PREPARATION OF SPHEROPLASTS FROM *VIBRIO COMMA*

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ABSTRACT

CHATTERJEE, B. R. (Baylor University College of Medicine, Houston, Texas), AND ROBERT P. WILLIAMS. Preparation of spheroplasts from *Vibrio comma*. J. Bacteriol. **85**:838-841. 1963.—Spheroplasts were prepared from several strains of *Vibrio comma* by lysozyme treatment combined with freezing and thawing of the organisms. The optimal concentration of lysozyme was 50 μg/ml, although some spheroplasts formed at a concentration of 10 μg/ml. Higher concentrations (200 μg/ml) caused lysis of cells along with spheroplast formation. Treatment was carried out in broth cultures containing 15% sucrose, and if the osmotic tension was lowered the spheroplasts lysed. Some motile, spherical cells were present in every preparation. Addition of 3% glycine to broth cultures resulted in rapid transformation of the vibrios into large, spherical bodies. However, these were actively motile, and were not sensitive to a lower osmotic tension. Therefore, they could not be considered as spheroplasts.

Unlike most gram-positive bacteria, gram-negative organisms usually are not susceptible to the solitary action of lysozyme. Various combinations of lysozyme and other agents are used to lyse these organisms or to convert them into spheroplasts. Repaske (1956) used ethylenediaminetetraacetate and lysozyme to prepare spheroplasts from *Escherichia coli*; Zinder and Arndt (1956) used media of high or low pH. These agents apparently acted upon the outer, lipoprotein layer of the gram-negative organisms, exposing to the action of lysozyme the mucopeptide substrate of the enzyme. Kohn (1960) reported formation of spheroplasts from *E. coli* by addition of lysozyme immediately before or after freezing and thawing of the cells. Using Kohn's method, we have prepared spheroplasts from *Vibrio comma*.

Jeynes (1957) reported "protoplast" formation in vibrios by growth in a medium containing 3% glycine. However, these results were questioned by McQuillen (1960). Salton and Shafa (1958) produced spheroplasts from *V. metschnikovii* by growth in penicillin. Grula and Hartsell (1957) and Krishna-Murti and Sengupta (1960) reported that vibrios were not susceptible to lysozyme. The formation of spheroplasts by the action of lysozyme on vibrios, as well as the effect of glycine treatment, are described in this paper.

MATERIALS AND METHODS

The Inaba, Ogawa, and Makasar El Tor strains and a nonagglutinable strain of *V. comma* were used. The organisms were grown at 37 C in nutrient broth (Difco) adjusted to pH 7.2. Crystalline lysozyme (Mann Research Laboratories) of the desired concentration was dissolved in distilled water, sterilized by Seitz filtration, and stored in a refrigerator before use. Fresh lysozyme solutions were prepared daily for the experiments. A 60% (w/v) solution of sucrose was prepared in distilled water, autoclaved, and stored in a refrigerator as a stock solution.

Spheroplasts were prepared from 4-hr-old broth cultures. Sucrose and lysozyme were added, to final concentrations of 15% and 50 μg/ml, respectively, to the broth cultures in polyethylene tubes. After 1 min, the tubes were immersed in a Dry Ice-acetone bath (−80 C), and the contents were frozen. Frozen cultures could be stored in a deep freeze chest (−20 C) for several weeks. For spheroplast formation, the frozen cultures were thawed under running, warm tap water while the tubes were gently shaken. After complete thawing, the cultures were placed on a shaking machine and incubated at 37 C. Development of spheroplasts was followed by examination of cover slip preparations from the cultures in a Zeiss phase-contrast microscope.

Glycine treatment was also carried out on

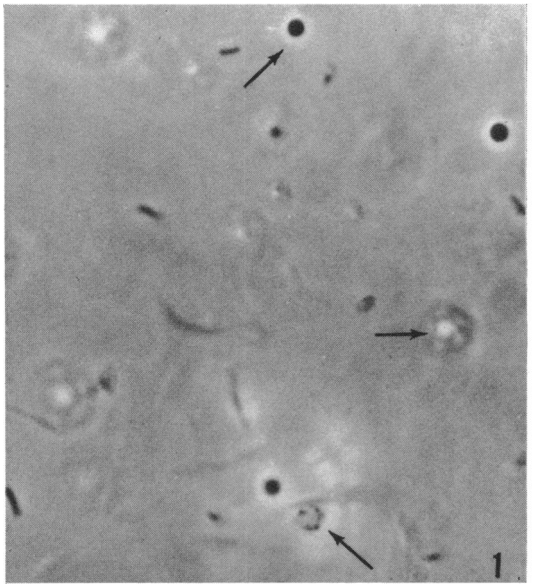
4-hr-old broth cultures. A stock solution of glycine (Calbiochem) in nutrient broth was added to the cultures to make a final concentration of 3% (w/v). The cultures were shaken gently on a mechanical shaker at 37 C and examined at intervals as described above.

RESULTS

Morphology of lysozyme spheroplasts. After 10 min of incubation, spherical bodies began appearing in the thawed cultures. The cells were nonmotile and apparently devoid of cell walls. Spherical forms continued to develop for up to 2 hr of incubation, after which time the spheres began to degenerate. Granules of various sizes and numbers appeared inside the spheres, and they gradually became more transparent with undefined outlines (Fig. 1). Ultimately, the spheroplasts ruptured, leaving ghosts and clusters of granules (Fig. 1). A few motile, spherical cells were present in all preparations. These cells were similar to those appearing after glycine treatment.

Effect of lysozyme concentration. As shown in Table 1, low concentrations of lysozyme effect some formation of spheroplasts. The effect improves with increasing concentrations of the enzyme. From concentrations of 50 to 200 μg/ml, spheroplast formation appears to be uniform. At the higher concentration, some cells lyse, and complete conversion of all organisms to spheroplasts did not occur. The ratio figures of Table 1 are not absolutely quantitative because vigorous motility of many vibrios made accurate counting impossible. Attempts to fix the cells in molten-agar suspensions were unsuccessful, because upon gelation of the agar the spheroplasts lysed.

Osmotic sensitivity of lysozyme spheroplasts. If the thawed suspensions undergoing spheroplast formation were not supplemented with sucrose, the cells rapidly lysed, and the cultures then contained some intact, motile vibrios and a few of the motile, spherical cells. Similarly, spheroplasts prepared as described, when harvested by centrifugation and resuspended in



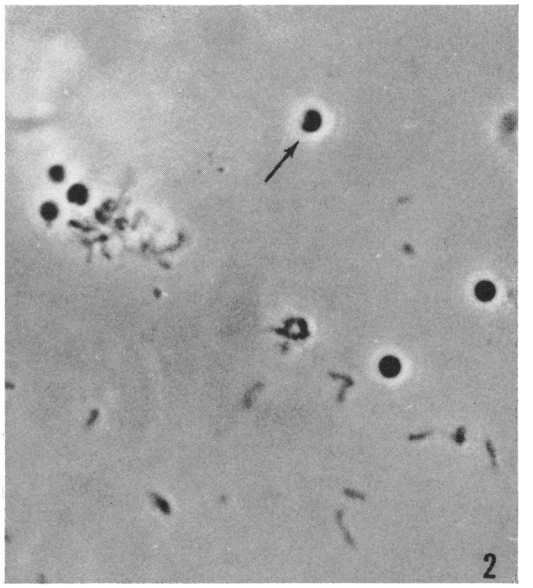


FIG. 1. *Phase-contrast micrograph of spheroplasts prepared from Vibrio comma by lysozyme treatment combined with freezing and thawing. The top arrow points to a typical spheroplast; the middle arrow, to a spheroplast lying out of the plane of focus; and the lowest arrow, to an old, degenerated spheroplast showing an empty ghost containing dark granules. Motility of the cells made photography difficult, and many spheroplasts lie at different planes of focus.* × *1,000.*

FIG. 2. *Spherical bodies prepared from Vibrio comma by treatment with 3% glycine. Arrow points to an incomplete sphere that is characteristic of gram-negative spheroplasts prepared by penicillin treatment.* × *1,000.*

TABLE 1. *Effect of lysozyme concentration upon spheroplast formation in Vibrio comma*

Concentration of lysozyme	Ratio of spheroplasts to intact cells*
μg/ml	%
10	30:70
25	50:50
50	75:25
100	80:20
200	90:10†

* Ratio obtained by counting several fields in the cover slip preparations and averaging them.

† Many cells lysed before spheroplast formation. Spheroplast formation was effected as described in the text.

nutrient broth, immediately lysed, again leaving in the suspension a few motile, spherical cells.

Effects of glycine treatment. Within 2 to 3 hr after addition of glycine, all the organisms in the culture were converted into large, spherical cells. These appeared identical to spheroplasts of gram-negative organisms (Fig. 2), but they were very motile. Except for the addition of 3% glycine, these spherical cells were formed in a medium of ordinary osmotic tension, and, unlike spheroplasts prepared by lysozyme treatment, they did not lyse when suspended in nutrient broth. We resuspended the cells in nutrient broth to eliminate the possibility that glycine might in some way have raised the osmotic tension of the medium. The spherical cells obtained by glycine treatment remained intact in nutrient broth suspensions for over 24 hr, but they could not be subcultured as reported by Jeynes (1957).

DISCUSSION

Repaske (1960) speculated that lysis of gram-negative and gram-positive organisms by lysozyme involved the same substrate. Gram-negative cells were more resistant to the enzyme because of a protective, lipoprotein outer layer in their cell walls. However, much less is known about the structure of the cell wall of gram-negative organisms than of gram-positive. The experiments of Grula and Hartsell (1957) suggested that different species of gram-negative organisms varied in their susceptibility to lysozyme. Mandelstam (1961) demonstrated that there was a mucopeptide layer in the cell wall of *E. coli*, and that the isolated mucopeptide was susceptible to lysozyme action. Therefore, successful spheroplast formation in gram-negative organisms required exposure of the susceptible mucopeptide layer to lysozyme.

Kohn (1960) attributed spheroplast formation by lysozyme in *E. coli*, after freezing and thawing of the organisms, to the cracks and fissures produced by the physical treatment in the lipoprotein and mucopolysaccharide layers of the cell wall. The rapid damage of the cell wall by freezing and thawing exposed large areas of the mucopeptide layer to the action of lysozyme. Freezing and thawing did not effect lysis in older bacterial cultures, and the presence of intact organisms and motile, spherical cells in our preparations was probably due to the complete lack of or the partial action of lysozyme upon older cells in the cultures. Purkayastha and Williams (1960) reported that lysozyme treatment released some hexosamine from whole cells of *Serratia marcescens*, thus suggesting an effect of lysozyme on the mucopeptide layer of these gram-negative organisms. However, the combination of freezing and thawing plus lysozyme did not produce spheroplasts from *S. marcescens*.

The spherical cells produced by glycine treatment could not be classified as spheroplasts. They were not susceptible to lowering of the osmotic tension, and they were persistently and actively motile. Contrary to Jeynes (1957), we could not grow the vibrios in media containing glycine even if the concentration was lowered to 0.5%. However, although glycine did not produce spheroplasts, the amino acid undoubtedly weakened the cell wall, as evidenced by the formation of spherical cells. The action of glycine was attributed by Park (1958) to inhibition of synthesis of cell-wall precursors. We have used combined glycine and lysozyme treatment to produce spherical cells from *Bacillus anthracis* (Chatterjee and Williams, 1961).

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