Formation of Spheroplasts from *Bacillus anthracis*

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Received for publication 14 November 1964

**ABSTRACT**

CHATTERJEE, B. R. (Baylor University College of Medicine, Houston, Tex.), and ROBERT P. WILLIAMS. Formation of spheroplasts from *Bacillus anthracis*, J. Bacteriol. 89:1128-1133. 1965.—Spheroplasts were prepared from *Bacillus anthracis* by combined treatment with lysozyme and glycine. Glycine, at a final concentration of 3%, was added to cultures of *B. anthracis* in nutrient broth that had grown at 37 C for 16 to 18 hr under 50% CO₂. After additional incubation under CO₂ for 2 hr, lysozyme, at the appropriate concentration (50 to 100 μg/ml), and sucrose, to a concentration of 15%, were added, and incubation was continued for 2 to 6 hr in CO₂. At the end of this period, incubation in CO₂ was discontinued. Spheroplasts formed after incubation in air for 6 to 12 hr. Lysozyme alone exhibited the same effect when added at much higher concentrations (500 to 2,000 μg/ml) to cultures growing under CO₂. No spheroplasts formed when cultures were treated with glycine alone. Treatment with lysozyme was more effective on smooth strains than rough. Cells from young cultures were more susceptible to lysozyme than older cells. CO₂ apparently was essential for formation of spheroplasts from *B. anthracis.*

Fischer, as cited by Weibull (1956), reported the extrusion of protoplasmic material from certain bacteria, including *Bacillus anthracis*, when the organisms were placed in various concentrations of salt. He named the phenomenon "plasmoypsis." Many years later, Tomesik and Guex-Holzer (1952) and Weibull (1953) reported that, under certain conditions, lysozyme digested the cell wall of *B. megaterium*, releasing spherical structures into the medium. Weibull (1953) termed the spherical structures "protoplasts." True protoplast formation in *B. anthracis* has not been reported. Stähelin (1953) reported spherical transformation of *B. anthracis* by suspension of cells in different concentrations of salt. He called these bodies "gymnoplasts." Gladstone and Johnston (1955) described spherical transformations of anthrax organisms after treatment with high concentrations of lysozyme. Neither the spherical structures described by Stähelin nor those reported by Gladstone and Johnston could be designated as protoplasts, judging from the authors' characterization of the bodies. This paper describes the formation of osmo-sensitive spherical bodies from *B. anthracis* by combined treatment with glycine and lysozyme. The generally accepted definition of a protoplast (Brenner et al., 1958)

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requires that the cell-wall components be absent from these bodies. Since chemical analysis showed small amounts of cell-wall materials still remaining in our preparation, we shall use the term "spheroplast" to characterize these bodies.

**MATERIALS AND METHODS**

**Organisms.** Most of the investigations were carried out with a smooth and a rough variant of the Vollum strain of *B. anthracis*. Differences in susceptibility to lysozyme between smooth and rough strains were investigated with strains of *B. anthracis* designated Ba-5, -19, -23, -25, -26, and -28. These cultures were obtained from the collection of K. I. Burdon, Department of Microbiology, Baylor University College of Medicine. Paired smooth and rough forms from the same strain were obtained by picking appropriate colonies after plating parent cultures on bicarbonate agar and incubating at 37 C for 24 hr (Roth and Williams, 1964).

**Media and conditions of growth.** Nutrient broth (Difco) was supplemented with 0.7% NaHCO₃. This medium was buffered at pH 7.0 to 7.3 by addition of KH₂PO₄, 5.23 g per liter, and KH₂PO₄, 4.08 g per liter. Organisms were grown for 24 hr at 37 C in the nutrient broth; then 0.1 ml of culture was inoculated into 25 ml of the nutrient broth contained in a 100-ml filtration flask. The flasks were tightly stoppered with rubber stoppers, 50% CO₂ was added, and the cultures were incubated with gentle shaking at 37 C.

**Chemicals.** Concentrated solutions of lysozyme (Mann Research Laboratories, New York, N.Y.) were prepared in water and were sterilized by
filtration. Glycine (Calbiochem) was prepared in 10% solution in nutrient broth (Difco), sterilized by autoclaving at 15 psi for 15 min, and stored as the stock solution. An aqueous solution of 60% sucrose (Baker Analyzed Reagents, Phillipsburg, N.J.) was autoclaved and stored as a stock solution. Glucosamine was obtained from Calbiochem. All other chemicals used were Baker analyzed reagent grade.

Preparation of spheroplasts. Turbidimetric measurements indicated both the smooth and rough strains to be growing logarithmically after 16 to 18 hr of incubation under 50% CO₂. At this stage of growth, glycine in nutrient broth was added to the cultures to give a final concentration of 3%. The CO₂ content of the flask was replenished to 50%, and shaking continued for another 2 hr at 37 C. At the end of this period, the culture was supplemented with sucrose to a final concentration of 15%, and lysozyme was added at the specified concentrations (50 to 100 μg/ml). The CO₂ content of the flask was again replenished to 50%, and incubation continued at 37 C without shaking for 2 to 6 hr. At the end of this period, the flasks were opened and incubation was continued in air for another 6 to 12 hr. Formation of spheroplasts occurred during this latter period. The exact time of appearance of spheroplasts varied from strain to strain.

Estimation of amino sugars. The method of Cardell (1953) was used. Microscopy and photography. Preparations were examined with a Zeiss phase-contrast microscope as wet cover slip mounts under oil immersion. Pictures were taken through the same microscope with a Bausch and Lomb microscopic camera and Kodak Tri-X panchromatic film. A Petroff-Hausser counting chamber was used to determine the number of cells in the preparation.

Results

Effect of glycine alone. There are many reports of the successful use of glycine to produce spheroplasts from bacteria (Jeynes, 1961). The effect of this amino acid upon B. anthracis was investigated. A 3% concentration of glycine, reported to be most effective for spheroplast formation, inhibited the growth of anthrax organisms. Therefore, the cells were first grown in nutrient broth under 50% CO₂ for 16 to 18 hr. Then glycine was added to the cultures at concentrations of 0.5, 1.0, 2.0, 3.0, 5.0, and 7.0%; sucrose was added to a concentration of 15%; and the CO₂ was replenished to 50%. Definite changes appeared in the cells 2 hr after addition of glycine. Bulbous herniations or fusiform swellings were seen, as well as spherical or ovoidal cells. Continued incubation in glycine did not further enhance these effects. The optimal concentration of glycine effecting the changes was 3%. These results suggested the amino acid had weakened the cell wall, and that the cells might be susceptible to further treatment with lysozyme. Effect of lysozyme on glycine-treated cells. Broth cultures of smooth and rough strains were incubated for 16 to 18 hr under 50% CO₂; glycine was added to a concentration of 3%; and, after incubation for an additional 2 hr, lysozyme was added in different concentrations. The cultures also were supplemented with sucrose to a concentration of 15%. Care was taken to replenish the concentration of CO₂ to 50% after each addition. There were no appreciable changes until incubation proceeded under CO₂ for 12 to 18 hr after the addition of lysozyme. At this time, a small number of spherical cells appeared. The bacillary chains separated with the release of many single rods. There was also a noticeable decrease in the total number of bacilli. Several experiments with various concentrations of lysozyme failed to increase the yield of spherical cells.

Continued incubation under CO₂ was thought to be responsible for the decrease in number of cells. To examine this assumption, the period of incubation under CO₂ after the addition of lysozyme was varied from 0 to 18 hr. This period was followed by incubation in air for an additional 6 to 12 hr. The experiments showed that incubation under CO₂ beyond 6 hr after the addition of lysozyme was not only unnecessary but detrimental, causing more lysis of cells. Incubation under CO₂ for 2 to 6 hr after the addition of lysozyme was best, and usually resulted in optimal transformation of bacilli into spherical forms during subsequent incubation in air.

A period of incubation under CO₂ after addition of lysozyme was crucial for formation of spherical forms; none was produced if this procedure was omitted. The optimal length of incubation under CO₂ varied from strain to strain, but was less for smooth strains. When used in combination with glycine and CO₂, the optimal concentration of lysozyme varied between 50 to 100 μg/ml for different strains.

The appearance of the spherical cells is shown in Fig. 1. They appear to have no cell wall, and their size varies from 2 to 8 μ in diameter. Dark granules of varying size and number are distributed randomly within the spheres. Placed in the proper environment, they remain viable for up to 24 hr, as determined by their appearance under phase microscopy. Viable cells showed constant cytoplasmic motion; dead cells, none. On centrifugation at 2,000 X g, for 10 min, the spheroplasts would remain in suspension at
different depths of the supernatant fluid, whereas the intact bacilli settle in the bottom. The spheroplasts could be largely separated from the intact bacilli by such a method. Dilution of the suspending medium, as well as vigorous manipulation, lysed the spheres.

No division or reversion to bacillar forms occurred. However, accessory spherical bodies occasionally budded out from the parent cell (Fig. 2a and 2c). The process never went to completion, and the parent cell usually collapsed. The characteristics of these spherical cells conformed to those described by Weibull (1953) for protoplasts, although, as described below, chemical tests for the presence of amino sugars, a constituent of cell walls, did not support this conclusion.

The cytoplasmic contents and granules in the spherical bodies were frequently seen to be in constant, random motion. Just before rupture, the motion became more vigorous. Rupture released the cytoplasmic contents, leaving behind the granules and the ghost of the cytoplasmic membrane which could often be seen as a faint, round shadow.

Individual photographs (Fig. 3) suggested that more than one spherical form might arise from a single rod. However, the whole process of emergence of the spherical forms from the rods was not followed, and the photograph might represent origin from a chain of three rods. McQuillen (1960) reported that more than one protoplast could emerge from a single rod.

Content of amino sugars in the spherical cells. To determine whether the cell wall was completely removed from the spherical cells, the hexosamine content of these cells was compared to that of whole cells. Data from four experiments (Table 1) showed that the spherical cells still contained hexosamine, although the amount was 70 to 85% less than that contained in whole cells. Efficiency of conversion of whole cells to spherical

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Content of hexosamine (mg/mg, dry wt)</th>
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<tbody>
<tr>
<td></td>
<td>Whole cells</td>
</tr>
<tr>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>1</td>
<td>23.7</td>
</tr>
<tr>
<td>2</td>
<td>28.2</td>
</tr>
<tr>
<td>3</td>
<td>18.0</td>
</tr>
<tr>
<td>4</td>
<td>24.1</td>
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</tbody>
</table>

* The smooth form of the Vollum strain of B. anthracis was used. Spherical forms were prepared as described in Table 2 with 100 μg/ml of lysozyme. Determinations were made on preparations separated by centrifugation. Values for spherical forms were corrected approximately for intact cells remaining in the preparations by assuming that 20% of the weight of whole cells in the mixture was cell wall. The percentage of whole cells in each sample of spherical forms was estimated, the amount of dry weight contributed by these cells to the preparation was determined, and 20% of the figure calculated. The latter figure was used to correct the observed values for weight and content of hexosamine in mixed preparations and to obtain the values shown in the table for spherical forms.
forms varied from experiment to experiment. Since the spherical cells still contained elements of the cell wall, they could not be designated protoplasts, but should be termed spheroplasts.

**Spheroplast formation by action of lysozyme alone.** When used alone without pretreatment of cells with glycine, 50 to 100 μg/ml of lysozyme had no effect on the bacilli. However, when used in extremely high concentrations of 500 to 2,000 μg/ml, lysozyme produced similar changes. The concentration of lysozyme required for the effects again varied from strain to strain. Incubation of the cultures in CO₂ also was essential. The spheroplasts formed by high concentrations of lysozyme appeared identical to those described in the previous section. Solutions of lysozyme heated to boiling did not bring about these changes, indicating that the action was enzymatic.

**Effect of lysozyme on smooth and rough forms.** There was a consistent difference in the effect of lysozyme on the smooth and rough strains. This difference was investigated with use of paired smooth and rough strains, each pair being obtained from a single, different parent organism. Following the procedure for incubation under CO₂, including pretreatment with glycine, the strains were exposed to different concentrations of lysozyme for various lengths of time. The cultures were incubated in air for 6 hr after the periods of incubation in CO₂ with lysozyme.

Results of cell counts made after treatment with lysozyme for 2 and 18 hr are shown in Table 2. A difference in susceptibility to lysozyme is evident. Smooth strains treated with 1,000 μg/ml of lysozyme for 2 hr are almost totally lysed, and the preparations show only long, filamentous strands (Fig. 4a). As the concentration of lysozyme is reduced, spheroplasts appear. Their number decreases with lower concentrations of the enzyme, until, at 50 μg/ml, they number about 50% of the cells.

Rough strains were more resistant to lysozyme. Concentrations of 1,000 μg/ml caused clumping of rough cells, and it was difficult to obtain an accurate count (Fig. 4b). An approximate count indicated that formation of spheroplasts in the rough strain at a concentration of 1,000 μg/ml of lysozyme was approximately equivalent to the effect obtained with 250 μg/ml of enzyme in the smooth. At a concentration of 50 μg/ml, almost no formation of spheroplasts occurred in the rough strain.

When examined after treatment with lysozyme for 18 hr, the rough strain showed similar changes to those which were observed after treatment of the smooth strain for 2 hr. After 18 hr, however, the smooth strain was completely lysed by concentrations of lysozyme above 100 μg/ml. The Vollum strain was used for the experiments shown in Table 2. Similar results were obtained with five of the six other paired smooth and rough strains examined. Strain Ba-19 was an exception, and the rough variant was more susceptible to lysozyme than the smooth.

**Effect of varying the concentration of CO₂.** CO₂ was an important factor in formation of spheroplasts. Its influence was investigated by incubating cultures in various concentrations of the gas and determining the effect upon spheroplasts.

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**Table 2. Effect of lysozyme upon smooth and rough strains of Bacillus anthracis**

<table>
<thead>
<tr>
<th>Concn of lysozyme</th>
<th>Percentage of spheroplasts found among bacillary forms</th>
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<tbody>
<tr>
<td></td>
<td>Treated with lysozyme for 2 hr</td>
<td>Treated with lysozyme for 18 hr</td>
</tr>
<tr>
<td></td>
<td>Smooth</td>
<td>Rough</td>
</tr>
<tr>
<td>μg/ml</td>
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<tr>
<td>1,000</td>
<td>79%</td>
<td>12%</td>
</tr>
<tr>
<td>500</td>
<td>82%</td>
<td>8%</td>
</tr>
<tr>
<td>250</td>
<td>71%</td>
<td>9%</td>
</tr>
<tr>
<td>100</td>
<td>54%</td>
<td>2%</td>
</tr>
<tr>
<td>50</td>
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* Cultures were incubated at 37 C in nutrient broth under 50% CO₂ for 16 to 18 hr. Glycine was added to a concentration of 3%, CO₂ was replenished, and incubation was continued for 2 hr. The cultures were supplemented with sucrose to a concentration of 15%, and lysozyme of the specified concentration was added. After treatment with lysozyme for the length of time indicated, the cultures were incubated in air for 6 hr. Cell counts were then made in a Petroff-Hauser chamber. The results tabulated were obtained with the smooth and rough forms of the Vollum strain.

† Accurate counts were impossible in these cases.

‡ Because of aggregation of cells, this count is only approximate.
Table 3. Effect of lysozyme upon cells of different ages

<table>
<thead>
<tr>
<th>Age of culture</th>
<th>Effectiveness of various concn of lysozyme for formation of spheroplasts</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>12.5 μg/ml</td>
</tr>
<tr>
<td>12 hr</td>
<td>10% spheroplasts; many intact bacilli</td>
</tr>
<tr>
<td>24 hr</td>
<td>Few spheroplasts; mostly intact bacilli</td>
</tr>
<tr>
<td>48 hr</td>
<td>No spheroplasts</td>
</tr>
</tbody>
</table>

* The smooth variant of the Vollum strain was used. Conditions of growth, incubation under CO₂, and addition of glycine were the same as those cited in Table 2. Cultures were treated for 4 hr with lysozyme under CO₂, followed by incubation in air for 6 hr.

formation. Cells of the smooth variant of the Vollum strain were grown in nutrient broth for 16 to 18 hr under the desired concentration of CO₂, glycine was added to a concentration of 3%, the CO₂ was replenished to the previous concentration, and incubation was continued for another 2 hr. Sucrose was added, and the cultures were treated with 50 μg/ml of lysozyme for 2 hr under the same concentration of CO₂. This period of treatment was followed by incubation in air for 6 hr.

When the concentration of CO₂ was 10%, only a few spheroplasts formed, and the cultures contained a large number of intact rods. At a concentration of 100%, there was marked cell lysis, and again only a few spheroplasts formed. The number of spheroplasts increased at a concentration of 25%, but there were still a
large number of intact bacilli. The optimal concentration of CO₂ was 50%, when about 50% of the cells were converted into spheroplasts. This concentration was used for all of the other investigations.

Effect of age of culture upon formation of spheroplasts. Lysozyme is known to have its most pronounced effects upon young cells. This fact was verified by exposing cultures of different ages to the glycine-lysozyme-CO₂ treatment. Various concentrations of lysozyme were used to examine the effect of the enzyme. The results are shown in Table 3. Lysozyme appeared to have maximal effect upon 12-hr cells, at which time a concentration of 12.5 µg/ml produced some spheroplasts. After growing for 48 hr, the cells were quite resistant to lysozyme, and 50 µg/ml converted only 13% of the organisms into spheroplasts. A growth period of 16 to 18 hr, as used in our experiments, appeared to be near the optimum. At this time, cells were growing logarithmically. Cultures grown for 24 hr were entering the stationary phase; whereas those grown for 48 hr were in the phase of decline.

DISCUSSION

In morphological appearance and behavior toward physical treatments, the spherical forms resemble true protoplasts. However, the retention of 14 to 30% of the content of hexosamine of whole cells characterizes the cells as spheroplasts. Both glycine and lysozyme influenced formation of spheroplasts in *B. anthracis* under the conditions described. Although glycine alone was not effective, the amino acid appeared to weaken the cell wall, making it susceptible to the action of lysozyme. Park (1958) suggested that glycine might have an effect similar to penicillin or polymyxin and that it acted as a metabolic block inhibiting synthesis of cell-wall materials. A dual metabolic and enzymatic attack upon the cell wall resulted in formation of spheroplasts.

Lysozyme alone in very high concentrations induced formation of spheroplasts if the proper concentration of CO₂ was maintained. Earlier failures to discover spheroplast formation in *B. anthracis* could be attributed to lack of appreciation of the importance of CO₂. The effect of the gas seemed complex, and our discovery of its essential role in the process was fortunate.

As reported by Gladstone and Johnston (1955), the effective concentration of lysozyme varied from strain to strain, as well as between smooth and rough forms. Although we completed only a few experiments, it did appear that smooth strains were usually more susceptible to lysozyme than rough. This finding might reflect some difference between the cell-wall structure of virulent and avirulent strains.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant AI-01535 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


