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In vitro Observations of the Mode of Action of Streptomycin.*

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Despite wide clinical interest in the use of streptomycin, the mode of action of this substance on bacteria is as yet poorly understood. The present report deals with 3 aspects of the action of streptomycin on bacteria *in vitro*: (1) morphological effects of streptomycin on growing cultures of various organisms; (2) the bactericidal action of streptomycin on resting bacteria; and (3) the effect on the rate of growth of bacteria of subinhibitory concentrations of streptomycin.

Effect of Streptomycin on Morphological Appearance of Bacteria. Little attention has been directed toward possible alterations in bacterial morphology as a result of growth in the presence of streptomycin. Welch, Price, and Randall[1] observed huge and bizarre forms of *E. typhosa* in cultures exposed to streptomycin. However, Miller and Bohnhoff[2] found no morphological changes in meningococci or gonococci grown in the presence of streptomycin.

In the present study, a single stock laboratory strain of each of the following organisms was employed: *Aerobacter aerogenes, Escherichia coli, Proteus ammoniae, Pseudomonas aeruginosa, Shigella sonnei, Eberthella typhosa, Salmonella typhimurium, Vibrio comma, Micrococcus tetragenus, Staphylococcus aureus, Bacillus anthracis, Bacillus mycoides, Bacillus subtilis,* and *Bacillus megatherium.* Preliminary tests were performed in heart infusion broth by the serial dilution technic to determine the range of sensitivity of each organism to streptomycin. The degree of bacteriostasis was estimated by the presence or absence of turbidity after 24 hours' incubation. In the final tests, 10^{-2} dilutions of overnight growths of organisms in broth were incubated at 37°C in heart infusion broth in the presence of various amounts of streptomycin, which, on the basis of the preliminary study, were expected to be slightly, moderately, or strongly bacteriostatic. Replicate tubes were prepared for each organism with the several streptomycin concentrations, as well as control tubes. At 0, 3 and 5 to 6 hours' incubation, the tubes were centrifuged for 20 minutes and Gram-stained smears of the bacterial sediment prepared. In each instance, a streptomycin-free culture was smeared and stained on the same slide as the streptomycin-containing culture. The stained smears were examined independently by 2 observers, one of whom was unaware of the purpose or technic of the study.

The effect of streptomycin on the size, shape and staining qualities of bacteria was variable, being marked with some Gram-negative bacilli, slight with Gram-positive cocci, and absent with Gram-positive bacilli. The most extreme alterations in appearance were found with the strain of *Shigella sonnei.* Cells of this organism, when exposed to streptomycin, became elongated (up to 3 or 4 times as long as the control cells), appeared swollen, and stained irregularly. (Fig. 1). These alterations were more pronounced with a slightly bacteriostatic amount of streptomycin (1 γ per ml) than with a higher, more strongly bacteriostatic concentration (5 γ per ml). Cells of changed appearance were somewhat more frequent after 6 hours' incubation than after 3 hours.

Cells of the strain of *S. typhimurium,* when exposed to streptomycin, became swollen and elongated (up to 2 or 3 times the normal length), and assumed coccoid forms. These changes were equally prominent after 3 and 6 hours' incubation. The proportion of altered cells to the total was greater with

* This investigation was supported by grants from the Lederle Laboratories Division, American Cyanamid Company, and from the United States Public Health Service.

1 Welch, H., Price, C. W., and Randall, W. A., *J. Am. Pharm. Assn.*, 1946, **35**, 155.

2 Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 485.

FIG. 1.

Shigella sonnei (× 675), Gram-stained smears after 6 hours' incubation at 37°C in heart infusion broth. Left: In the presence of streptomycin, 1.0 γ per ml. Right: Control, without streptomycin.

slightly bacteriostatic concentrations of streptomycin (3 and 6 γ per ml) than with a strongly bacteriostatic concentration (12 γ per ml).

Definite, but lesser, degrees of alteration in cellular appearance were observed with the strains of *Aerobacter aerogenes* and *Proteus ammoniae*. In both instances incubation with streptomycin for either 3 or 6 hours resulted in a greater proportion of moderately elongated and swollen cells than were observed in control cultures incubated in the same manner without streptomycin. Again the changes were somewhat more frequent in slightly bacteriostatic amounts of streptomycin (1 γ and 3 γ per ml) than in more strongly bacteriostatic concentrations (6 γ and 12 γ per ml).

The other Gram-negative organisms exhibited little or no alteration in appearance. *Vibrio comma* appeared somewhat more pleomorphic in the presence of streptomycin than in its absence. No consistent changes were observed in the appearance of *Escherichia coli, Eberthella typhosa,* and *Pseudomonas aeruginosa.*

A few swollen and deeply-stained cells of *Staphylococcus aureus* and *Micrococcus tetragenus* were found in cultures containing streptomycin but such cells were also seen with nearly the same frequency in control cultures. The Gram-positive bacilli studied exhibited no consistent alterations in shape, size, or staining as a result of incubation with

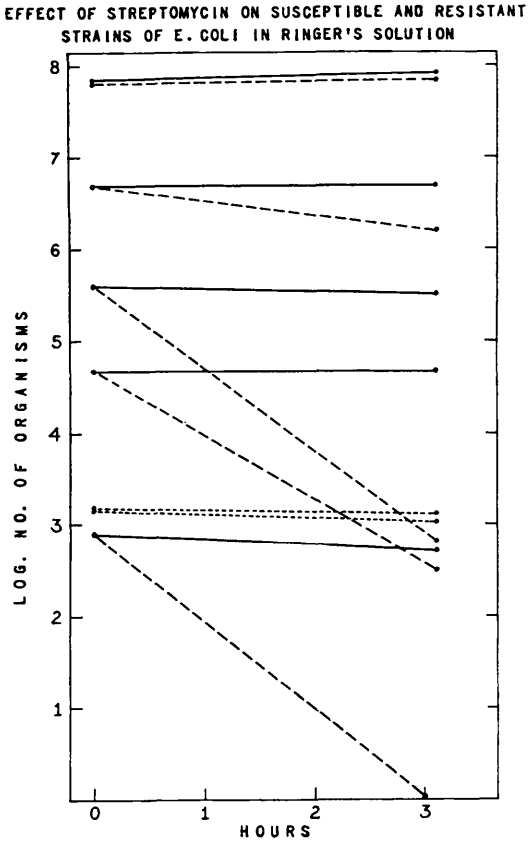

FIG. 2.

Bactericidal action of streptomycin on sensitive and resistant strains of *E. coli* suspended in Ringer's solution at 37°C. Solid lines: Sensitive strain, without streptomycin. Broken lines: Sensitive strain, with streptomycin, 25 γ per ml. Dotted lines: Resistant strain, without streptomycin, and with streptomycin 25 γ per ml.

streptomycin.

Bactericidal Action of Streptomycin on Resting Bacteria. Hamre, Rake, and Donovick[3] reported that streptomycin was bactericidal for *K. pneumoniae* in Ringer's solution. The effect was related to the concentration of streptomycin employed. A greater concentration was required for bactericidal action when the bacterial cells were in the resting state than when they were actively growing. Resting cells surviving 3 or 5 hours' incubation with streptomycin subsequently were not found more resistant than controls. It was suggested, therefore, that the mode of action of streptomycin on washed, resting cells is different from its action on multiplying organisms. Klein and Kimmelman[4] also noted that streptomycin was bactericidal for washed suspensions of *Shigellae* and that the survivors did not show increased resistance. The present studies were undertaken to describe more fully the bactericidal action of streptomycin on resting bacteria and to ascertain whether or not its mode of action under these circumstances differed from its action on multiplying organisms.

A stock laboratory strain of *E. coli* was used. The sedimented organisms from a 6-hour growth in heart-infusion broth were washed twice in Ringer's solution (pH 7.0), and resuspended in Ringer's solution after dilution to the desired bacterial concentrations. Streptomycin in Ringer's solution was added to some of the tubes and the volumes of both control and drug-containing tubes were adjusted. Incubation was carried out in a water bath at 37°C for 3 hours. Explants were made at 0 and 3 hours in a simple synthetic medium[5] to which were added agar, glucose (1%), Bacto tryptose (1%) and sodium thioglycolate (0.05%). Preliminary tests indicated that in this medium concentrations of streptomycin up to 5 γ per ml had no inhibitory effect on the number of developing colonies as compared with controls. In heart-infusion broth assays, the strain was inhibited by 1.6 γ per ml or less of streptomycin.

The results of one set of experiments are shown in Fig. 2. The bactericidal action of streptomycin was related, in general, to the concentration of organisms. Bacterial suspensions of 1000 per ml or less were sterilized in 3 hours by 25 γ per ml of streptomycin; suspensions of 10 million or more cells per ml were not affected by this concentration of streptomycin in the same time interval. Intermediate numbers of organisms were affected proportionately. The activity of 50 γ per ml of streptomycin was greater and that of 10 γ per ml was correspondingly less than the effects indicated with 25 γ per ml. The amount of streptomycin required to sterilize a resting suspension of *E. coli* within 3 hours was approximately 25 times as great as the bactericidal concentration for a similar number of organisms incubated in heart-infusion broth.

An attempt was made to increase the resistance of *E. coli* by repeated exposure of resting organisms to sublethal concentrations of streptomycin. After growth in heart-infusion broth the organisms were washed twice, resuspended in Ringer's solution containing 10 γ per ml of streptomycin, and incubated for 3 hours at 37°C. A loopful of the bacterial suspension was then streaked on an agar plate. After incubation the growth from the agar was again washed and resuspended for 3 hours in Ringer's solution containing 10 γ per ml of streptomycin. This procedure was carried out through 12 passages. A control strain was treated in an identical manner except for the omission of streptomycin. At the completion of the series, both strains were tested for resistance to streptomycin. Neither exhibited any increased resistance to the bactericidal action of streptomycin, as compared with unexposed control strains, when tested in the resting state or when incubated in heart-infusion broth. These results, which are in agreement with the reports of others, suggest a difference in the mode of action of streptomycin on resting and on multiplying organisms.

To further elucidate the mechanism of the mode of action of streptomycin, observations

[3] Hamre, D., Rake, G., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 25.

[4] Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

[5] Sahyun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Infect. Dis.*, 1936, **58**, 28.

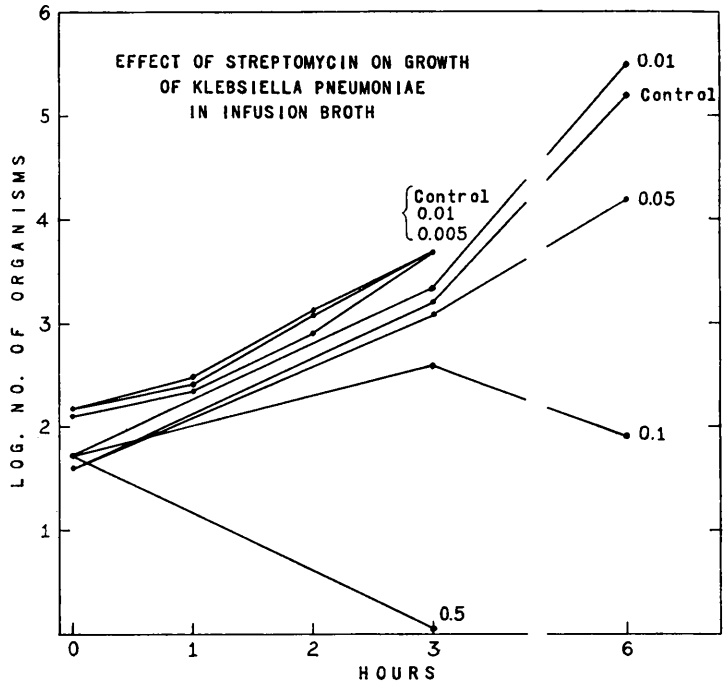

FIG. 3.

Rate of growth of *K. pneumoniae* in heart-infusion broth at 37°C in presence of bactericidal, bacteriostatic, and subinhibitory concentrations of streptomycin. Numbers indicate γ per ml of streptomycin.

were made of the behavior of a strain of bacteria made resistant to streptomycin and subsequently exposed in the resting state to the antibiotic. Accordingly, the stock strain of *E. coli* was made resistant by repeated passage in increasing concentrations of streptomycin in tryptose-phosphate broth. After 12 daily passages, the organisms grew freely in 150 γ per ml of streptomycin. The resistant organisms were then suspended in Ringer's solution and exposed to streptomycin by the procedure already described. A representative result is shown in Fig. 2. This strain was completely resistant to 25 γ per ml of streptomycin—an amount of streptomycin which was bactericidal for a similar number of nonresistant organisms. The mechanism of bacterial resistance to chemotherapeutic agents is presumed to be intimately related to the mechanism of the antibacterial action of such drugs. These results, therefore, suggest that the mode of action of streptomycin on nonmultiplying organisms is essentially similar to its action on multiplying organisms.

Effect of Subinhibitory Concentrations of Streptomycin on Rate of Growth. On the basis of *in vivo* studies in mice, the suggestion was advanced that subinhibitory concentrations of streptomycin might have a stimulatory effect on the rate of growth of *E. typhosa*.[1] This possibility was investigated *in vitro* using a stock strain of *K. pneumoniae*. The organisms were grown overnight in heart-infusion broth, suitably diluted, and inoculated in broth containing various amounts of streptomycin. Explants in tryptose-phosphate agar were made at intervals after incubation at 37°C, and the subsequent growth of colonies counted. The results of 2 experiments are shown in Fig. 3. With this strain of bacteria, 0.5 γ per ml of streptomycin was bactericidal in 3 hours, and 0.1 and 0.05 γ per ml were moderately or slightly bacteriostatic. Lesser concentrations of streptomycin did not appreciably modify the rate of growth of the organisms as compared with the drug-free controls. Colony counts

on explants made at hourly intervals during the early phases of growth likewise yielded no evidence of an initial stimulus to the rate of growth from the presence of subinhibitory concentrations of streptomycin.

Comment. The morphological changes in bacteria resulting from streptomycin appear to be less marked than those which occur with exposure to penicillin.[6-9] Like the latter, however, they occur with concentrations of streptomycin which are only slightly bacteriostatic. Alterations in morphology are not regularly associated with bacteriostasis. The growth of all of the organisms studied was inhibited by streptomycin, in concentrations varying between 1 and 50 γ per ml, but only a few strains exhibited abnormalities in size and shape.

Because of its pronounced effect on bacterial morphology, as well as its failure to sterilize nonmultiplying cultures, penicillin is presumed to interfere directly with bacterial fission. In the case of streptomycin it is suggested that the observed morphological changes may be secondary effects resulting from disturbances of intermediary metabolism rather than primary effects on fission. Such an hypothesis is also consistent with the observed bactericidal action of streptomycin on resting, nonmultiplying cells.

The failure of resting cells to develop resistance to streptomycin and the greatly increased concentrations of antibiotic required to effect sterilization in the resting state, as contrasted with the multiplying state, suggest that streptomycin may act on a different locus or by a different mechanism under the 2 conditions of study. However, cells made resistant to streptomycin under conditions of growth are also resistant in the resting state. This observation appears to support the view that the mechanism of action of streptomycin on resting and on multiplying cells is fundamentally the same, the observed differences in behavior being accounted for on the basis of differences in the rate of bacterial metabolism.

Summary. 1. Streptomycin in bacteriostatic concentrations produced morphological changes in some Gram-negative bacilli but not in others. Gram-positive cocci were only slightly affected, and Gram-positive bacilli were not affected. 2. Streptomycin was bactericidal for *E. coli* in the resting state, the effect being related both to the size of the inoculum and the concentration of the antibiotic. 3. Strains of *E. coli* resistant to streptomycin under conditions permitting growth were also resistant in the resting state, but exposure of susceptible strains to streptomycin in the resting state did not result in increased resistance. 4. No stimulation of the rate of growth of *K. pneumoniae* was observed *in vitro* by subinhibitory concentrations of streptomycin.

Grateful acknowledgment is made to Elizabeth Watson for assistance with the morphological studies.

6 Gardner, A. D., *Nature*, 1940, **146**, 837.

7 Miller, C. P., Scott, W. W., and Moeller, V., *J. Am. Med. Assn.*, 1944, **125**, 607.

8 Thomas, A. R., and Levine, M., *J. Bact.*, 1945, **49**, 623.

9 Alture-Werber, E., Lipschitz, R., Kashdan, F., and Rosenblatt, P., *J. Bact.*, 1945, **50**, 291.

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Similarities in Electron Micrographs of Purified Lansing and SK Poliomyelitis Virus.

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This note is written to correct the impression which was erroneously given[1] that purified SK poliomyelitis virus consists of asymmetric particles of the type described by

1 Loring, H. S., Marton, L., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 291.