MYCOBACTERIAL CELL WALLS

I. Methods of Preparation and Treatment with Various Chemicals

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Abstract

Takeya, Kenji (Kyushu University, Fukuoka, Japan) and Kazuhito Hisatsune. Mycobacterial cell walls. I. Methods of preparation and treatment with various chemicals. J. Bacteriol. **85**:16–23. 1963.—Several methods of preparation of mycobacterial cell walls were examined, and the grinding method with glass powder, using Dry Ice, was found to give fairly good cell-wall preparations. "Paired fibrous structures" were clearly seen on the purified cell wall. The appearance of the cell wall as revealed by the electron microscope was not altered by digestion with trypsin, pronase, or pronase in 5% alcoholic solution, nor by treatment with 95% alcohol, acetone-alcohol mixture, or ether-alcohol mixture. By treatment with alcoholic KOH solution, the fibrous structure was removed. The remaining thin layer of the cell wall was tentatively designated the "basal layer" of the mycobacterial cell wall. The fibers appeared also to be removed by chloroform treatment. Nagarse digestion seemed to solubilize some constituents of the cell wall. The cell wall lost its shape and rigidity after lysozyme digestion.

Immunological properties of mycobacterial cell walls have recently been intensively studied by several investigators (Ribi, Larson, and Wicht, 1958; Kanai and Youmans, 1960; Kanai, Youmans, and Youmans, 1960; Kotani et al., 1961; Larson, Ribi, and Wicht, 1961). Kotani et al. (1959) also performed quantitative analysis of the chemical composition of the cell wall of the BCG strain of *Mycobacterium tuberculosis*. In previous publications, fine structure of the mycobacterial cell wall was investigated by the present authors (Takeya et al., 1958, 1961).

Attempts to elucidate the morphological constituents of the mycobacterial cell wall, their chemical composition, and also their biological significance have been undertaken in this series of reports.

Materials and Methods

Mycobacterial cells. M. phlei was used throughout the experiment. The organisms were grown on the surface of modified Sauton synthetic media (containing sodium glutamate, 8.0 g; glycerine, 60.0 ml; K_2HPO_4, 0.5 g; ammonium iron citrate, 0.05 g; citric acid, 2.0 g; $MgSO_4$, 0.5 g; distilled water to 1,000 ml) for 8 days at 37 C. Cells were collected by filtering the contents of the culture flasks through filter paper, and were washed by centrifugation three times with distilled water.

Electron microscopy. Samples were homogeneously suspended in distilled water, and drops of the suspension were placed on a thin collodion film deposited on a 3% agar plate. After an appropriate period, excess fluid from the drop was sucked up, and the collodion film was floated on distilled water for dialyzing out impurities. After completion of dialysis, the film was removed on copper-mesh screen. The specimens were shadowed with chromium and examined with a JEM 5C electron microscope.

Results

Cell-wall preparation method. Several methods for disintegration of cells were examined. Cell walls prepared by the ultrasonic vibration method, by the grinding method with glass beads, or by the Waring Blendor method (Lamanna and Mallette, 1954) were found to contain many cytoplasmic remnants or to be rather fragmentary, as revealed by careful electron microscopy. Finally, the grinding method with glass powder, using Dry Ice, was proved to give fairly good cell-wall preparations and was adopted in this experiment.

Approximately 50 g of washed semidried

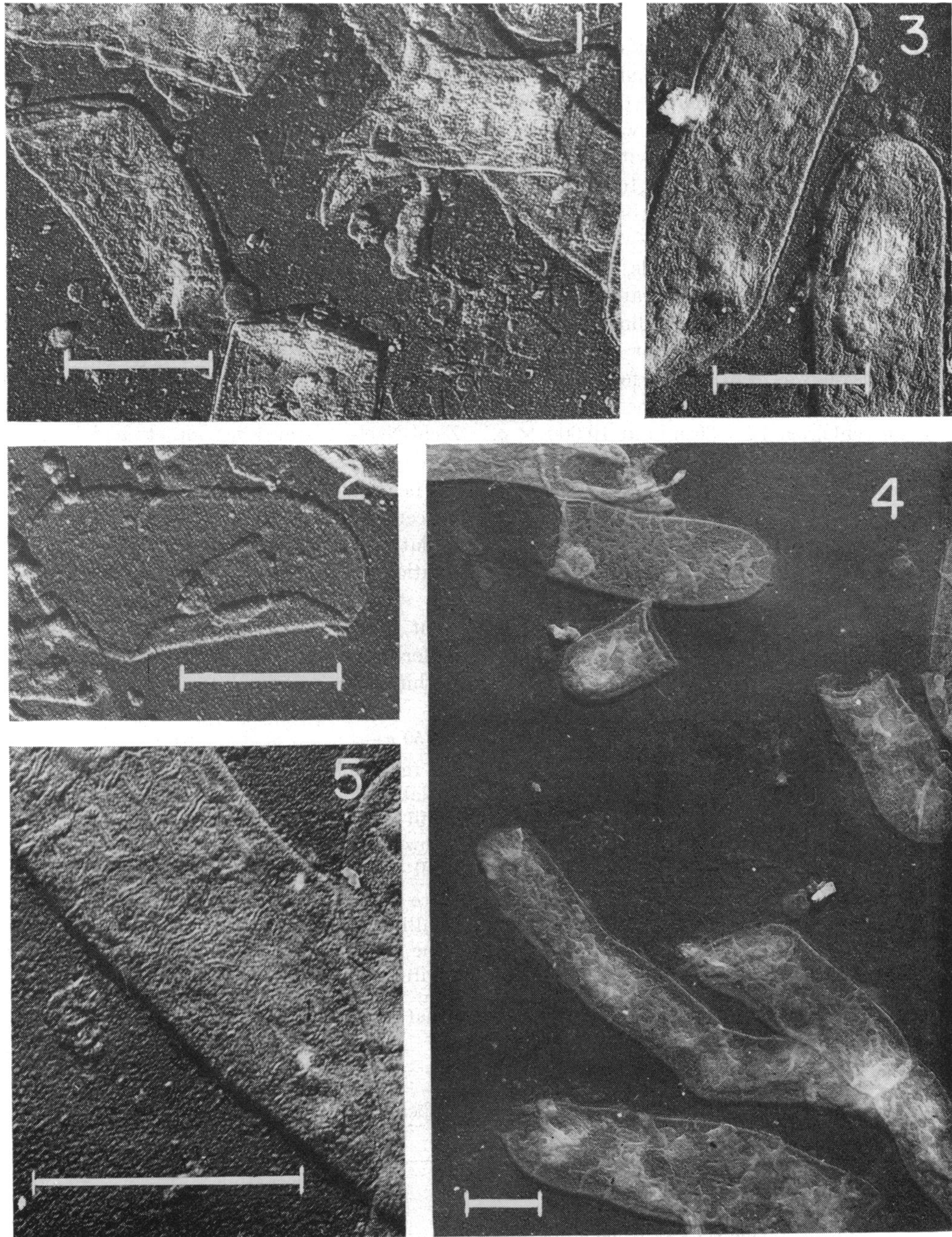


FIG. 1. *Crude cell-wall preparation obtained by centrifugation at 10,000 × g for 30 min. × 38,000. Magnification mark = 0.5 μ in all figures.*

FIG. 2. *Membranous structure, probably representing a fragment of cytoplasmic membrane, found in the crude cell-wall preparation obtained by centrifugation at 23,000 × g for 30 min. × 42,000.*

FIG. 3. *Crude cell walls treated with 0.4% deoxycholate solution. The appearance of the cell wall is not altered. × 40,000.*

FIG. 4. *Purified cell-wall preparation. × 20,000.*

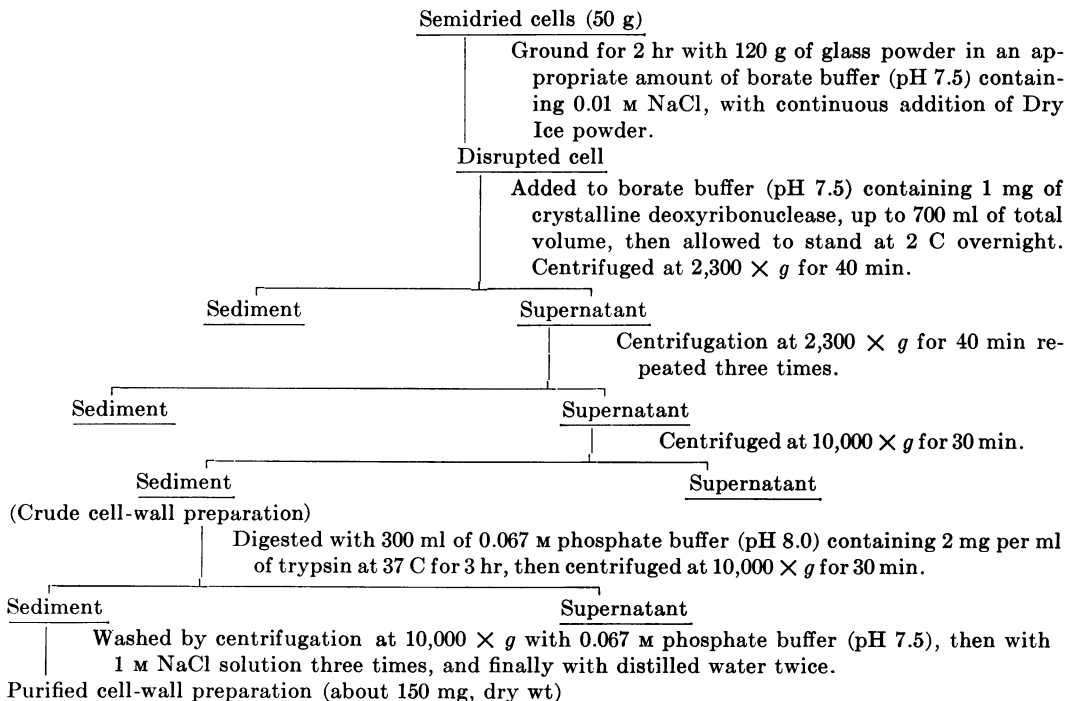
FIG. 5. *Paired fibrous structures clearly seen on a cell wall. × 77,400.*

cells were mixed with 120 g of glass powder (Minnesota Mining and Manufacturing Co., Saint Paul) with the addition of an appropriate volume of borate buffer (pH 7.5) containing 0.01 M NaCl. This pastelike mixture was vigorously ground in a ball mill for 2 hr with continuous addition of small blocks of Dry Ice, enough to maintain the mixture in a frozen state just like ice cream.

After disintegration of the cells, the material was suspended in 500 ml of borate buffer (pH 7.5) containing 1 mg of crystalline deoxyribonuclease, and let stand overnight at 0 to 2 C. The suspension was centrifuged four times for 40 min at 2,300 $\times$ g, and the supernatant was again centrifuged for 30 min at 10,000 $\times$ g. The sediment was designated "crude cell-wall preparation" (Fig. 1). When centrifuged at 23,000 $\times$ g for 30 min, membranous structures, probably representing free cytoplasmic membranes (Fig. 2), were frequently observed in the crude cell-wall preparation. Although treatment with 0.5% polyoxyethylene sorbitan monooleate (Tween 80) solution (Ribi et al., 1958) has the advantage of eliminating intact acid-fast bacilli in one centrifugation, the color of the cell-wall preparation turned from yellow to white, and electron microscopy of the preparation gave the impression that the cell wall was covered with amorphous substances. These facts may indicate that some components of the cell wall would be removed by Tween 80 treatment. Therefore, treatment with Tween 80 was omitted, and successive centrifugations, as mentioned below, were carried out to exclude intact cells and cells containing cytoplasmic residues.

The crude cell-wall preparation was washed three times by centrifugation with 1 M NaCl solution, and then twice with distilled water. Smears of this preparation were examined with Ziehl-Neelsen stain; no intact acid-fast bacilli were observed.

The crude cell-wall preparation was washed by centrifugation three times with 0.3% deoxycholate solution or 0.4% dodecyl sulfate solution. These treatments did not alter the fine structure of the cell wall (Fig. 3). Treatment with 0.2% trypsin in 0.067 M phosphate buffer (pH 8.0) for 3 to 5 hr at 37 C, followed by washing by centrifugation once in phosphate



SCHEME 1. *Procedure used for isolation of purified cell wall preparation.*

buffer and twice in distilled water, also did not affect the appearance of the cell wall, as expected from the results of the previous experiment (Takeya et al., 1961).

According to the results obtained above, the following preparative method was finally employed to obtain a "purified cell-wall preparation." The crude cell-wall preparation was digested with 0.2% trypsin in 0.067 M phosphate buffer (pH 8.0) for 3 hr at 37 C, with continuous stirring. The sediment obtained by centrifugation at 10,000 $\times$ g for 30 min was resuspended by use of a Teflon homogenizer, and washed by centrifugation once with phosphate buffer, three times with 1 M NaCl solution, and then twice with distilled water. The final sediment was suspended in a small amount of distilled water and lyophilized. The yield of the purified cell wall was approximately 150 mg (dry wt) from 50 g (semidry wt) of cells. The color of the dried purified cell wall was yellowish. Electron microscopy of this preparation revealed that cell walls contained little or no cytoplasmic impurities (Fig. 4). Probably because of the high lipid content of the cell walls, they often aggregate and adsorb inorganic impurities. Paired fibrous structures, the presence of which was supposed to have taxonomic significance (Takeya et al., 1958, 1961), are clearly seen on the cell wall (Fig. 5). These fibers, however, were not necessarily so numerous as found on the "ghost cells" obtained by the action of mycobacteriophages or by the treatment of bactericidal drugs, in which the entire area of the cell wall was compactly covered with the fibers (Takeya et al., 1961). Finer fibrillar texture could sometimes be seen underneath the paired fibers.

When cells heated at 100 C for 30 min were used as a starting material, the crude cell-wall preparation, obtained by the same procedure as described above for living cells, was found by electron microscopy to contain many impurities. The impurities, however, could easily be removed by treatment with 0.2% trypsin in 0.067 M phosphate buffer (pH 8.0) for 3 hr at 37 C. Paired fibrous structures were also seen on the cell walls thus obtained (Fig. 6). However, in this experiment, the cell-wall preparation obtained from heat-killed cells was not used for further treatments.

Treatments of cell wall with proteolytic enzymes. The result of trypsin digestion has already been described above. On the other hand, the paired fibers on the cell wall were found to be rather fragmentary when chloroform was added as the preservative to the trypsin solution (one drop per ml), and the cell wall was treated for 24 hr (Fig. 7).

The cell wall was digested with 1,000 μg of pronase per ml of 0.067 M phosphate buffer (pH 7.0) for 3 hr at 37 or 60 C. The pronase digestion failed to alter the appearance of the cell wall, even when 5% alcohol was added to the pronase solution (Fig. 8).

Digestion of the cell wall with nagarse, a crystalline protease produced by *Bacillus subtilis*, was also carried out. The cell wall was digested with nagarse at 10,000 units per ml of 0.067 M phosphate buffer for 3 hr at 37 C. With this treatment, the cell wall became rather thinner, giving an appearance that some constituents of the cell wall were solubilized. A reaction for detecting reducing sugar was positive in the supernatant obtained by nagarse digestion.

Treatment of the cell wall with organic solvents. The cell wall was treated with 95% alcohol, a mixture of acetone and alcohol (1:1, v/v), or a mixture of ether and alcohol (1:1, v/v) for 24 hr at 37 C. The color of the cell wall treated with these solvents turned to white, but the appearance as revealed by the electron microscope was not changed.

On the other hand, as mentioned before (Takeya et al., 1961), treatment with alcoholic KOH solution succeeded in removing the paired fibrous structures on the cell wall, leaving a thin layer of the cell wall (Fig. 9). The method of treatment employed in this experiment was as follows: 380 mg of cell wall were suspended in 280 ml of 0.5% alcoholic KOH solution and incubated for 48 hr at 37 C. After incubation, the suspension was centrifuged at 26,000 $\times$ g for 30 min, and the sediment was washed by centrifugation twice with 95% alcohol and then twice with distilled water. The remaining thin layer of the cell wall was tentatively designated the "basal layer" of mycobacterial cell wall. The final yield of the basal layer was approximately 80 mg, and the color was white. The surface of the layer appeared to be rather rough.

Paired fibrous structures of the cell wall also appeared to be removed by chloroform treatment. After treatment with chloroform for 24 hr at 37 C, the suspension was centrifuged for

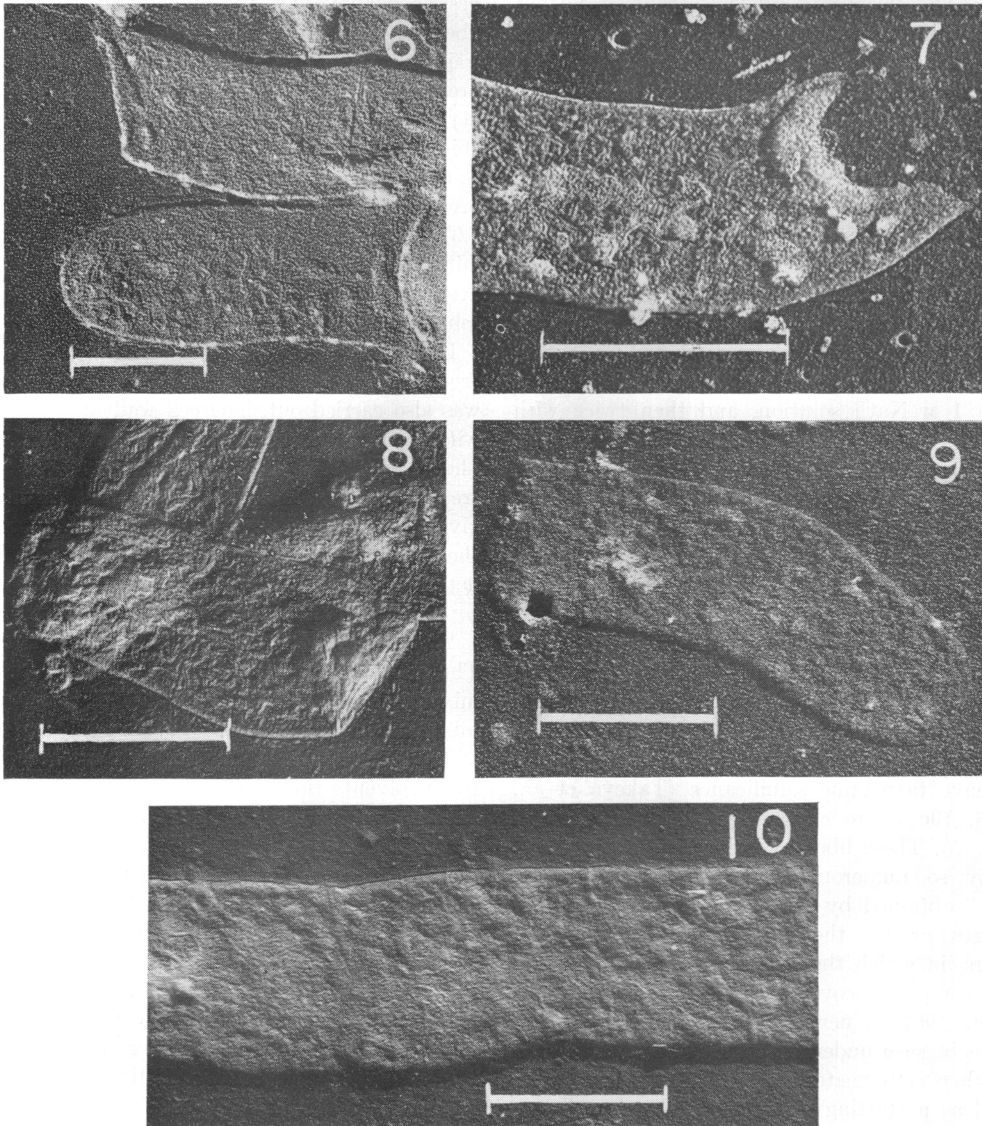


FIG. 6. *Cell walls obtained from heat-killed cells.* × *35,000. Magnification mark = 0.5 μ in all figures.*

FIG. 7. *Cell wall treated with trypsin containing chloroform. The paired fibers appear to become fragmentary.* × *65,000.*

FIG. 8. *Cell walls treated with pronase in 5% alcohol. The appearance of the cell wall is not changed.* × *48,000.*

FIG. 9. *Cell wall treated with alcoholic KOH solution. Paired fibrous structures are removed. The remaining thin layer still preserves original shape of the cell wall.* × *46,000.*

FIG. 10. *Cell wall treated with chloroform. Paired fibrous structures are removed. The remaining layer is thicker than the layer obtained by the treatment with alcoholic KOH solution.* × *48,000.*

30 min at 1,000 × *g*. The lower chloroform layer was aspirated out, and the remaining treated cell wall was washed by centrifugation twice with 95% alcohol and then twice with distilled water. Paired fibrous structures were removed by this treatment (Fig. 10), but treated cell walls appeared to be rather thicker than the basal layer obtained from alcoholic KOH treatment.

Treatment with lysozyme. The cell walls were

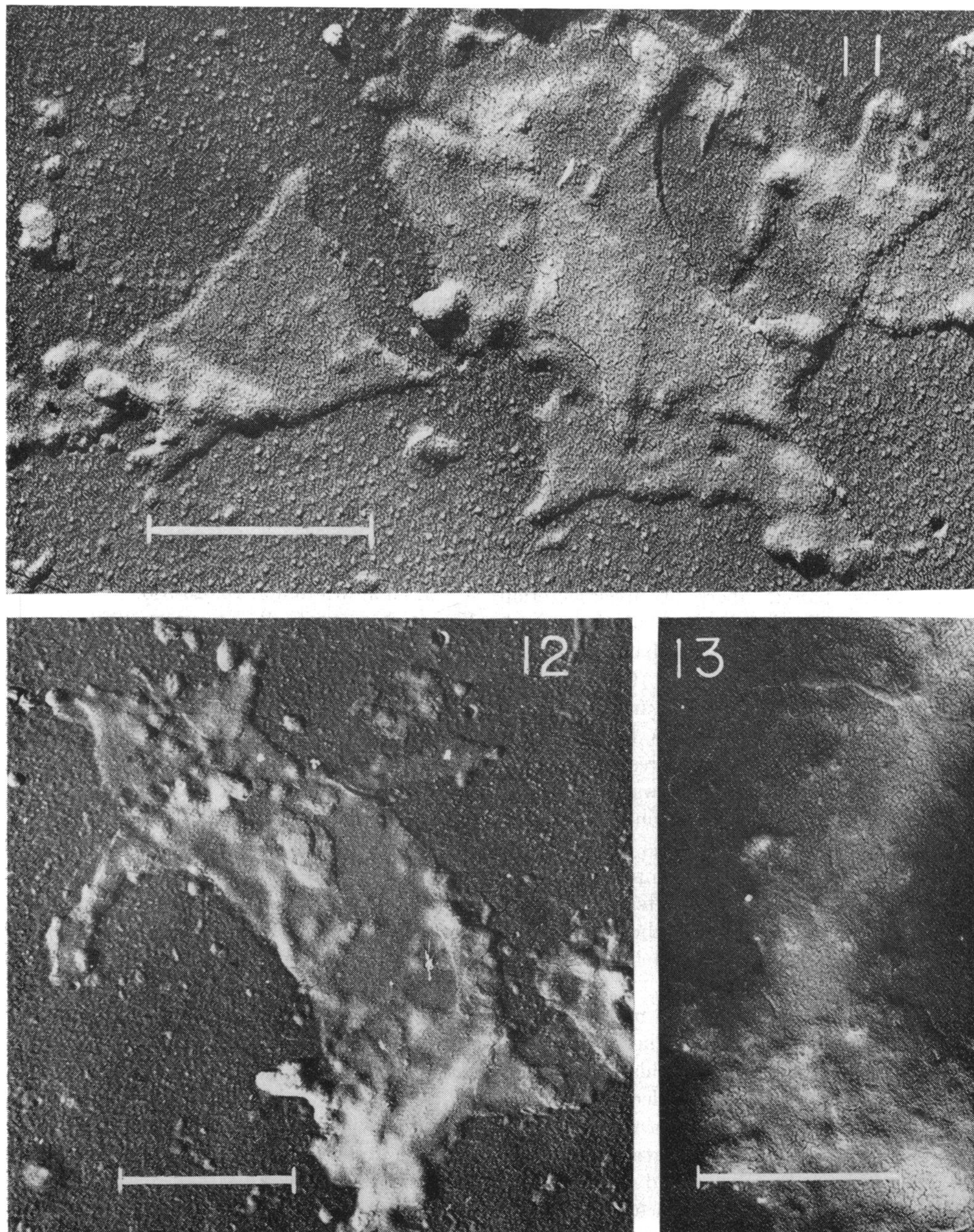


FIG. 11 and 12. *Cell walls digested by lysozyme. The cell walls seem to have lost their rigidity and tend to be amorphous.* Fig. 11, × *60,000;* Fig. 12, × *48,000. Magnification mark = 0.5 μ in all figures.*

FIG. 13. *Cell walls treated with chloroform and then digested by lysozyme. The cell walls have become completely amorphous masses.* × *56,000.*

treated with 1,000 μg of crystalline egg lysozyme per ml of 0.067 M phosphate buffer (pH 7.0) containing 0.01% $MgCl_2$ for 3 hr at 37 C, under continuous shaking. After incubation, the treated cell walls were washed in the same way as described for trypsin treatment. The lysozyme-treated cell wall appeared to lose its own rigidity and tend to be rather amorphous (Fig. 11 and 12). The supernatant obtained after treatment was found to contain Elson-Morgan

reaction-positive substance and reducing sugars. This indicates that the cell-wall mucopeptide was degraded by lysozyme digestion.

Essentially the same kinds of results were obtained by the lysozyme digestion of chloroform-treated cell wall. In this case, the digested cell walls appeared to turn into entirely amorphous masses (Fig. 13).

Discussion

The "paired fibrous structures" found on the cell wall obtained in this experiment were resistant to treatment with trypsin, pronase, alcohol, alcohol-acetone and alcohol-ether mixture, but were destroyed by treatment with alcoholic KOH and chloroform. These results indicate that the main component of the paired fiber is lipidic in nature. Fine fibrils found on the cell surface of *Streptomyces violaceoruber* by Hopwood and Glauert (1961) had dimensions similar to the paired fibers of mycobacterial cell wall. Although the presence of the paired fibers was first considered to be characteristic to *Mycobacteriaceae* (Takeya et al., 1961), it may be found to be a common structure in *Actinomycetales*, depending upon results from further investigations. The thin layer resulting from alcoholic KOH treatment of cell walls will coincide with the "cell-wall preparation" chemically analyzed by Cummins and Harris (1958). This layer, however, is obviously a component of the cell wall, as seen from the results obtained in this experiment. Accordingly, the layer was tentatively designated the "basal layer" of the mycobacterial cell wall.

Protoplasts or spheroplasts of mycobacterial cells have not been obtained hitherto by lysozyme treatment. However, in this study, the mycobacterial cell wall was revealed by electron microscopy to lose its rigidity and become amorphous after lysozyme treatment. Moreover, chemical analysis disclosed that Elson-Morgan reaction-positive substances and reducing sugars were split off in the supernatant of the lysozyme-treated cell-wall suspension. Therefore, it is assumed that the main component of the outer layer of the mycobacterial cell wall is composed of material insensitive to lysozyme, while the inner layer is sensitive to the enzyme and is responsible for the shape and rigidity of the cell wall, as reported in the case of the cell walls of gram-negative bacteria. The basal layer seems to correspond to the inner rigid layer of the cell wall of *Escherichia coli* reported by Weidel, Frank, and Martin (1960). The data to confirm this are presented in the accompanying paper (Takeya, Hisatsune, and Inoue, 1963).

Acknowledgments

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