ABSENCE OF A CHLORAMPHENICOL-INSENSITIVE PHASE OF STREPTOMYCIN ACTION

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ABSTRACT

PLOTZ, PAUL H. (Harvard Medical School, Boston, Mass.) AND BERNARD D. DAVIS. Absence of a chloramphenicol-insensitive phase of streptomycin action. J. Bacteriol. **83**:802–805. 1962.—Previous findings have suggested the possibility that the damaging effect of streptomycin on the bacterial cell membrane is only preliminary to an intracellular lethal action. Since the membrane effect requires protein synthesis, a two-stage action might be recognized if the second stage did not require protein synthesis. Indeed, it has been reported that *Escherichia coli* cells, pretreated with streptomycin, are not protected by chloramphenicol from subsequent killing by streptomycin in a nitrogen-free medium. However, we were unable to confirm this report or to observe any chloramphenicol-insensitive phase of streptomycin action in a growth medium. It is, therefore, not yet possible to decide whether the lethal event occurs at the membrane or within the cell.

It has been reported from this laboratory (Anand, Davis, and Armitage, 1960) that growing *Escherichia coli* exhibits an immediate primary uptake of streptomycin, followed after a lag by a gradually increasing secondary uptake. During the first phase the drug damages the growing cell membrane (Anand et al., 1960; Anand and Davis, 1960; Dubin and Davis, 1961) and, subsequently, attaches to an increasing number of intracellular sites that have become freely accessible (Plotz, Dubin, and Davis, 1961). It is not known whether the irreversible, lethal effect of the drug results directly from its action on the membrane or from a subsequent intracellular action.

An approach to this question was suggested by the observation that chloramphenicol, which prevents the lethal action of the drug (Anand and Davis, 1960), also prevents its secondary uptake (Anand et al., 1960). This finding implies that protein synthesis is required for the damage to the membrane that precedes the intracellular uptake. If an intracellular action of the drug is essential for its lethal effect, and if this action does not require further protein synthesis, chloramphenicol might not protect cells that had been damaged by streptomycin but not yet killed. Indeed, this possibility has been supported by the report of Hurwitz and Rosano (1960) that chloramphenicol does not prevent streptomycin-treated cells from being killed by further exposure to streptomycin in a nitrogen-free medium. We have, therefore, tested the protective effect of chloramphenicol under these conditions and also in a growth medium.

MATERIALS AND METHODS

Organisms. *E. coli* ML-35 (cryptic and constitutive for β-galactosidase), kindly furnished by J. Monod, was used since its behavior with streptomycin has been extensively studied in this laboratory. *E. coli* B was used for experiments in nitrogen-free medium since it had been employed by Hurwitz and Rosano (1960).

Media. In the experiments in growth medium, strain ML-35 was inoculated into mineral-citrate medium A (Davis and Mingioli, 1950) with 0.5% glucose. After overnight incubation the culture was diluted 1:100 with fresh medium and the cells were allowed to reach exponential growth before streptomycin was added. All incubations were at 37 C on a New Brunswick rotary shaker with flasks large enough to insure adequate aeration.

In the experiments in a nitrogen-free medium, a tryptic digest tube was inoculated with cells of strain B from an agar slant and allowed to stand overnight at 37 C. In the morning, cells were washed once with water and suspended in 1% NaCl–0.011 M Na lactate solution (Hurwitz and Rosano, 1960) containing appropriate drugs. The suspensions were incubated with shaking at 37 C.

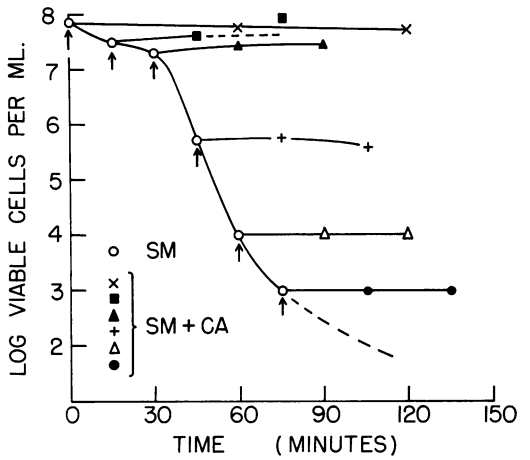

FIG. 1. *Rapid protection by chloramphenicol against killing by streptomycin in growth medium. Streptomycin (30 μg/ml) was added to exponentially growing Escherichia coli ML-35 at 0 time. At intervals the viable count was determined, and 10-ml samples were transferred (arrows) to flasks containing sufficient chloramphenicol (in 0.2 ml water) to yield a final concentration of 20 μg/ml. These flasks were similarly incubated, and samples were removed for viable counts after 30 and 60 min.*

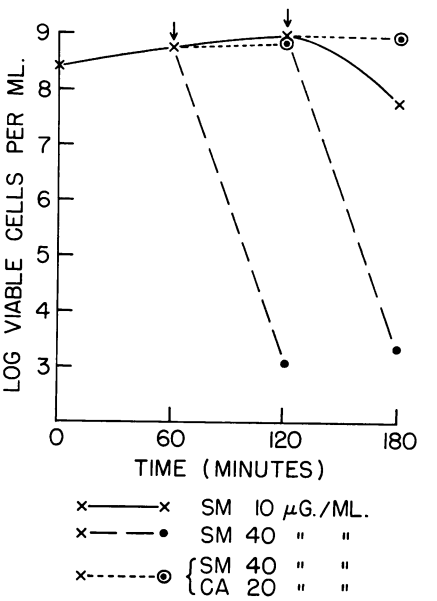

FIG. 2. *Effect of sublethal exposure to streptomycin on subsequent killing and protection. Streptomycin (10 μg/ml) was added to exponentially growing Escherichia coli ML-35 at 0 time. At 1-hr intervals 10-ml samples were transferred (arrows) to two flasks. Both contained sufficient streptomycin to raise the concentration to 40 μg/ml, and one also contained chloramphenicol (final concentration, 20 μg/ml). Viability was determined after 1 hr of further incubation.*

Viable counts. Samples were diluted in medium A without glucose, and 0.1-ml portions were spread immediately on tryptic digest–1% agar plates. The colonies were counted after overnight incubation at 37 C.

Drugs. Streptomycin sulfate (Squibb) and chloramphenicol (Parke, Davis) were used.

RESULTS

Experiments in growth medium. In an attempt to produce streptomycin-damaged but viable cells, a growing culture of strain ML-35 was exposed to streptomycin at a concentration (40 μg/ml) that caused, after a lag, an exponential decline in viability. At various times, portions were removed and incubated further with chloramphenicol. Whether the chloramphenicol was added at the same time as streptomycin, during the lag, or at intervals during the exponential decline, it protected from further killing all survivors at the time of its addition (Fig. 1). These results provide no evidence for the presence at any time of a significant number of damaged cells capable of being killed by further exposure to streptomycin in the presence of chloramphenicol.

It seemed possible that, with this concentration of streptomycin, the transition from membrane damage to lethal intracellular action might be too rapid for detection. Accordingly, cells were exposed to a lower concentration of streptomycin (10 μg/ml), which caused a decline in viable number only after more than 2 hr. During this 2-hr period, the viable number increased about fourfold. Samples were taken at 1 and 2 hr and exposed to a higher concentration of streptomycin (40 μg/ml), with and without chloramphenicol. As Fig. 2 shows, this sublethal pretreatment with streptomycin failed to affect the ability of chloramphenicol to protect the cells from subsequent killing by a higher concentration of streptomycin.

Experiments in nitrogen-free medium. The above results appear to be in conflict with those of Hurwitz and Rosano (1960), who reported that, after nonlethal exposure to streptomycin, *E. coli* was no longer protected by chloramphenicol from the lethal effect of further exposure to

TABLE 1. *Effect of streptomycin (SM) pretreatment on subsequent streptomycin killing and chloramphenicol (CA) protection in lactate solution**

Drug (μg/ml)		Viable cells/ml
CA	SM	
Preincubated with SM		
0	0	11×10^5
0	40	6.0×10^5
30	0	15×10^5
30	40	7.0×10^5
Preincubated without SM		
0	0	18×10^5
0	40	7×10^5
30	0	16×10^5
30	40	12×10^5

* Washed cells of *Escherichia coli B* were suspended in NaCl-lactate solution. Half the suspension was incubated for 1 hr with SM (10 μg/ml), and the rest without the drug. (The viable count after incubation was 47×10^5 in the SM flask and 48×10^5 in the control flask.) Each suspension was divided in two, centrifuged, and washed twice with either distilled water or water containing 30 μg/ml of chloramphenicol (for those cells to be subsequently incubated with it). The cells were resuspended in NaCl-lactate solution, distributed into flasks containing drugs as indicated, and incubated for 2 hr more. Samples were then taken for viable counts, which represent the average of two plates. Comparison should be restricted to the paired samples within a block (with and without streptomycin), which represent identical inocula.

streptomycin. They observed this effect, however, in a nitrogen-free medium. Although it was difficult to see the advantage of testing the effect of chloramphenicol in a medium already incapable of supporting net protein synthesis, their results were so relevant to our problem that we tried to duplicate the experiment as closely as possible. Accordingly, cells in the stationary phase were harvested from an overnight culture in tryptic digest broth, washed with water, and incubated in NaCl-lactate solution with or without drugs. After 1 hr the cells were washed twice with water and suspended again in NaCl-lactate solution with the indicated drugs.

Our results in this solution differed in several respects from those reported by Hurwitz and Rosano (1960). Whereas they observed that, with pretreated cells, 40 μg/ml of streptomycin during the second incubation decreased the survival from about 50% to less than 0.2%, we found only a slight decrease, from 10-40% to 5-20% (Table 1). Furthermore, this slight effect of streptomycin was essentially the same in control cells that had not been pretreated with streptomycin. To be sure, chloramphenicol did not protect against the slight killing effect observed; but the absence of protection was observed equally with cells that had not been pretreated with streptomycin. This pattern stands in marked contrast to the complete protection chloramphenicol provides against the extensive killing by streptomycin in a medium adequate for growth.

DISCUSSION

Hurwitz and Rosano's experiment was carried out in an unbuffered, nitrogen-free medium which cannot support net protein synthesis. Under these conditions, cells die at an appreciable rate even in the absence of drugs. In such an experiment we have observed only a slight, chloramphenicol-insensitive killing by streptomycin; and by adding a drug-free control flask during the first incubation, we have shown that this killing did not depend on pretreatment with a low concentration of streptomycin. It is likely that in this already lethal, unbuffered medium the slight additional killing in the streptomycin-containing flasks proceeds by mechanisms unrelated to those operating under growth conditions. These findings therefore do not appear relevant to the problem of the mechanism of action of streptomycin.

The results of the experiments under growth conditions showed that, after streptomycin treatment of widely varying extent, the survivors can be protected by chloramphenicol from further killing by streptomycin. These findings fail to provide any evidence for a stage in the lethal action of streptomycin that does not require protein synthesis. Three possibilities seem plausible: (i) the primary action on the membrane, which requires protein synthesis, is itself lethal; (ii) a second intracellular step is essential for the lethal action and also requires protein synthesis; or (iii) the second step may not require protein synthesis but may follow so rapidly, after the drug has entered through damaged membrane, that

there is not a detectable population of cells caught between the two stages.

The present results do not narrow down the mechanism of action of streptomycin; but the opposite results, demonstrating a chloramphenicol-insensitive stage of streptomycin action, would have eliminated the possibility that the primary membrane damage is itself the lethal event. This possibility must, therefore, be considered open.

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