dry weight of adsorbent. Elution of activity was accomplished after 1-hour standing at 4°C by addition of 2 *M* NaCl in buffer to the adsorbent, followed by centrifugation.

Further purification of the delta hemolysin was achieved on a column of diethylaminoethyl (DEAE) cellulose equilibrated with 0.02 *M* phosphate buffer at pH 7.0. The hemolysin was eluted from the column against a linear gradient of NaCl (0–0.5 *M*) in phosphate buffer prepared in a Varigrad.[2] About 40 mg of protein was applied to the column and 10 ml fractions were collected, the active material appearing in the first of several peaks eluted.

A 30-fold increase in specific activity was achieved by this method when crude hemolysin was compared to that eluted from the DEAE column. One strong line of precipitation was observed in Ouchterlony plates when purified hemolysin was incubated with a rabbit-produced antiserum to crude material. However, Jackson and Little (5) and Gladstone and Yoshida (6) have observed that protein components of normal serum strongly inhibit hemolytic activity and the latter workers were also unable to demonstrate antigenicity. We think it unlikely that the single line of precipitation we have observed with our preparations is due to anything other than delta hemolysin.

Results. Purified Newman and E-delta hemolysins lysed human erythrocytes to a

* Supported by the Medical Research Council.

[1] The Newman strain was obtained from Dr. G. P. Gladstone, Sir William Dunn School of Pathology, Oxford University, and the E-delta from Professor S. D. Elek of St. George's Hospital Medical School, London, England.

[2] Buchler Instruments, Inc., Fort Lee, New Jersey.

TABLE I. Relationship of Degree of Hemolysis to Organic Phosphorus Released by Delta Hemolysin from Erythrocytes.

Erythrocyte species	Hemolytic titer (HU/ml)[a]	Total P released (%)[b]
Man	256	82
Guinea pig	64	57
Rabbit	64	54
Bovine	32	64
Goat	32	43
Sheep	32	39
Horse	16	30

[a] The titer of hemolysin, given as hemolytic units/ml, is the highest dilution which lyses 50% of the red cells.

[b] Phosphorus content of controls has been subtracted from each value.

greater degree than those of other species. Both hemolysins were strongly inhibited by 0.1% rabbit and human serums and to a lesser extent by 0.1% human gamma globulin, bovine albumin, and gelatin, which is in keeping with Jackson's observations (5) and those of Gladstone (6,7). Hemolytic activity was also inhibited fourfold by cholesterol at a concentration of 2 mg/ml.

Phospholipid extracts of various erythrocyte species were prepared according to the method of Rose and Oklander (8). Sphingomyelin, phosphatidylinositol (fraction I of Folch), phosphatidylserine, and phosphatidylcholine, obtained from the Sigma Chemical Co., St. Louis, Mo., were used as substrates. Delta hemolysin activity in the presence of these substrates was assayed as follows: One ml of a 1 mg/ml suspension of phospholipids in 0.01 *M* Tris buffer at pH 7.0 was placed in a test tube to which 1 ml of purified hemolysin containing 250 hemolytic units/ml of activity was added (9). In some experiments, ethylenediaminetetraacetic acid disodium (EDTA) or $MgCl_2$ was included at a final concentration of 0.001 *M*. After incubation of the reaction mixture for 1 hour at 37°C, 2 ml of 10% trichloroacetic acid was added. The contents of the tube were centrifuged and the supernatant fluid was assayed for organic phosphorus by the method of Fiske and Subbarow (10).

In Table I, seven species of erythrocytes are arranged in order of decreasing sensitivity to the delta hemolysin. Erythrocytes of man were most sensitive to the hemolysin and from their extracts the largest amounts of organic phosphorus were released. Horse red cells were quite resistant with only 30% of the total phosphorus liberated. It thus appears that with the possible exception of bovine cells, hemolytic activity and release of organic phosphorus are significantly correlated.

In another experiment (Table II), delta hemolysin activity was compared with that of beta hemolysin in the presence and absence of Mg^{2+} ions and EDTA. Results obtained with beta hemolysin show a 30–40% enhancement of its phospholipase activity in the presence of Mg^{2+}. The inclusion of 0.001 *M* EDTA resulted in a twofold inhibition of beta hemolysin activity. By contrast, neither Mg^{2+} ions nor EDTA had any appreciable effect upon delta hemolysin activity compared to the control.

We also incubated sphingomyelin, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine with the delta hemolysin. The hemolysin liberated 20 μg of phosphorus from the phosphatidylinositol, 2.3 μg from phosphatidylserine, a trace from phosphatidylcholine and none from sphingomyelin. We carried out limited kinetic studies with hemolysin from the E-delta strain using Sigma phosphatidylinositol as substrate. When hemolysin concentration was plotted against the reaction velocity, a straight line was obtained. The activation energy, determined from an Arrhenius plot, was 18,750

TABLE II. Liberation of Organic Phosphorus from Extracts of Sheep Erythrocytes by Staphylococcal Hemolysins,

	P (μg) released by			
	Beta strains		Delta strains	
Additions[a]	R-1	Foggie	Newman	E-delta
None	38	44	45	30
Mg^{2+}	54	58	40	32
EDTA	20	27	39	32

[a] EDTA and Mg^{2+} ions are present in 0.001 *M* concentrations.

cal, within the range of 1000–25,000 cal characteristic of most enzymes as determined by Sizer (11).

Discussion. On the basis of the evidence presented, it is likely that the delta hemolysin is an enzyme. In view of the fact that aqueous organic phosphorus is released from phospholipid substrates by the hemolysin, it may have a mode of action similar to that of phospholipase C. Unlike the beta hemolysin, which is a phospholipase C and releases water-soluble phosphorylcholine from sphingomyelin, the delta hemolysin does not attack this substrate. Its activity is not affected by Mg^{2+} ions and is uninhibited by EDTA in contrast with the beta hemolysin. Although its substrate in the erythrocyte has not yet been clearly identified, the delta hemolysin releases water-soluble organic phosphorus from phosphatidylinositol and to a lesser degree from phosphatidylserine. We have detected the presence of small amounts of phosphatidylinositol in the erythrocytes used in this study when their extracts were chromatographed using Marinetti's technique (12). The same species of erythrocytes have also been shown to contain phosphatidylinositol and in addition, phosphatidylserine, according to a recent report by Nelson (13).

Summary. Purified delta hemolysin from the Newman and E-delta strains of *S. aureus* liberates aqueous organic phosphorus from phospholipid extracts of various species of mammalian erythrocytes. Of several phospholipids investigated as substrates, phosphatidylinositol is most susceptible to degradation by the delta hemolysin. Phosphatidylserine was to a lesser extent attacked by the enzyme. In contrast with the beta hemolysin of *S. aureus,* delta hemolysin does not hydrolyze sphingomyelin and its activity is unaffected by EDTA or Mg^{2+} ions.

1. Doery, H. M., Magnusson, B. J., Gulasekharam, J., and Pearson, J. E., J. Gen. Microbiol. **40**, 283 (1965).

2. Wiseman, G. M. and Caird, J. D., Can. J. Microbiol. **13**, 369 (1967).

3. Dolman, C. E. and Wilson, R. J., Can. J Public Health **31**, 68 (1940).

4. Tiselius, A., Hjerten, S., and Levin, O., Arch. Biochem. Biophys. **65**, 132 (1956).

5. Jackson, A. W. and Little, R. M., Can. J. Microbiol. **4**, 435 (1958).

6. Gladstone, G. P. and Yoshida, A., Brit. J. Exptl. Pathol. **48**, 11 (1967).

7. Gladstone, G. P. and van Heyningen, W. E., Brit. J. Exptl. Pathol. **38**, 123 (1957).

8. Rose, H. G. and Oklander, M., J. Lipid Res., **6**, 428 (1965).

9. Wiseman, G. M., J. Pathol. Bacteriol **89**, 187 (1965).

10. Fiske, C. H. and Subbarow, Y., J. Biol. Chem. **66**, 375 (1925).

11. Sizer, I. W., Advan. Enzymol. **3**, 35 (1943).

12. Marinetti, G. V., J. Lipid Res. **3**, 1 (1962).

13. Nelson, G. J., Biochim. Biophys. Acta **144**, 221 (1967).

Received Jan. 19, 1968. P.S.E.B.M., 1968, Vol. 128.

The Effect of SC 15396 on Gastrin Stimulated Pancreatic Secretion (33030)

G. F. STENING AND MORTON I. GROSSMAN

Research, Veterans Administration Center, Los Angeles, California 90073 and Departments of Medicine and Physiology, UCLA School of Medicine, Los Angeles, California 90024

Bedi *et al.* (1) described the inhibitory effects of 2-phenyl-2-(2-pyridyl) thioacetamide (SC 15396, G. D. Searle Co.) on gastric secretory responses to both pure gastrin II and the synthetic peptide, pentagastrin. They were satisfied that at low doses its antagonism was specific for gastrin and the pentapeptide, and they termed the substance antigastrin. Cook and Bianchi (2) also showed that SC 15396 was an inhibitor of gastrin induced gastric secretion in rats and dogs. Others (3) used larger doses and suggested that the effect is not specific because inhibition was demonstrated against both insulin and histamine induced gastric acid secretion.

Gastrin (4) stimulates pancreatic secre-