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# METHICILLIN-INDUCED LYSOZYME-SENSITIVE FORMS OF STAPHYLOCOCCI[1]

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## ABSTRACT

ALDRICH, K. M. (The University of Kansas, Lawrence), AND C. P. SWORD. Methicillin-induced lysozyme-sensitive forms of staphylococci. J. Bacteriol. **87**:690–695. 1964.—*Staphylococcus aureus* and *S. epidermidis* grown in the presence of sublethal amounts of methicillin were converted to enlarged spheres within 2 to 4 hr, as shown by phase microscopy, Gram stain, and electron microscopy. Addition of lysozyme to cells incubated in the presence of methicillin, and to methicillin-induced spheres suspended in hypotonic saline, caused lysis of methicillin-treated cells but not of untreated cells.

Staphylococci are not easily lysed by mechanical or chemical means (Marmur, 1961). Penicillin inhibits mucopeptide and cell-wall synthesis of *Staphylococcus aureus* (Park and Strominger, 1957) and can cause formation of osmotically fragile cells from *Escherichia coli* (Lederberg, 1956). Methicillin (dimethoxyphenyl penicillin) also inhibits mucopeptide synthesis in staphylococci (Rogers and Jeljaszewicz, 1961). Egg-white lysozyme depolymerizes polysaccharides in the mucopeptide portion of the cell wall and results in lysis of sensitive organisms such as *Micrococcus lysodeikticus* and *Bacillus megaterium* (Salton, 1953; Brumfitt, Wardlaw, and Park, 1958). Osmotically fragile bodies of *Streptococcus faecalis* var. *liquefaciens* have been produced by combined treatment with penicillin and lysozyme (Bleiweis and Zimmerman, 1961).

This study was initiated to explore the possibility of forming osmotically fragile staphylococci to facilitate cell fractionation and extraction of nucleic acid.

[1] A preliminary report of these data was presented at the 63rd Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, 5–9, May 1963.

## MATERIALS AND METHODS

*Cultures. Staphylococcus epidermidis* M47SR was originally isolated from a patient with a mixed *Klebsiella* and staphylococcus infection; *S. aureus* M71SR was isolated from a human carbuncle (Shikashio, 1962).

*Determination of sublethal amount of methicillin.* Sensitivity of each staphylococcus strain to methicillin was determined by the tube dilution method. Volumes (2 ml) of twofold serial dilutions of methicillin in Penassay (PA) Broth ranging from 100 to 0.012 μg/ml were inoculated with 0.1 ml of a $10^{-3}$ dilution of an 18-hr culture. After incubation at 37 C for 24 hr, the highest concentration of antibiotic in which growth occurred was called the sublethal end point. This concentration of methicillin was used for staphylococcus sphere formation.

*Production of methicillin-induced spheres.* A modification of Lederberg's (1956) procedure for *E. coli* spheroplast induction by penicillin was followed. Portions (10 ml) of an 8- to 10-hr PA broth culture were added to 30 ml of PA broth containing methicillin to give a final concentration of the predetermined sublethal amount (0.78 μg/ml). Cultures were incubated at 37 C. When a larger quantity of cells was needed, volumes were increased according to a ratio of one part culture to three parts PA-methicillin broth. Formation of spheres was followed by phase microscopy, Gram stain, and optical density. When optical density was followed, cultures were incubated in a set of specially designed side-arm Erlenmeyer flasks. Standardized Pyrex cuvettes (18 × 125 mm) were fused onto 250-ml Erlenmeyer flasks, approximately 1 in. from the bottom in a horizontal position. Optical density was read hourly at 620 mμ on a Coleman model 14 Universal spectrophotometer.

*Addition of lysozyme to cultures of methicillin-induced spheres.* Spheres were prepared in side-arm flasks containing PA broth supplemented

with methicillin. Controls contained no methicillin. At time zero and after exposure to methicillin for 1, 2, 3, and 4 hr, sterile lysozyme (0.025 mg/ml, final concentration; egg-white, three times crystallized; Calbiochem) was added to each flask, and optical densities were read periodically.

*Electron microscopy.* After 4 hr of incubation in PA broth and prior to lysozyme addition, samples were taken from control cells (no methicillin) and from cells exposed to methicillin, and prepared for electron microscopy. After an additional 2 hr of incubation, samples were taken from control cells with lysozyme but without methicillin and from methicillin- and lysozyme-treated cells. Samples of cell suspensions were placed on specimen screens with carbon membranes. The specimens were allowed to air-dry, washed once with distilled water, air-dried, and shadowed at a 4:1 angle with a platinum-palladium (80:20) mixture. Micrographs were taken with an RCA EMU-3F2 electron microscope at an accelerating voltage of 50 kv.

*Lysis of methicillin-induced spheres by lysozyme in hypotonic saline-citrate.* Flasks containing 200 ml of spheres and flasks containing control cells without methicillin were prepared. After 4 hr of incubation at 37 C, the methicillin-induced spheres and control cells were sedimented at 1,300 × *g* for 30 min at 4 C, washed once in 0.05 M saline-citrate, and resuspended in 50 ml of 0.05 M saline-citrate (pH 7). Portions (10 ml) of each stock suspension were added to flasks containing 30 ml of saline-citrate and tenfold dilutions of lysozyme to give final concentrations from 0.25 to 0.00025 mg/ml. Controls contained no lysozyme. During incubation at 37 C, 7-ml samples were removed, and optical densities read hourly at 600 mμ.

## RESULTS

*Formation of methicillin-induced staphylococcal spheres.* Optical density changes and phase microscopy were used to follow the formation of spheres. Phase microscopy showed that cells exposed to a sublethal amount of methicillin for 2 hr became enlarged, occurred in pairs, small clusters, or singly, and were less refractile than normal cells. A decrease in number of spheres and the presence of ghosts and debris indicated lysis. Lysis observed with phase microscopy correlated with decreases in optical density. In contrast to methicillin-treated cells, cells not exposed to methicillin were of normal size, grouped in grape-like clusters, and highly refractile. Gram stains also revealed that methicillin-treated cells were larger than untreated cells. The majority of treated cells retained the ability to stain gram-positive, although some gave intermediate or negative reactions. When lysis occurred, gram-negative debris could be seen. Optical densities of methicillin-treated cultures differed from those of untreated cells (Fig. 1). Maximal optical densities and associated cellular enlargement indicated that the peak yield for sphere production was usually between 3 and 6 hr after methicillin exposure. Decreases in optical densities and lysis of spheres began about 6 hr after methicillin exposure with *S. epidermidis*, and at 4.5 hr with *S. aureus*. Maximal lysis was not observed microscopically or spectrophotometrically until 7 or 8 hr after exposure.

*Lysis of methicillin-induced spheres.* Although gram-positive cocci such as *M. lysodeikticus* and other micrococci are sensitive to lysozyme, staphylococci are generally not sensitive. However, staphylococci exposed to methicillin for several hours were rendered lysozyme-sensitive. Since maximal sphere formation occurred about 4 hr after incubation with methicillin, this time was selected to expose the spheres to lysozyme. Figure 1 illustrates the effect of lysozyme on cultures of methicillin-treated *S. epidermidis*. After incubating cells with methicillin for 4 hr, the addition of lysozyme caused rapid lysis of methicillin-treated cells but not of untreated cells. Experiments with *S. aureus* produced similar results.

Figure 2A shows untreated *S. aureus* cells at 4 hr. This field is representative and shows intact cells with a few cells that appear to be undergoing lysis. Figure 2B shows control cells which were treated at 4 hr with lysozyme for an additional 2 hr. The cells are of normal size, and lysis has not noticeably increased over that of untreated cells. Figure 2C shows cells treated with methicillin for 4 hr. The cells are enlarged in comparison with the untreated cells. Some lysis can be seen at 4 hr, although the rate, compared with controls, is not high. The spheres maintained this approximate level of integrity for an additional 2 to 3 hr, as shown by optical density determinations, phase microscopy, and Gram staining. Figure 2D shows cells that were treated for 4 hr with methicillin and subsequently exposed to

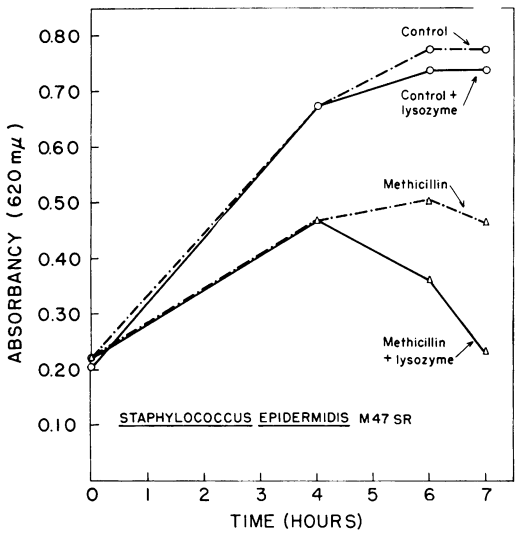


FIG. 1. *Addition of lysozyme to 4-hr cultures of methicillin-treated and untreated Staphylococcus epidermidis.*

lysozyme for 2 hr. Cells treated with both methicillin and lysozyme show increased lysis in comparison with methicillin-treated cells or lysozyme-treated cells. Similar results were obtained for *S. epidermidis*.

The period of onset of lysozyme sensitivity of cells exposed to methicillin was investigated. Figure 3 shows the effect of adding lysozyme at varying intervals to cells incubated in PA broth containing methicillin. While the results with *S. epidermidis* and *S. aureus* differ slightly, initial sensitivity to lysozyme was evident between 1 and 2 hr of methicillin treatment, and optimal lysozyme effect was achieved after 4 hr of exposure to methicillin. The onset of sensitivity to lysozyme correlated with the onset of enlargement of cells, as observed by phase microscopy.

*Lysis by lysozyme in hypotonic saline-citrate.* When methicillin-induced spheres (after 4 hr of methicillin exposure) were centrifuged and resuspended in a hypotonic solution, such as 0.05 M saline-citrate (pH 7) or distilled water, appreciable lysis was not observed. However, when methicillin-treated cells were incubated with lysozyme in hypotonic saline-citrate, lysis was increased. Figure 4 illustrates the effect of varying amounts of lysozyme on methicillin-treated *S. epidermidis* suspended in 0.05 M saline-citrate (pH 7). Methicillin-treated cells showed a greater drop in optical density after addition of lysozyme than did methicillin-treated controls. Untreated cells and cells exposed only to lysozyme showed little lysis in comparison with cells treated with both methicillin and lysozyme. Similar experiments with *S. aureus* yielded comparable results.

## DISCUSSION

Formation, enlargement, and sensitivity to lysozyme of methicillin-induced spheres can be correlated with observations by other workers. Enlargement of the staphylococci, observed by phase and electron microscopy and Gram stains, was noticed about 2 hr after methicillin exposure and reached its peak after 4 hr. Similarily, Suganuma (1962) observed enlargement of cells and thin cell walls in electron micrographs of 1- to 2-hr penicillin-treated *S. aureus*. Strominger (1962) reported that accumulation of uridine nucleotides (cell-wall and mucopeptide precursor) after penicillin addition was an early and striking effect. Half-maximal accumulation occurred about 15 min after penicillin addition; maximal accumulation occurred after 2 hr. This correlates with the observed enlargement and optical density changes in methicillin-induced spheres. Enlargement of methicillin-induced spheres is probably a reflection of interrupted cell-wall biosynthesis. The cell wall is obviously altered because the cell has (i) swollen, probably due to loss of rigidity of the cell wall, and (ii) lost its resistance to lysozyme. Apparently there is enough cell wall present, although modified, to prevent the cell from lysing in hypotonic solutions. To what extent the cell wall retains its rigidity and structure after methicillin treatment is unknown.

The nature of the lysozyme action on methicillin-treated cells has not been determined. Lysozyme sensitivity was first detected spectrophotometrically 1 to 2 hr after methicillin exposure, and optimal lysozyme effect occurred after 4 hr of exposure. This onset of sensitivity is probably a reflection of cell-wall changes observed in staphylococci which had been exposed to methicillin for 2 hr. The electron micrographs provide additional evidence that methicillin-treated cells are rendered sensitive to lysozyme. Possibly pretreatment with methicillin causes a rearrangement or alteration of the cell wall such that lysozyme-sensitive linkages are formed or made available. Bleiweis and Zimmerman (1961) reported that lysozyme-resistant *Streptococcus faecalis* var.

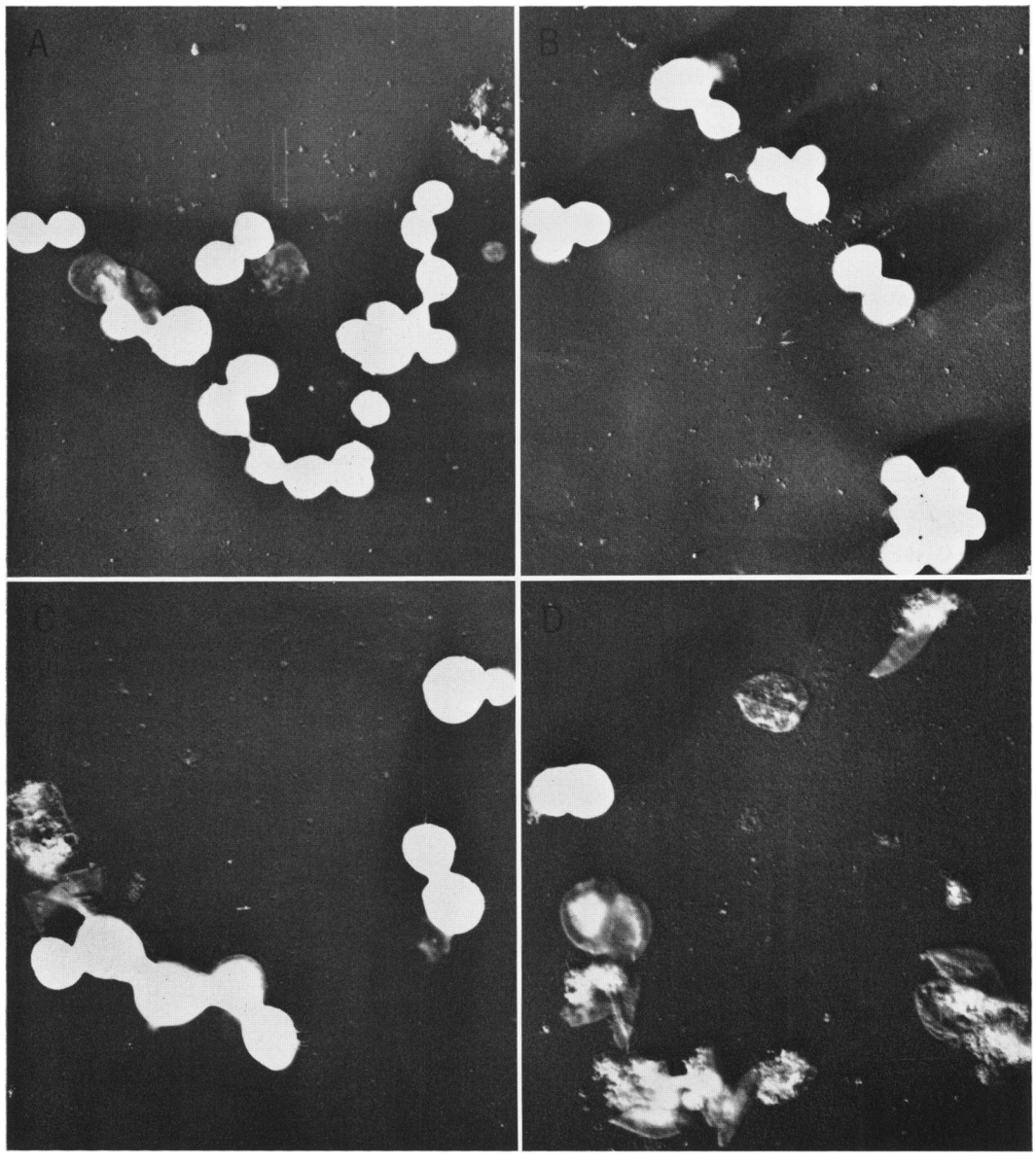


FIG. 2. *Electron micrographs of Staphylococcus aureus. Magnification, ×14,000. (A) Untreated cells. (B) Lysozyme-treated cells. (C) Methicillin-treated cells. (D) Cells treated with both methicillin and lysozyme.*

*liquefaciens* was rendered sensitive to lysozyme by penicillin treatment. Susceptibility or resistance to lysozyme has been correlated with absence or presence, respectively, of cell-wall teichoic acid (McQuillen, 1960). Cell walls of *B. megaterium* and *M. lysodeikticus*, lacking teichoic acid, are solubilized by lysozyme; cell walls of *B. subtilis* and *S. aureus*, which contain teichoic acid, show varying degrees of lysozyme resistance. Removal of teichoic acid from isolated cell walls of *S. aureus* Copenhagen rendered resistant walls sensitive to lysozyme (Mandelstam and Strominger, 1961). Methicillin is known to inhibit cell-wall mucopeptide synthesis (Rogers and Jeljaszewicz, 1961), but it is not known whether it inhibits synthesis or incorporation of teichoic acid into the cell-wall

polymer. On the basis of reports by other workers and our experimental data, it appears that methicillin inhibits a cell-wall component(s), possibly teichoic acid, and thereby renders the cell sensitive to lysozyme.

Staphylococci are difficult to lyse by the procedures commonly used for other bacterial species, and mechanical means of cell disruption may degrade deoxyribonucleic acid (DNA; Marmur, 1961). The combined methicillin-lysozyme treatment facilitates staphylococcal lysis, while avoiding the dangers associated with mechanical disruption. Additional data not presented here show that this procedure offers a means of extracting more highly polymerized DNA in greater yield than from the same quantity of untreated cells.

These studies also indicate the possibility of a combined effect of low levels of therapeutic penicillin (or derivatives) and lysozyme from host cells in overcoming infections. The significance of lysozyme as a host defense factor is obscure. However, it is known that leukocytes and serum from rabbit, guinea pig, and man contain lysozyme among their bactericidal and bacteriostatic substances (Myrvik and Weiser, 1955; Amano et al., 1956; Muschel, Carey, and Baron, 1959). The formation of osmotically fragile bodies from gram-negative organisms such as *E. coli* and *Salmonella typhosa* by fresh guinea pig serum containing specific antibody, complement, and lysozyme, and by combined use of polymyxin and guinea pig serum lysozyme was reported by Muschel et al. (1959). Sublethal or low levels of penicillin or methicillin might also render staphylococci sensitive to lysozyme in serum or leukocytes, and result in the formation of osmotically fragile bodies susceptible to lysis.

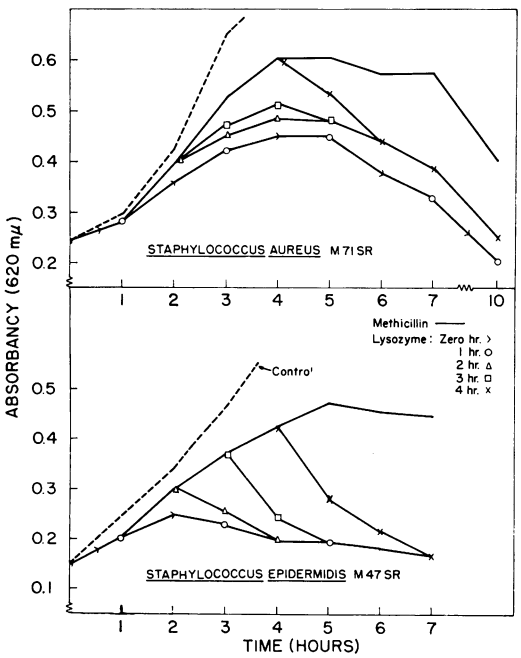


FIG. 3. *Onset of lysozyme sensitivity of methicillin-treated cells.*

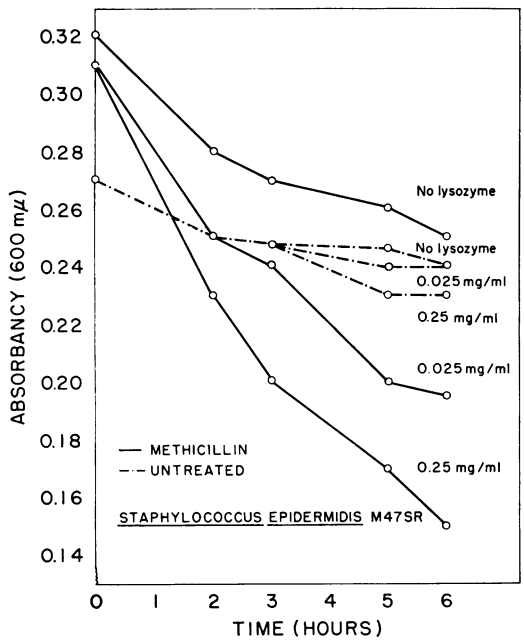


FIG. 4. *Effect of lysozyme on methicillin-treated cells suspended in hypotonic saline-citrate.*

## ACKNOWLEDGMENT

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