Effect of *Prymnesium parvum* Toxin, Cetyltrimethylammonium Bromide and Sodium Dodecyl Sulphate on Bacteria

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SUMMARY

At low concentrations (0.03 to 3 $\mu\text{g.}/10^9$ cells), purified prymnesium toxin lysed penicillin and lysozyme-EDTA spheroplasts of *Escherichia coli* and *Pseudomonas fluorescens* and protoplasts of *Micrococcus lysodeikticus* and *Bacillus subtilis*. Intact *E. coli* and *P. fluorescens* were unaffected by the toxin (3.3 $\mu\text{g.}/\text{ml.}$), but *E. coli* B was lysed by prymnesium toxin in the presence of EDTA. The activities of selected detergents against spheroplasts and EDTA-treated *E. coli* B were compared with the action of prymnesium toxin.

INTRODUCTION

The toxins produced by the phytoflagellate *Prymnesium parvum* have a wide range of biological activity, including lysis of various mammalian erythrocytes (Yariv & Hestrin, 1961; Bergmann & Kidron, 1966) and nucleated cells such as Ehrlich ascites cells (Dafni & Shilo, 1966; Dafni, 1969), HeLa cells, and normal liver and amnion cells (Shilo & Rosenberger, 1960). Common to all these toxin effects is damage to the cytoplasmic membrane and one result of this is leakage of intracellular constituents into the medium. Prymnesium extracts, including the highly purified preparations, lead to changes in the potassium and sodium content of Ehrlich ascites cells with very rapid leakage (within 20 sec.) of most of the intracellular potassium (Dafni, 1969). Furthermore, the primary effect of the prymnesium ichthyotoxin on immersed fish seems to be the loss of selective permeability of the gill epithelial cells towards various substances, including macromolecules such as trypan blue or radio-iodinated albumin (Ulitzur & Shilo, 1966).

This study describes the effects of prymnesium toxin on bacteria (intact organisms, protoplasts, spheroplasts, and also species of *Mycoplasma*). The similarity of chemical and physical properties of various detergents (cetyltrimethylammonium bromide and sodium dodecyl sulphate) to those of prymnesium toxin (Ulitzur & Shilo, 1966, 1970) made their testing in this experimental system of interest.

METHODS

Prymnesium parvum toxin. The intracellular toxic principle ('toxin B') was extracted and purified as described previously (Ulitzur, 1969; Ulitzur & Shilo, 1970). The purified 'toxin B' preparation had a specific activity of 3000 haemolytic units (h.u.)/ $\mu\text{g.}$, and was dissolved in methanol. In the experimental systems, the final methanol

concentration was never greater than 0.1 %; this concentration did not affect the bacteria tested. For alkaline treatment of the toxin, 0.5 N-NaOH (in methanol) was mixed with toxin and incubated for 30 min. at 20° and then neutralized by addition of 1 N-NaCl (in methanol). For several experiments, 'toxin B' was separated into six components by thin-layer chromatography (Ulitzur & Shilo, 1970).

Bacterial strains and growth conditions. The bacterial strains used were *Escherichia coli* B, *E. coli* K12, *E. coli* ML35 (a lactose permeaseless mutant with constitutive β -galactosidase ($i^{-}z^{+}y^{-}$)), *Pseudomonas fluorescens* MD9 (an isolate described by Rosenberger & Shilo, 1961), *Bacillus subtilis* W168, and *Micrococcus lysodeikticus* (all from the collections of the Departments of Bacteriology and Microbiological Chemistry of the Hebrew University-Hadassah Medical School, Jerusalem). Bacteria were grown in nutrient broth (Difco) at 37° with shaking and harvested at the logarithmic phase of growth by centrifugation at 10,000 g for 15 min. at 20°. They were washed and resuspended in 0.05 M buffer (pH 7.6).

Mycoplasma capri and *M. laidlawii* (obtained from S. Razin, Department of Clinical Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem) were grown as described by Razin (1963).

Preparation of spheroplasts and protoplasts. Lysozyme-EDTA spheroplasts of *Escherichia coli* and *Pseudomonas fluorescens* were prepared as described by Repaske (1958). Protoplasts of the Gram-positive organisms were obtained by incubation of 2×10^{10} bacteria/ml. in buffer (0.01 M-tris, 0.05 M-NaCl and 1 M-sucrose at pH 8) with the addition of 100 μ g. lysozyme (Fluka) and 20 μ g. DNase (Calbiochem) per ml. After incubation of this mixture for 30 min. at 30°, phase contrast microscopy showed that at least 90% of the organisms were converted into protoplasts. The protoplasts were centrifuged at 5000 g for 20 min. at 4° and resuspended in the buffer. Penicillin spheroplasts of *E. coli* and *P. fluorescens* were prepared by growth in nutrient broth containing 0.6 M-sucrose. When the bacterial population reached 5×10^7 organisms/ml., 1000 u. penicillin G/ml. (potassium salt; Rafa, Jerusalem) were added. Microscopic examination showed that, after 2 to 3 h. incubation, more than 90% of the organisms were converted into spheroplasts. Spheroplasts and protoplasts were stored at 4°.

Determination of lytic effect of prymnesium toxin on intact and osmotically sensitive bacteria. Assay mixtures containing 10^9 spheroplasts, protoplasts, or mycoplasma organisms/ml. in 6 ml. of various isotonic buffers and different concentrations of prymnesium toxin or detergents were incubated at 35° for 1 h. or at 8° for 17 h. The degree of lysis was determined by following the decrease in turbidity in a Klett-Summerson photometer (filter 42). Untreated cell suspensions were used as controls. Complete (100%) lysis was obtained by adding 9 ml. water to 1 ml. of bacterial suspension (10^{10} cells) and incubating the diluted suspension for 30 min. at 20°. This system was also used for testing the lytic effects of prymnesium toxin, sodium dodecyl sulphate (SDS, Fluka) and cetyltrimethylammonium bromide (CTAB, Fluka) on intact *Escherichia coli* B and *E. coli* K12 in the presence or absence of 2×10^{-4} M-sodium ethylenediaminetetra-acetate (EDTA).

Viable count of bacteria. The bacterial suspension was incubated for 3 h. at room temperature in 0.05 M-tris buffer (pH 7.6) with prymnesium toxin. The control contained the same mixture with suitable concentrations of methanol instead of toxin. After incubation the bacteria were diluted and plated on nutrient agar (Difco). Colonies were counted after incubation for 24 h. at 37°.

Assay of β -galactosidase activity. Hydrolysis of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) by *Escherichia coli* ML 35 was carried out as described by Wallenfels, Lehman & Malhotra (1962).

RESULTS

The effect of prymnesium toxin on bacterial spheroplasts and protoplasts and on *Mycoplasma*. Prymnesium toxin lysed penicillin and lysozyme-EDTA spheroplasts of *Pseudomonas fluorescens* and *Escherichia coli* as shown in Fig. 1. The minimal dose which lysed 50% of the organisms was found to be independent of the method used for preparing the spheroplasts. The toxin also lysed protoplasts of *Bacillus subtilis* and

Fig. 1

Fig. 2

Fig. 1. The lytic activity of prymnesium toxin on spheroplasts, protoplasts and mycoplasma organisms. *Escherichia coli* and *Pseudomonas fluorescens* spheroplasts were suspended in 0.3M-sucrose in 0.06M-tris buffer (pH 8). *Bacillus subtilis* and *Micrococcus lysodeikticus* were suspended in 1M-sucrose and 0.005M-NaCl in 0.01M-tris buffer (pH 8), and the mycoplasma organisms were suspended in 0.04M-sucrose and 0.25M-NaCl in 0.02M-tris buffer (pH 8). The degree of lysis was determined after 1 h. at 35° as described in Methods. PL = protoplasts prepared with lysozyme; SL = spheroplasts prepared by the lysozyme-EDTA method; P = spheroplasts prepared with penicillin.

Fig. 2. The effect of pH on the lytic activity of prymnesium toxin. The incubation time which gave lysis of 50% of *Pseudomonas fluorescens* lysozyme-EDTA spheroplasts was determined in the presence of different toxin concentrations in a solution of 0.3M-sucrose at different pH values (in 0.02M-sodium phosphate buffer) at 35°.

Micrococcus lysodeikticus and *Mycoplasma laidlawii* organisms (Fig. 1). The sensitivity of mycoplasma organisms to the toxin was more than 500 times that of the *E. coli* spheroplasts. *Micrococcus capri* was even more sensitive: 0.05 nm. toxin/ml. lysed 50% of the *M. capri* population under the same conditions. The sensitivity of *Escherichia coli* spheroplasts to prymnesium toxin was more than 10 times greater than their sensitivity to CTAB and 500 times greater than their sensitivity to SDS on a weight basis.

Figure 2 shows that the lytic activity of the toxin on *Pseudomonas fluorescens* sphero-

plasts increased progressively when the pH of the system increased from 6 to 8. Figure 3 shows that toxin pretreated with NaOH had practically no lytic effect on *P. fluorescens* spheroplasts when incubated at 35°. However, such pretreated toxin retained some of its original lytic activity when tested at 8°. Tabor, Tabor & Rosenthal (1961) showed that divalent cations (Mg^{2+} and Ca^{2+}) and polycationic substances (spermine) stabilized osmotically sensitive spheres in hypotonic media. We found that spermine or 3',3-diaminodipropylamine markedly protected *Mycoplasma laidlawii* against the lytic activity of the prymnesium toxin. It was found that, while 0.5 µg. toxin/ml. gave 50% lysis in the absence of these bases, 25 µg./ml. were required in the presence of spermine (0.001M) and 30 µg./ml. in the presence of 3',3-diaminodipropylamine (0.0015M).

Fig. 3

Fig. 4

Fig. 3. The lysis of *Pseudomonas fluorescens* spheroplasts by NaOH-treated prymnesium toxin. The lytic activity was determined before (control) and after the toxin had been treated with NaOH (as described in Methods); the extent of lysis was determined at 35° after 60 min. and at 8° after 17 h., a time after which no further lysis occurred.

Fig. 4. The effect of prymnesium toxin on EDTA-treated *Escherichia coli* B. At a final concentration of 10^9 organisms/ml. *E. coli* B were suspended in 0.3M-sucrose in 0.05M-tris buffer (pH 8) containing 0.66 µg. prymnesium toxin/ml. EDTA (2×10^{-4} M) and lysozyme (10 µg./ml. were added to the mixture as indicated by arrows. The rate of lysis at 22° was determined in a Perkin-Elmer spectrophotometer (Model UV 137) at 420 nm. against a cuvette containing only tris-sucrose buffer.

The lytic activity of four of the six haemolytic components separated by thin-layer chromatography (t.l.c.) of 'toxin B' (Ulitzur & Shilo, 1970) was tested on *Pseudomonas fluorescens* spheroplasts. All four components showed lytic activity, and for each the ratio of lytic to haemolytic activity was similar.

The effect of prymnesium toxin on intact cells. Untreated logarithmic-phase *Escherichia coli* ML35 and *Pseudomonas fluorescens* bacteria were resistant to prymnesium toxin. No decreases in viable counts occurred when these bacteria were treated with $3.3 \mu\text{g. toxin/ml.}$ at 35° for 3 h. This concentration of toxin is 100 times that sufficient to lyse spheroplasts of *E. coli*. Incubation of *E. coli* ML35, a strain cryptic for β -galactosidase, with $3.3 \mu\text{g. toxin/ml.}$ under similar conditions did not result in any detectable cell membrane damage as determined by the ability of such cells to hydrolyse ONPG.

Fig. 5. The lytic effect of prymnesium toxin, SDS, and CTAB on EDTA-treated *Escherichia coli* B (a) and *E. coli* K 12 (b). Both strains of *E. coli* were studied in systems without and with EDTA ($2 \times 10^{-4} \text{ M}$) and lysis measured after 60 min. at 35° . *E. coli* B was suspended in sucrose-tris buffer as described in Fig. 4, and *E. coli* K 12 was suspended in 0.05 M -tris buffer (pH 8).

It is likely that intact bacteria are resistant to prymnesium toxin because cell-wall components block access of the toxin to the bacterial membrane. EDTA treatment of *Escherichia coli* has been shown to destroy cell-wall barriers towards certain antibiotics, such as actinomycin D (Leive, 1965a), polymyxin B and novobiocin, as well as towards detergents, such as cetylpyridinium bromide and sodium tetradecyl sulphate, and towards complement (Muschel & Gustafson, 1968). EDTA-treated cells were therefore tested for their sensitivity to prymnesium toxin. When suspended in

0.05M-tris buffer (pH 5), *E. coli* B organisms, but not *E. coli* K12 were lysed in the presence of 2×10^{-4} M-EDTA. The sensitivity of EDTA-treated *E. coli* B organisms to prymnesium toxin was therefore tested in the presence of stabilizing concentrations of sucrose (0.3 M). Figure 4 shows that prymnesium toxin alone had no effect on intact *E. coli* B suspended in the sucrose-tris buffer solution. When EDTA (2×10^{-4} M) was added to these toxin-treated bacteria, the turbidity decreased. Addition of lysozyme to toxin-treated *E. coli* B had no effect, but subsequent addition of EDTA, which converted the cells into spheroplasts, caused rapid lysis.

The two detergents (SDS and CTAB) tested for their lytic effect on *Escherichia coli* B showed a four- to fivefold increase of activity in the presence of EDTA (Fig. 5a), similar to the action of prymnesium toxin. *Escherichia coli* K12, on the other hand, was found to be more resistant to the lytic activity of prymnesium toxin and SDS even in the presence of EDTA (Fig. 5b).

DISCUSSION

The highly purified prymnesium toxin preparation has been shown to be one of the most active lysins described, having a specific haemolytic activity more than 3000 times that of digitonin or lysolethicin (Ulitzur & Shilo, 1970). It has been suggested that the strong affinity of the toxin towards biological membranes results from its chemical structure which, as with synthetic detergents and lysophospholipids, consists of lipid (fatty acids) and polar (protein and phosphate) moieties (Ulitzur & Shilo, 1970). Unlike most of the ionic detergents which exert lytic activity on intact bacteria (Baker, Harrison & Miller, 1941; Hotchkiss, 1946; Salton, 1951), prymnesium toxin only acts upon bacteria whose cell walls are partially removed and thus resembles some non-ionic detergents and antibacterial agents (such as actinomycin D and complement) which are not capable of affecting certain intact bacteria (Leive, 1965a; Muschel & Gustafson, 1968).

The resistance of intact bacteria to lysis by prymnesium toxin may be related to the impermeability of the cell wall to the large micelles of the toxin existing in aqueous solutions. On the other hand, it is known that some detergents can affect the metabolism of an organism without affecting its viability (Hotchkiss, 1946). The fact that *Escherichia coli* ML35 treated with prymnesium toxin did not become permeable to ONPG indicates that, if indeed it occurs at all, toxin damage to the intact cell membrane was small.

Treatment of bacteria with lysozyme-EDTA or growth in the presence of penicillin, treatments which expose the cell membrane or part of it, rendered the bacteria susceptible to lysis on subsequent exposure to prymnesium toxin. However, this lytic effect on the osmotically sensitive bacteria was very disparate, in contrast to the consistent effect on different sorts of vertebrate erythrocytes; for instance, mycoplasma organisms are highly sensitive to prymnesium toxin, while *Escherichia coli* spheroplasts are more resistant.

Malamy & Horecker (1964) have shown that after spheroplast formation by treatment with lysozyme-EDTA, but not by growth in the presence of penicillin, the alkaline phosphatase bound in the periplasm is liberated. In spite of the differences in the nature of spheroplasts formed by lysozyme-EDTA and penicillin treatment, no noticeable differences in sensitivity were observed between the two kinds of spheroplasts towards prymnesium toxin. It appears that the resistance of intact bacteria to

lysis by prymnesium toxin must depend on the presence of a 'barrier' in the cell wall against the toxin, and not on any particular stability of the cell membrane. This would explain the sensitivity of *Escherichia coli* B to toxin (as well as to SDS and CTAB) in the presence of EDTA. As is known, EDTA treatment of *E. coli* liberates a considerable portion of the cell wall lipopolysaccharide (Leive, 1965*b*), rendering the cell penetrable to a number of substances which cannot enter the intact organism (or hardly so). Muschel & Gustafson (1968) have found that EDTA-treated *E. coli* showed higher sensitivity to ionized detergents, polymyxin B, and complement.

The findings described here indicate that both the haemolysin and the lysis of osmotically sensitive bacteria could be the same toxic principle. This suggestion is based on the similar response to alkaline treatment of both activities, in contrast to the ichthyotoxic activity which is not affected by alkaline treatment (Shilo, 1967). Furthermore, it was found that four out of six of the haemolysins separated by t.l.c. showed lytic activity towards *Pseudomonas* spheroplasts corresponding to their haemolysin content, while no fixed relationship was observed between their haemolytic and ichthyotoxic activities (Ulitzur & Shilo, 1970).

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