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Evidence for a Streptomycin Permease

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ABSTRACT

HURWITZ, CHARLES (Veterans Administration Hospital, Albany, N.Y.), AND CARMEN L. ROSANO. Evidence for a streptomycin permease. J. Bacteriol. **90**:1233–1237. 1965.—The hypothesis that an induced streptomycin permease is required for entry of the antibiotic into cells is further supported by the finding that proflavine and borate, which inhibit transcription, also block the lethal action of streptomycin. Furthermore, if ribonucleic acid (RNA) synthesis is permitted to proceed in the presence of streptomycin and chloramphenicol, and chloramphenicol is then replaced with either proflavine or borate, these inhibitors of transcription no longer block the lethal action of streptomycin. This finding is interpreted to mean that, if induced messenger RNA for streptomycin-permease is formed before transcription is blocked, inhibitors of transcription no longer block the lethal action of streptomycin by preventing formation of the permease.

In previous reports (Hurwitz and Rosano, 1960, 1962*a*, *b*), it has been shown that the chloramphenicol block of the lethal action of streptomycin, and of the entry of labeled streptomycin into sensitive cells, can be circumvented if the cells receive a prior exposure to nonlethal concentrations of streptomycin. To explain this phenomenon, it has been proposed (Hurwitz and Rosano, 1960, 1962*a*) that a streptomycin-induced permease is required to permit passage of the antibiotic past a permeability barrier.

Induction of a permease in present day terminology implies de novo synthesis of specific messenger ribonucleic acid (mRNA) (transcription) followed by the synthesis of specific protein at ribosomal sites (translation). If the hypothesis is correct, chloramphenicol blocks the lethal action of streptomycin by preventing formation of the permease at the ribosomal sites, and circumvention of the chloramphenicol block by a prior exposure to nonlethal doses of streptomycin occurs because permease is synthesized before chloramphenicol is added.

The hypothesis would be greatly strengthened if it could be shown that an inhibitor of mRNA synthesis behaved similarly to chloramphenicol. A block of protein synthesis at the level of transcription should also block the lethal action of streptomycin, since an inducible permease could not be synthesized. Likewise, if induced mRNA could be synthesized before the addition of an inhibitor of transcription, one would expect the blocking effect by such an inhibitor on the lethal action of streptomycin to be circumvented also, providing the inhibitor had no effect on translational aspects of protein synthesis.

The finding by Woese et al. (1963) that proflavine blocks synthesis of mRNA in *Escherichia coli* provided the means to perform the experiments postulated above. As reported elsewhere (Hurwitz and Rosano, Biochim. Biophys. Acta, *in press*), proflavine blocks transcription of the lactose operon without affecting translation once the proper mRNA has been formed.

MATERIALS AND METHODS

E. coli B was grown in nutrient broth under aeration at 37 C and was harvested in the exponential-growth phase at a concentration of about 3×10^8 per milliliter. The cells were washed once by centrifugation and were then suspended in prewarmed nutrient broth. (The washing procedure extends the lag period before the onset of loss of viability resulting from exposure to streptomycin, and therefore allows more precise control of the events in the following procedures. Under these conditions, the cells were also slightly less sensitive to proflavine).

Viable counts were performed in quadruplicate on nutrient agar after dilution in saline to avoid carry-over of streptomycin adsorbed to cells (Hurwitz, Rosano, and Landau, 1962).

RESULTS

Studies with proflavine. Proflavine at 17.5 μg/ml was found to be nonlethal but bacteriostatic for at least 40 min for *E. coli* under the conditions described in Materials and Methods.

Under these conditions, it was also found that proflavine blocked the lethal action of streptomycin (Fig. 1). These findings would be expected if proflavine blocks the formation of streptomycin permease by blocking the formation of induced mRNA.

As was shown previously (Anand and Davis, 1960; Anand, Davis, and Armitage, 1960), chloramphenicol blocks the lethal action of streptomycin and the entry of streptomycin into sensitive cells. Since chloramphenicol blocks protein synthesis but permits RNA synthesis to proceed (Hahn et al., 1957), it was considered possible that mRNA for a streptomycin permease might be formed by exposing cells to streptomycin and chloramphenicol. In the presence of chloramphenicol, formation of the permease itself would be blocked while the induced mRNA should be at least partially protected from degradation (Nakada and Magasanik, 1964). Addition of proflavine and removal of chloramphenicol should now control two events: no more mRNA should be formed; and protein synthesis should proceed, only those proteins being synthesized for which preformed mRNA exists. If an induced streptomycin permease is a reality, under these conditions one would expect the cells to be killed by streptomycin in the presence of proflavine. It should be recalled, as shown in Fig. 1, that proflavine, added at the same time as streptomycin, effectively blocks the lethal action of the latter.

The experiment was performed as follows. *E. coli* B cells were grown, harvested, and suspended as described in Materials and Methods. To the cell suspension were added 30 μg/ml of chloramphenicol and 2.5 μg/ml of streptomycin. The suspension was then incubated at 37 C under aeration for 10 min. Proflavine at 17.5 μg/ml was then added, and the cells were washed twice by centrifugation from nutrient broth containing proflavine, to remove the chloramphenicol. The cells were then suspended in nutrient broth containing proflavine, and the suspension was divided into two portions. Streptomycin at 2.5 μg/ml was added to one. Samples were removed at intervals for viable counts.

As seen in Fig. 2, the cells were susceptible to the lethal action of streptomycin in the presence of proflavine, and proflavine itself under these conditions was still bacteriostatic. From these results it would appear that the block of the lethal action of streptomycin by proflavine can, like the block by chloramphenicol, be circumvented under proper conditions. Under the conditions of this experiment it appears that when induced mRNA for a streptomycin permease is formed before the addition of proflavine, streptomycin can cause irreversible loss of viability in the presence of proflavine.

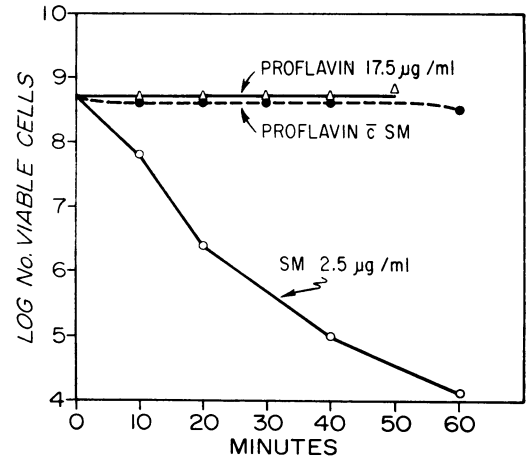

FIG. 1. *Effect of proflavine on the lethal action of streptomycin. Escherichia coli B cells growing exponentially in nutrient broth were washed once by centrifugation and were resuspended in nutrient broth. After resuspension, proflavine was added at 17.5 μg/ml and streptomycin at 2.5 μg/ml, as indicated.*

It was further reasoned that, if mRNA for the permease was preformed under these conditions, the concentration of streptomycin required for loss of viability should be markedly lowered, and no lag in loss of viability should occur after addition of streptomycin. A lag in onset of loss of viability always occurs when cells are exposed to streptomycin without prior treatment (Hurwitz et al., 1962).

Streptomycin at concentrations of 0.05 μg/ml and lower has no effect on *E. coli* B under the conditions used in these experiments, and exposure of these cells to 1 μg/ml for 5 min has also been shown to be nonlethal (Hurwitz et al., 1962). When these cells were first exposed to 1 μg of streptomycin per ml for 5 min, and the cells were then washed free from the inducing dose, the induced cells were sensitive to 0.05 μg/ml, and no lag occurred before the lethal action of the antibiotic began. The results of such an experiment are shown in Fig. 3. Under these conditions, loss of viability can also be demonstrated at 0.025 μg/ml of streptomycin.

Studies with borate. Adams (1959) reported that borate blocks the formation of adaptive enzymes in *Pseudomonas striata*, and he postulated that this anion may exert this effect by blocking transcription. As shown in Table 1, borate effectively blocks RNA synthesis.

Experiments (Table 2) were also performed to

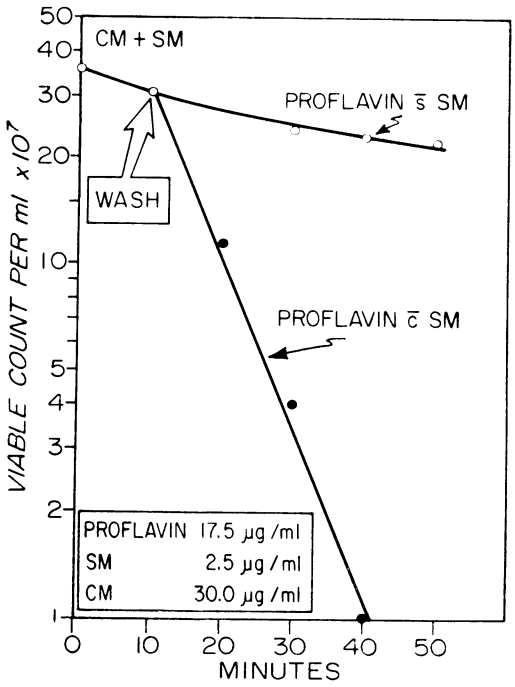

FIG. 2. *Circumvention of the proflavine block of the lethal action of streptomycin. Escherichia coli B cells growing exponentially in nutrient broth were exposed to chloramphenicol (30 µg/ml) and streptomycin (2.5 µg/ml) for 10 min at 37 C. The cells were then washed by centrifugation from nutrient broth containing proflavine and were resuspended in this medium. The suspension was divided into two parts, and streptomycin at 2.5 µg/ml was added to one.*

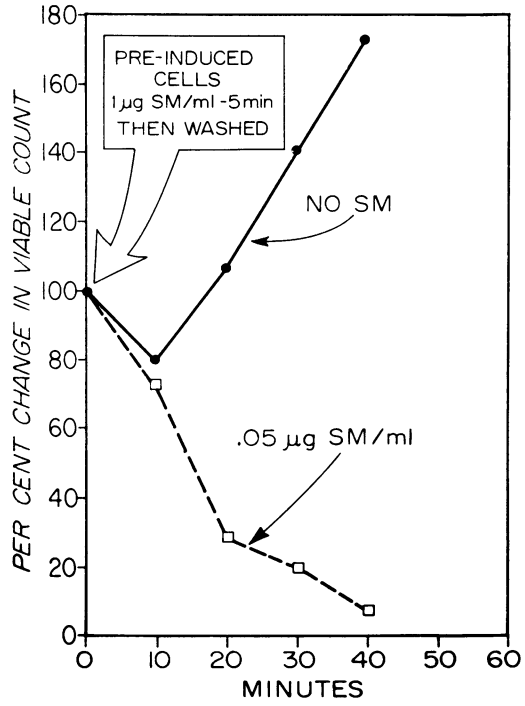

FIG. 3. *Escherichia coli B cells growing exponentially in nutrient broth at 4 × 10⁸ cells per milliliter were exposed to 1 µg of streptomycin per ml for 5 min with no decrease in viability. The cells were then washed by centrifugation and resuspended in prewarmed nutrient broth. One portion of the cellular suspension was then exposed to 0.05 µg of streptomycin per ml, and the remainder was incubated further without added streptomycin.*

show that borate can replace proflavine in comparable experiments; i.e., it is also bacteriostatic at 0.2 M (column B), it blocks the lethal action of streptomycin (column C), and the block can be circumvented by a prior exposure to streptomycin under conditions suitable for mRNA induction (column D). Similar results were obtained under the conditions used in the proflavine experiments, i.e., when the cells were induced with chloramphenicol and streptomycin rather than with streptomycin alone. With borate, it was not necessary to use prewashed cells. Further support for the conclusion that borate blocks the lethal action of streptomycin by preventing induced mRNA synthesis comes from our finding that borate does not block in vitro synthesis of polyphenylalanine as directed by polyuridylic acid.

TABLE 1. *Effect of borate on RNA synthesis**

Time	RNA/6 × 10⁸ cells	
	Control	Plus borate (0.2 M)
min	*µg*	*µg*
0	420	420
15	460	400
30	540	420
45	590	420
60	620	420

* *Escherichia coli* B cells growing exponentially in nutrient broth (3.0 × 10⁸ per milliliter) were exposed to 0.2 M sodium borate (*p*H 7.0); 2-ml samples of the cells were first washed with cold 5% trichloroacetic acid and then were extracted with hot 5% trichloroacetic acid. The RNA content of the extracts was determined by the orcinol method.

DISCUSSION

The hypothesis that a streptomycin-inducible permease must be synthesized to permit entry of streptomycin past a permeability barrier has been supported by the following findings: (i) chloramphenicol blocks the lethal action and entry of streptomycin (Anand and Davis, 1960); (ii) a permeability barrier to the entry of streptomycin has been demonstrated (Anand et al.,

TABLE 2. *Effect of borate on the lethal action of streptomycin**

Time	Viable count			
	A	B	C	D
min				
0	3.0×10^8	3.0×10^8	3.0×10^8	3.0×10^8
5	—			1.1×10^8
10	—	3.3×10^8	3.7×10^8	9.5×10^7
20	4.2×10^5	3.8×10^8	3.6×10^8	3.5×10^7
30	3.5×10^5	4.4×10^8	3.1×10^8	1.6×10^7

* *Escherichia coli* B cells growing exponentially in nutrient broth were treated as follows: A, cells were exposed to 1 μg/ml of streptomycin; B, borate was added at 0.2 M; C, borate was added at 0.2 M, the suspension was shaken for 3 min, and streptomycin was then added at 1 μg/ml; D, streptomycin was added at 1 μg/ml 5 min before addition of borate at 0.2 M. Zero-time is time of addition of streptomycin in A and C, and time of addition of borate in B and D. Streptomycin at 1 μg/ml has no lethal effect during the 5-min exposure prior to zero-time in D.

1960; Hurwitz and Rosano, 1962*b*); (iii) the chloramphenicol block of the lethal action of streptomycin can be circumvented; i.e., cells can be killed by streptomycin in the presence of chloramphenicol if the cells receive a prior exposure to nonlethal doses of streptomycin (Hurwitz and Rosano, 1960, 1962*a*); (iv) the chloramphenicol block of the entry of streptomycin can be likewise circumvented (Hurwitz and Rosano, 1962*b*); (v) the longer the cells receive a prior exposure to streptomycin, the more rapid is the rate of entry of streptomycin after the addition of chloramphenicol (Hurwitz and Rosano, 1962*b*); (vi) the higher the concentration of the lethal dose of streptomycin, the shorter is the lag before the onset of loss of viability (Hurwitz et al., 1962); (vii) regardless of the concentration of streptomycin, a lag of at least 2.5 min always precedes the onset of loss of viability after exposure to lethal doses (Hurwitz et al., 1962).

The present work further supports the permease hypothesis by showing that a block of transcription also blocks the lethal action of streptomycin, and that this block of the lethal action can be circumvented by conditions consistent with the prior synthesis of induced mRNA for the permease.

The validity of the use of proflavine (or borate) and chloramphenicol to control transcriptional and translational aspects of induced protein synthesis will be further documented in a separate report of experiments in which the procedur used to titrate these aspects of the action c β-galactoside operon of *E. coli* B cells (Hu and Rosano, 1965).

In a previous report (Hurwitz and Rc 1962*a*), the proposal was made that resistan streptomycin may result from the loss of a of sensitive cells to make a permease. The fi by Flaks et al. (1962) and Speyer, Lengyel Basilio (1962) that ribosomes derived resistant cells are also resistant to the effe streptomycin on in vitro protein synthesi peared to rule out this possibility. However possible that more than one mechanism c sistance may exist. The multigenic, mul resistance (Watanabe and Watanabe, may prove to operate by a different mecha than the one-step resistance controlled single gene. In addition, further attention be paid to the report by Sager (1965) tha ribosomes isolated from resistant *Chlamydom* in which resistance is carried as an extran inheritance factor, are sensitive. Rosen (1964) reported the finding of episome-med resistant strains of *E. coli* from which sen ribosomes are derived, and Gundersen (reported the finding of a dominant epison mutator factor for resistance in *E. coli*. likelihood that resistance in these cells result from loss of ability to synthesize a mease should be investigated.

Gorini and Kataja (1964) and Davies, Gi and Gorini (1964) showed that streptor can cause misreading of the code in bo vivo and in vitro systems. It has been inf from these findings that the lethal actic streptomycin may result from the formati faulty protein produced in the presence of s tomycin. However, the following considera make it seem unlikely that misreading o code is the cause of the lethal action of str mycin.

The finding (Hurwitz and Rosano, 1962 that cells can be killed by streptomycin i presence of chloramphenicol, after prior exp to the former, would rule out the possibility an excessive amount of faulty protein v result in death. The presence of chloramphe would prevent formation of even faulty pr Furthermore, if streptomycin is removed b the addition of chloramphenicol, the lo viability stops (Hurwitz and Rosano, 19 This latter finding indicates that no toxic le misread protein could have been synthe before the addition of chloramphenicol. Th appears that neither excessive amounts of

read protein, nor small but toxic amounts of misread protein, could contribute to the lethal action of streptomycin in the presence of chloramphenicol.

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