JOURNAL OF BACTERIOLOGY, May, 1965
Vol. 89, No. 5

Lysis of Bacterial Protoplasts and Spheroplasts by Staphylococcal α-Toxin and Streptolysin S

ALAN W. BERNHEIMER AND LOIS L. SCHWARTZ
Department of Microbiology, New York University School of Medicine, New York, New York

Received for publication 31 December 1964

ABSTRACT

BERNHEIMER, ALAN W. (New York University School of Medicine, New York, N.Y.), AND LOIS L. SCHWARTZ. Lysis of bacterial protoplasts and spheroplasts by staphylococcal α-toxin and streptolysin S. J. Bacteriol. **89**:1387–1392. 1965.—Protoplasts of *Bacillus megaterium*, *Sarcina lutea*, and *Streptococcus pyogenes*, and spheroplasts of *Escherichia coli* were lysed by staphylococcal α-toxin, whereas spheroplasts of *Vibrio metschnikovii* and *V. comma* were not. In the spectrum of its lytic action, streptolysin S qualitatively resembled staphylococcal α-toxin except for failure to lyse *S. pyogenes* protoplasts. In contrast to the two foregoing agents, streptolysin O did not lyse protoplasts and spheroplasts. The observations are interpreted in relation to similarities and differences in lipid composition of bacterial and mammalian cell membranes.

Staphylococcal α-toxin and the streptolysins are toxic agents of bacterial origin which are capable of lysing a variety of membrane-bound mammalian structures, as erythrocytes, platelets (Bernheimer and Schwartz, 1965), and lysosomes (Bernheimer and Schwartz, 1964). Their lytic action is best explained by assuming that they alter the molecular architecture of the membrane surrounding the mammalian cell or that of membranes of intracellular structures, or both. The concept that all biological membranes may have a similar basic molecular pattern (Robertson, 1964) suggests that not only mammalian but also bacterial cell membranes might be susceptible to the action of these toxins. With these considerations in mind, we examined the effects of staphylococcal α-toxin and streptolysins on selected species of bacteria that had been partially or completely deprived of their cell walls. Observations showing that spheroplasts of *Escherichia coli* undergo lysis in the presence of staphylococcal α-toxin were made initially in 1962 by M. Malamy in collaboration with the authors.

MATERIALS AND METHODS

Toxins. Staphylococcal α-toxin was prepared as described by Bernheimer and Schwartz (1963) and was estimated to be 70% pure. The solution used contained 2.65 mg of protein per ml and 40,000 hemolysin units per ml.

Streptolysin S was a lyophilized product containing 10,000 hemolysin units per mg, prepared as described elsewhere (Bernheimer, 1949).

Streptolysin O was a partially purified product described by Weissmann, Keiser, and Bernheimer (1963) and designated by them as Preparation C′. It was activated with 0.1% cysteine before use.

Spheroplasts and protoplasts. Spheroplasts of *Escherichia coli* W were prepared by treating washed log-phase cells with lysozyme and ethylenediaminetetraacetate (EDTA; Repaske, 1956; Malamy and Horecker, 1961). One volume of an overnight bacterial culture was added to 10 volumes of Neopeptone meat-infusion broth and incubated in a rotary shaker at 37 C. When the optical density (500 mμ) reached 1.5, 50 ml of culture were centrifuged, the cells were washed in 50 ml of 0.01 M tris-(hydroxymethyl)amino methane (Tris) buffer (*p*H 8.0) and suspended in 100 ml of 0.033 M Tris buffer (*p*H 8.0) containing 0.6 M sucrose. To this were added 0.5 mg of lysozyme (Armour Pharmaceutical Co., Kankakee, Ill.) and 1 ml of 0.1 M EDTA at *p*H 8.0. After standing in the cold for 25 min, at which time most of the cells had become transformed to spheroplasts, the supension was centrifuged, and the sediment was suspended in sufficient 0.6 M sucrose in 0.033 M Tris (*p*H 8.0) containing 0.01 M $MgCl_2$, to give an optical density of 0.5.

Penicillin-produced spheroplasts (Lederberg, 1956) of *E. coli* W were obtained by adding 1 volume of broth culture to 10 volumes of Neopeptone meat-infusion broth containing 0.6 M sucrose and 0.6 mg of benzylpenicillin per ml. After incubation for 90 min at 37 C on a rotary shaker, the spheroplast suspension was centrifuged, and the cells were suspended in sufficient 0.6 M sucrose in 0.033 M Tris (*p*H 8.0) containing 0.01 M $MgCl_2$, to bring the turbidity in to range of the spectrophotometer.

Protoplasts of *Streptococcus pyogenes* were prepared by exposing the cocci to the action of phage-associated lysin (Krause, 1958; Markovitz and Dorfman, 1962). One volume of an overnight

broth culture of strain C203S was added to 10 volumes of Neopeptone meat-infusion broth. After 5 hr at 37 C, 20 ml of culture were centrifuged, and the cocci were washed twice in 0.055 M KH_2PO_4-K_2HPO_4 (*p*H 7.0) and 0.067 M KCl. The cocci were suspended in 1 ml of "protoplasting solution," which consisted of 2 mg of phage-associated lysin dissolved in 1 ml of 0.055 M KH_2PO_4-K_2HPO_4 (*p*H 7.0), 1.2 M NaCl, 0.01 M $MgCl_2$, and 0.01 M cysteine. After 20 min at room temperature, the suspension was diluted to an optical density (500 mμ) of 0.5 by use of protoplasting solution lacking enzyme and cysteine.

Protoplasts of *Bacillus megaterium* KM were prepared by suspending in 10 ml of 0.033 M phosphate buffer (*p*H 7.2) the cells from a nutrient agar plate that had been streaked 2 days earlier. After centrifugation, the bacilli were suspended in 20 ml of 0.2 M sucrose in the same buffer, and 1.0 mg of lysozyme (Weibull, 1953) contained in 0.2 ml water was added. After standing at room temperature for 20 min, at which time most, but not all, of the organisms had undergone protoplast formation, 0.2 ml of 1 M $MgCl_2$ solution was added and the mixture was centrifuged. The sediment was gently suspended in sufficient 0.2 M sucrose, 0.033 M phosphate (*p*H 7.2), and 0.01 M $MgCl_2$ to bring the turbidity in range for measurement.

Sarcina lutea was grown in a shaking incubator at 30 C in 1% peptone (Difco), 0.1% yeast extract, (Difco), and 0.5% NaCl (*p*H 7.5). The cocci from 40 ml of culture were washed in 1 volume of 0.033 M phosphate buffer (*p*H 7.2) and suspended in 0.125 volume of 1.5 M sucrose in the same buffer. Lysozyme was added to a concentration of 100 μg/ml, and, after 10 to 15 min, the suspension was diluted in sufficient 1.5 M sucrose in 0.033 M phosphate (*p*H 7.2) containing 0.01 M $MgCl_2$, to give an optical density of 0.5 to 0.6.

Spheroplasts of *Vibrio metschnikovii* were produced with benzyl-penicillin by the procedure of Salton and Shafa (1958). The spheroplasts were suspended in 0.9% NaCl solution buffered with 0.01 M phosphate (*p*H 7.0). Spheroplasts of the Ogawa strain of *V. comma* were produced by means of antibody and complement (Shafa and Salton, 1959). Conversion to spheroplasts resulted when a mixture containing 0.6 ml of broth culture, 0.02 ml of immune rabbit serum, 0.2 ml of 0.9% NaCl solution, 0.1 ml of freshly thawed guinea pig serum, and 0.6 ml of 0.6 M sucrose were allowed to stand for 3 hr at room temperature.

Criterion of lysis. The principal procedure employed involved the addition of α-toxin, or other test material, in a volume of 50 μliters, to 700 μliters of spheroplast or protoplast suspension contained in a previously warmed cuvette with internal dimensions of 10 by 5 by 38 mm. The optical density at 500 mμ was continuously recorded for 30 min, or longer, in a Cary spectrophotometer equipped with a cell holder through which circulating water maintained the temperature of the cuvette at 35 C. A substantial decrease in optical density was interpreted as evidence of lysis, and phase-contrast microscopy corroborated this interpretation. In no instance was decrease in optical density attributable to settling of particles in the reaction mixtures.

RESULTS

Lysis of E. coli spheroplasts. In the presence of α-toxin, *E. coli* spheroplasts prepared with lysozyme and EDTA underwent extensive lysis. The results of a typical experiment are shown in Fig. 1, in which it can be seen that rate of lysis depended on toxin concentration. The lytic effect was prevented when a small amount (10 μliters) of antitoxin was included in the system.

In contrast to spheroplasts, the turbidity of whole cells of *E. coli* suspended in Tris buffer (0.01 M; *p*H 8.0) was not affected by α-toxin. To test the possibility that toxin might alter the cells without producing gross lysis, viable counts were done on toxin-treated and untreated whole cells. No reduction in number of viable cells was brought about by exposure to 90 μg of toxin per ml for 20 min at 37 C.

As a further test of the possibility that whole cells might be subtly altered by toxin, *E. coli* cells suspended in sucrose-Tris (*p*H 8.0), 0.6 and 0.033 M, respectively, were allowed to stand at 37 C for 20 min in the presence (90 μg/ml) and absence of toxin. After centrifuging and suspending in sucrose-Tris, the optical density of samples was followed in the presence of 0, 5, and 25 μg of lysozyme per ml. There was no difference in sensitivity to lysozyme of toxin-treated and untreated cells.

Spheroplasts prepared with penicillin were also lysed by α-toxin, but the decrease in optical density was not so great as that observed when lysozyme-EDTA spheroplasts were employed.

Experiments in which streptolysin S and

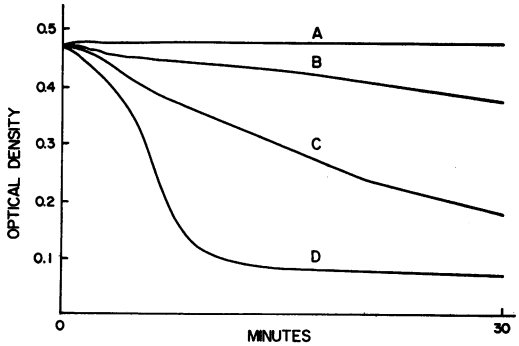

FIG. 1. *Optical density of suspension of Escherichia coli W spheroplasts in presence of α-toxin in concentrations of 0 (curve A), 10 (curve B), 30 (curve C), and 90 (curve D) μg/ml.*

TABLE 1. *Capacity of α-toxin and streptolysins S and O to lyse Escherichia coli spheroplasts*

Spheroplasting method	Test toxin	Concn of toxin	Decrease in optical density 30 min at 35 C
		μg/ml	%
Lysozyme-EDTA	Staphylococcal α-toxin	30	62
	Steptolysin S	50	20
	Streptolysin O	30	4
	None	—	0
Penicillin	Staphylococcal α-toxin	50	47
	Streptolysin S	50	25
	Streptolysin O	50	7
	None	—	0

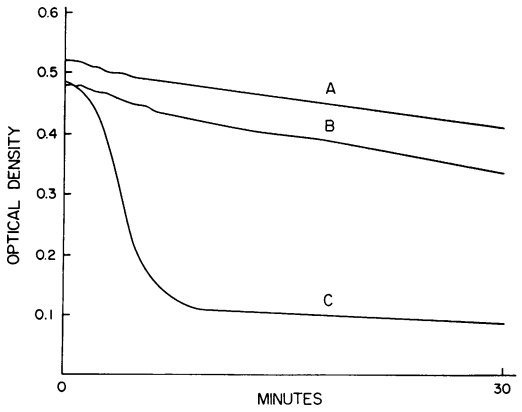

FIG. 2. *Optical density of suspension of Streptococcus pyogenes C203S protoplasts in presence of 0 (curve A), 10 (curve B), and 30 (curve C) μg of α-toxin per ml.*

streptolysin O were substituted for staphylococcal α-toxin revealed that the former, but not the latter, brings about a significant degree of lysis of *E. coli* spheroplasts prepared either with lysozyme and EDTA or with penicillin. The effects are illustrated in Table 1 in which it can be seen that streptolysin S, in the concentrations used, is a somewhat less effective agent than α-toxin in this system.

Lysis of streptococcal protoplasts. Streptococcal protoplasts were somewhat unstable at 35 C as indicated by a gradual decrease in turbidity. Rate of fall of optical density was sufficiently slow, however, to allow experiments to be carried out. It was found that 10 μg of α-toxin per ml produced no effect, but 30 μg/ml or more caused rapid lysis (Fig. 2). Neither streptolysin O nor S, in a concentration of 50 μg/ml produced a significant increase in rate of lysis over that of controls.

In contrast to protoplasts, exposure to α-toxin of whole streptococcal cells in broth resulted in no decrease in turbidity. Similarly, viable counts of unwashed streptococci that had been exposed to 50 μg of α-toxin per ml for 30 min at 37 C did not differ from viable counts of control suspensions.

Lysis of B. megaterium protoplasts. Protoplasts of *B. megaterium* underwent lysis in the presence of appropriate concentrations of α-toxin (Fig. 3 and 4). In experiments in which bacilli were substituted for protoplasts, no lysis occurred nor was any reduction in number of viable cells demonstrable.

Streptolysin S was found to be active on *B. megaterium* protoplasts to a greater degree, even, than α-toxin; as little as 1 μg/ml caused significant lysis (Fig. 5). Streptolysin O, in contrast, was inactive at 50 times this concentration. As with α-toxin, streptolysin S affected neither the turbidity nor the viability of cells with intact walls.

Lysis of Sarcina lutea protoplasts. Addition of α-toxin, in a final concentration of 90 μg/ml, to *Sarcina* protoplasts caused an increase in turbidity during the first 5 min, after which the optical density rapidly fell to 20% of its initial value in 30 min. At 35 μg/ml, similar but smaller effects were observed, the overall decrease in optical density being 18%. Streptolysin S, in a concentration of 50 μg/ml, caused a small (16%) but evidently significant fall in optical density in 30 min. Curves obtained with streptolysin O were not distinguishable from control curves.

Absence of lysis of spheroplasts of V. metschnikovii and V. comma. The turbidity of suspensions

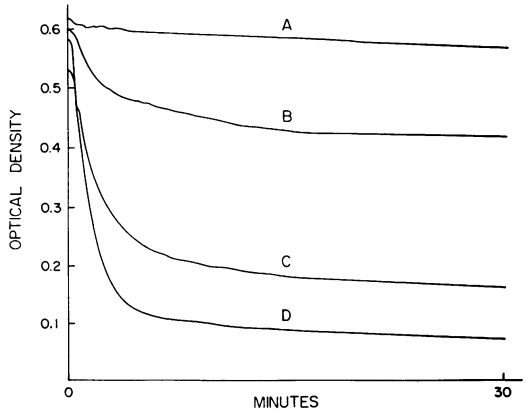

FIG. 3. *Optical density of suspension of Bacillus megaterium protoplasts in presence of 0 (curve A), 18 (curve B), 36 (curve C), and 90 (curve D) μg of α-toxin per ml.*

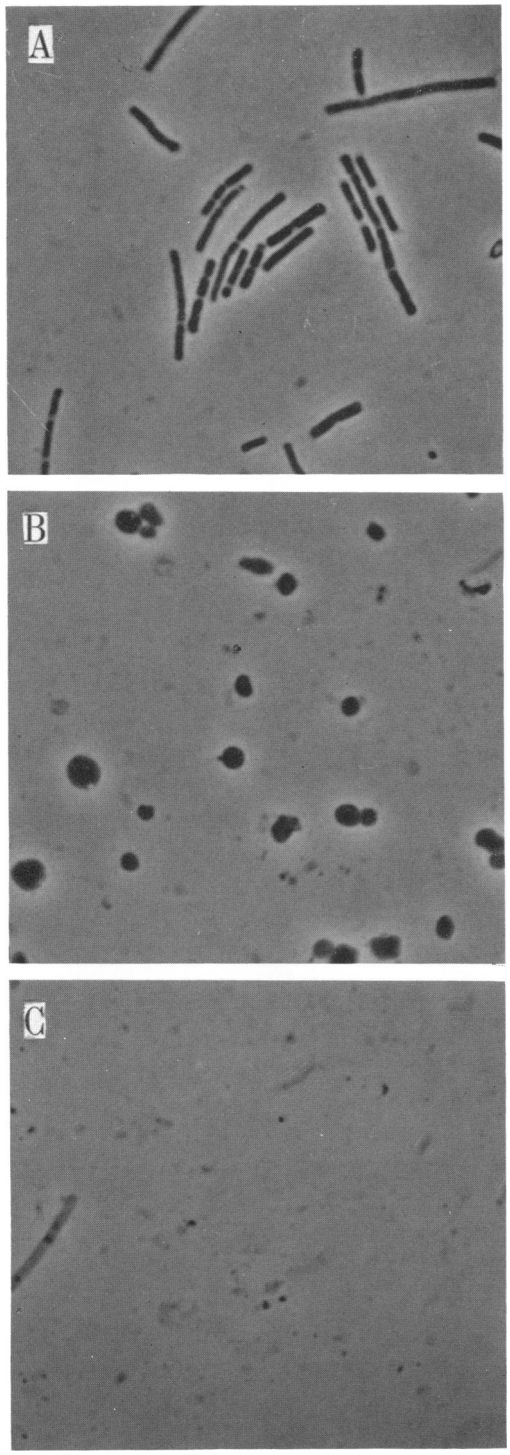

FIG. 4. *(A) Bacillus megaterium before exposure to lysozyme. (B) Protoplast preparation resulting from A and after 5 min at 35 C. (C) Disappearance of protoplasts after exposure to α-toxin (90 μg/ml) for 5 min at 35 C.*

of spheroplasts of the two species of *Vibrio* underwent no significant change in the presence of staphylococcal α-toxin (90 μg/ml), streptolysin S (50 μg/ml) or streptolysin O (50 μg/ml).

DISCUSSION

Much of the foregoing information is summarized in Table 2, from which the following conclusions can be drawn. (i) Staphylococcal α-toxin is capable of lysing protoplasts and spheroplasts of the bacterial species examined with the exception of *V. metschnikovii* and *V. comma*. (ii) In its effects, streptolysin S qualitatively resembles staphylococcal α-toxin except for inability to lyse protoplasts of the streptococci that produce it. (iii) Streptolysin O contrasts sharply with the two foregoing agents in failing to lyse protoplasts of any of the bacteria tested.

The last statement will be considered first as it is the least difficult to analyze. It has long been believed that the action of streptolysin O may involve specific binding to cellular cholesterol, chiefly because its various biological effects are inhibited by small amounts of this compound (Hewitt and Todd, 1939; Smythe and Harris, 1940; Bernheimer and Cantoni, 1945; Howard, Wallace, and Wright, 1953; Kellner et al., 1956; Weissmann et al., 1963) and because there are observations suggesting that cholesterol is the receptor for pneumolysin (Cohen, Halbert, and Perkins, 1942) which closely resembles streptolysin O in several important respects. On this basis, the absence of sterols of any kind from bacterial, in contrast to mammalian, cell membranes explains the insusceptibility of bacterial protoplasts and spheroplasts to streptolysin O.

In distinction to streptolysin O, the receptor (or substrate) of streptolysin S and staphylococcal α-toxin may be, or involve, phospholipid. The biological effects of streptolysin S are more or

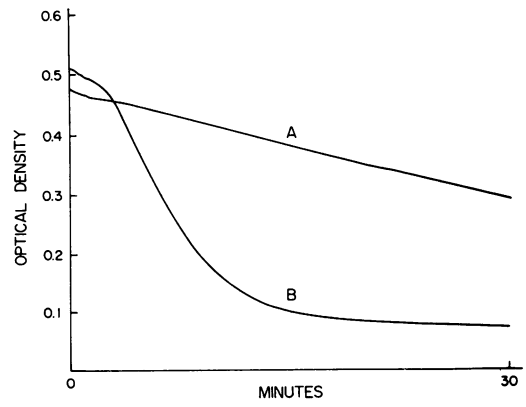

FIG. 5. *Optical density of suspension of Bacillus megaterium protoplasts in presence of 0.8 (curve A) and 8.0 (curve B) μg of streptolysin S per ml.*

TABLE 2. *Comparison of sensitivity to toxins of protoplasts and spheroplasts of various bacteria**

Nature of test material	Preparation of test material	α-Toxin	Streptolysin S	Streptolysin O
Escherichia coli spheroplasts	Lysozyme and EDTA	++	+	0
E. coli spheroplasts	Penicillin	+	+	0
Streptococcus pyogenes protoplasts	Phage-associated enzyme	++	0	0
Sarcina lutea protoplasts	Lysozyme	++	+	0
Bacillus megaterium protoplasts	Lysozyme	++	++++	0
Vibrio metschnikovii spheroplasts	Penicillin	0	0	0
V. comma spheroplasts	Antibody and complement	0	0	0

* Symbols: 0 = no lysis observed; + = significant lysis but reduction in optical density less than 50% at 50 μg/ml; ++ = half-maximal lysis caused by 20 to 50 μg/ml; +++ = half-maximal lysis caused by 2 to 19 μg/ml; ++++ = half-maximal lysis caused by 2 μg/ml.

less specifically inhibited by phospholipid (Hewitt and Todd, 1939; Stollerman, Bernheimer, and MacLeod, 1950), and streptolysin S has recently been shown to cause disorganization of the substructure of synthetic membranes similar to that caused by lysolecithin (Weissmann, Standish, and Bangham, *in preparation*), presumably by virtue of its capacity to react with phosphatidyl choline.

The case for staphylococcal α-toxin is more tenuous, but like streptolysin S it appears to be inhibited by phosphatidyl choline (Rowen, *personal communication*) and to affect synthetic membrane-like structures (Bernheimer, *unpublished data*). The occurrence of the same or similar phosphatides in both bacterial and mammalian cell membranes can explain the capacity of both agents to destroy both bacterial protoplasts and mammalian cells. It is noteworthy that *B. megaterium* KM protoplasts are extraordinarily sensitive to streptolysin S. Since the membrane phosphatide of this microorganism is almost exclusively phosphatidyl ethanolamine (Yudkin, 1962; Kodicek, 1963), it can be inferred that this substance may play a key role in the cytotoxic action of streptolysin S.

Repeated attempts to demonstrate significant effects of staphylococcal α-toxin and streptolysin S on healthy intact bacteria have been consistently negative. The simplest explanation of insensitivity of whole bacteria, in contrast to protoplasts, is that the unaltered bacterial cell wall physically prevents access of toxin to the underlying membrane, whereas in protoplasts and spheroplasts the membrane is vulnerable to the disorganizing action of toxin.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Life Insurance Medical Research Fund, by Public Health Service grant AI-02874-06 from the National Institute of Allergy and Infectious Diseases, and by Public Health Service Research Career Program Award 5K6-AI-14, 198-03.

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