CHLORAMPHENICOL-SENSITIVE AND -INSENSITIVE PHASES OF THE LETHAL ACTION OF STREPTOMYCIN[1]

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ABSTRACT

HURWITZ, CHARLES (Veterans Administration Hospital, Albany, N.Y.) AND CARMEN L. ROSANO. Chloramphenicol-sensitive and -insensitive phases of the lethal action of streptomycin. J. Bacteriol. **83**:1202–1209. 1962.—If chloramphenicol is added to sensitive *Escherichia coli* cells at the same time as streptomycin, the lethal effect of the latter drug is prevented. If the cells receive a prior exposure to streptomycin before the addition of chloramphenicol, the bacteria are then susceptible to killing by streptomycin in the presence of chloramphenicol.

These results are interpreted to mean that the lethal action of streptomycin can be divided into chloramphenicol-sensitive and chloramphenicol-insensitive stages. It is proposed that during the chloramphenicol-sensitive stage, a streptomycin-initiated protein synthesis occurs, and that this protein synthesis must precede the actual killing by streptomycin. Inorganic phosphate has no effect on the chloramphenicol-sensitive phase, but does prevent killing by streptomycin. Evidence is presented arguing against the formation of a leaky permeability barrier as being the primary cause of death of cells exposed to streptomycin.

Exposure of sensitive nonproliferating (Hurwitz and Rosano, 1958) or growing *Escherichia coli* cells (Hurwitz, Rosano, and Landau, 1962) to streptomycin results in a decline of viability as measured by loss of ability to produce macrocolonies after removal of the antibiotic by dilution. The decline in viable count, however, is always preceded by a lag period during which no change in viable count is observed. It therefore appeared that some events, initiated by

[1] A preliminary report of some aspects of this work has been published (Hurwitz and Rosano, 1960).

the presence of streptomycin, must precede the later lethal effect of the drug.

The means for investigating these events evolved from the interesting discovery by Anand and Davis (1960) and Anand, Davis, and Armitage (1960) that chloramphenicol blocks such effects of streptomycin on sensitive bacteria as the loss of viability, the enhancement of excretion of 5'-nucleotides, and the accumulation of C^{14}-label from C^{14}-streptomycin.

Chloramphenicol is a potent and rapid inhibitor of protein synthesis by *E. coli* (Wisseman et al., 1954). Streptomycin, which also inhibits protein synthesis, always permits some protein synthesis before the inhibition takes effect (Anand et al., 1960).

Since chloramphenicol blocks the effects of streptomycin on sensitive bacteria, it would appear that either the lethal action of streptomycin occurs only during chloramphenicol-sensitive protein synthesis, or that events sensitive to chloramphenicol and initiated by streptomycin must precede the lethal action of the latter antibiotic.

If the lethal effect of streptomycin can occur only during active protein synthesis, killing of cells exposed to streptomycin should abruptly cease on addition of chloramphenicol. If, on the other hand, killing by streptomycin occurs only after a streptomycin-initiated event has occurred, addition of chloramphenicol after a prior exposure of cells to streptomycin should result in the continued lethal effect of the latter. The following experiments were designed to discriminate between these two possibilities.

MATERIALS AND METHODS

E. coli B, sensitive to streptomycin, was used for most of the studies in this report. Nonproliferating cells were prepared by suspending saline-washed cells in 0.011 M lactate in 0.85% NaCl, pH 7.0. Prior to resuspension in the

saline-lactate medium, the cells were grown overnight in aerated nutrient broth. Growing cells were prepared by inoculating an aerated, overnight culture into nutrient broth. The increase in turbidity was measured with a Coleman model 7 photonephelometer. Growth was continued until the rate of increase was exponential (cell count about 3×10^8/ml). The cells were then rapidly concentrated by centrifugation and resuspension in one-half volume of fresh nutrient broth. The cell concentration was about 6×10^8 ml when used. Viable counts were determined in quadruplicate by surface plating on nutrient agar after dilution in 0.85% NaCl.

Streptomycin was obtained as streptomycin sulfate from E. R. Squibb and Sons. The concentrations of the antibiotic cited are not corrected for the content of sulfate. Chloramphenicol was obtained from Parke, Davis and Co.

RESULTS

Nonproliferating *E. coli* cells incubated with 10 μg of streptomycin/ml in 0.85% NaCl and 0.011 M lactate show a logarithmic decline in viability, but only after a lag period lasting about 1 hr (Hurwitz and Rosano, 1958).

If streptomycin exerts its lethal action only during active protein synthesis, no loss of viability should occur in the presence of chloramphenicol, even though the addition of the latter has been delayed until after the lag period is over. If, on the other hand, a streptomycin-initiated event must precede actual killing by streptomycin, it is logical to assume that this event would occur during this prolonged lag preceding the logarithmic decline in viability, and the cells would then be sensitive to the lethal action of streptomycin in the presence of chloramphenicol.

These possibilities can therefore be tested by exposing nonproliferating cells to streptomycin for 1 hr to allow the streptomycin-initiated event to occur, followed by addition of chloramphenicol to prevent further protein synthesis, and subsequent exposure of the cells to streptomycin in the presence of chloramphenicol to test whether the cells can then be killed. If there is no further loss of viability in the presence of chloramphenicol, the conclusion that the lethal effect of streptomycin can be exerted only in the presence of active protein synthesis would be valid. If there is a further loss of viabil-
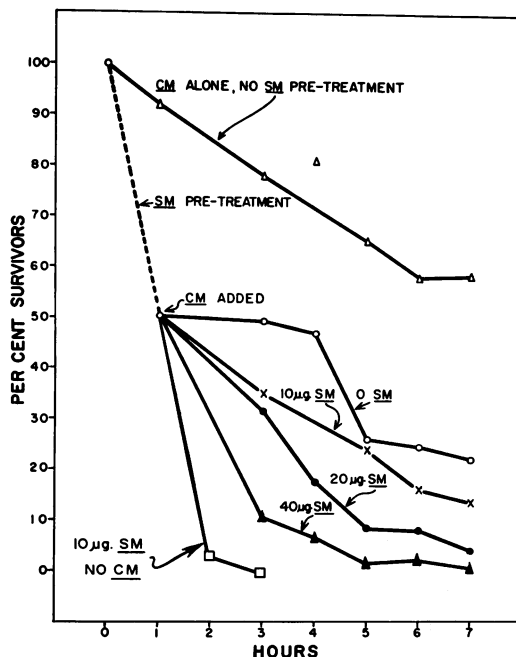

FIG. 1. *Effect of streptomycin concentration in the killing phase on viability of nonproliferating Escherichia coli B cells. Nonproliferating cells were prepared from overnight growth in nutrient broth. The cells were washed with saline by centrifugation and resuspended in 0.011 M lactate in 0.85% NaCl, pH 7.0. Chloramphenicol (30 μg/ml) was added after 1 hr of exposure to streptomycin (10 μg/ml). The cells were then washed with saline and resuspended in the lactate-saline medium containing chloramphenicol and the indicated concentrations of streptomycin. Viable counts were made in quadruplicate on nutrient agar after dilution in 0.85% NaCl, and are reported as per cent survivors. The dotted line, labeled SM pretreatment, indicates the over-all decline in viability, not the actual kinetics. The 50% loss of viability actually occurs within the last 10 to 15 min of the streptomycin pretreatment. SM = streptomycin; CM = chloramphenicol.*

ity in the presence of chloramphenicol, it can be concluded that a streptomycin-initiated event must precede the lethal effect of streptomycin.

In the following experiments, chloramphenicol (30 μg/ml) was added after 60 min exposure of the cells to streptomycin (10 μg/ml). The cells were then washed with 0.85% saline containing chloramphenicol to remove residual streptomycin. Samples of cells were then resuspended in

the same medium containing chloramphenicol and varying concentrations of streptomycin.

The cells which were pre-exposed to streptomycin in the absence of chloramphenicol were killed by streptomycin in the presence of chloramphenicol (Fig. 1). Since cells which have not received a prior exposure to streptomycin cannot be killed by streptomycin in the presence of chloramphenicol, it is concluded that the lethal action of streptomycin can occur in the absence of protein synthesis and that a streptomycin-initiated event must precede the lethal effect of the antibiotic.

In the following discussion, this initial streptomycin exposure in the absence of chloramphenicol is referred to as the "initiation phase." The subsequent period, during which loss of viability occurs owing to exposure to streptomycin in the presence of chloramphenicol, is referred to as the "killing phase."

In the experiment reported in Fig. 1, there is a decline of about 50% in viability after the first 60 min of exposure to streptomycin. This decline
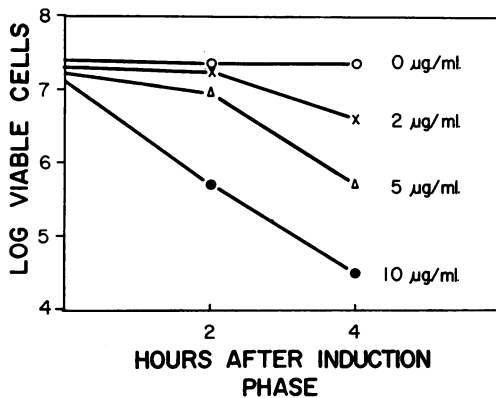

FIG. 2. *Effect of varying streptomycin concentrations during the initiation phase on the subsequent rate of loss of viability of Escherichia coli B cells in the presence of chloramphenicol. Nonproliferating cells in lactate-saline medium were exposed for 1 hr to the indicated concentrations of streptomycin. The cells were then washed with saline and resuspended in the lactate-saline medium containing chloramphenicol (30 μg/ml) and streptomycin (10 μg/ml). The viable counts shown in the figure illustrate the decline in viability during exposure to streptomycin and chloramphenicol (the killing phase) as a result of exposure to various concentrations of streptomycin during the initiation phase.*

indicates that some killing by streptomycin occurred before addition of the chloramphenicol. The decline in viability during this first 60 min of exposure varies from one experiment to another, in some experiments being as low as 10%. The extent of decrease in viability before addition of chloramphenicol does not alter the subsequent results.

The rate of loss of viability in the killing phase is proportional to the external concentration of streptomycin. Cells exposed to streptomycin during the initiation phase, but not during the killing phase, show no greater loss of viability in the presence of chloramphenicol than controls exposed to chloramphenicol alone. It is therefore evident from the data that the postulated streptomycin-initiated event itself cannot be lethal.

Effect of concentration of streptomycin during the chloramphenicol-sensitive initiation phase. In these experiments, the effect of concentration of streptomycin during the initiation phase on the subsequent rate of loss of viability in the killing phase was studied. Cells were initially exposed to various concentrations (0 to 10 μg/ml) of streptomycin during the initiation phase, under the same conditions as in the first experiment. The cells were washed and resuspended as before, except that in this instance all cells were then exposed to streptomycin (10 μg/ml) in the presence of chloramphenicol. The rate of killing in the presence of chloramphenicol was proportional to the concentration of streptomycin during the initiation phase (Fig. 2). Cells not pre-exposed to streptomycin (zero initial streptomycin concentration) were not killed by streptomycin in the presence of chloramphenicol. These results indicate that the rate at which the streptomycin-initiated event occurs is a function of the external streptomycin concentration.

Further studies on the initiation and killing phases. As has been shown previously (Hurwitz and Rosano, 1958), inorganic phosphate (6.7 × 10^{-2} M) prevents killing of aerated, nonproliferating *E. coli* cells by 10 μg/ml streptomycin. To determine whether phosphate acted in the initiation or the killing phase, the effect on each of the two phases was therefore examined.

In the first experiment of this series, the cells were exposed to 10 μg/ml streptomycin and 6.7 × 10^{-2} M phosphate for 1 hr in the saline-
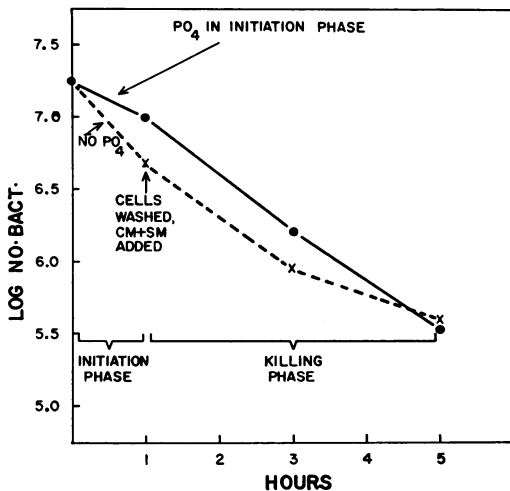
FIG. 3. *Effect of presence of inorganic phosphate during the initiation phase on the lethal effect of streptomycin. Nonproliferating cells in 0.011 M lactate and 0.85% NaCl (pH 7.0) were exposed to streptomycin (10 μg/ml), with or without inorganic phosphate (0.067 M) for 1 hr (initiation phase). The cells were then washed with saline by centrifugation and resuspended in lactate-saline medium containing chloramphenicol (30 μg/ml) and streptomycin (10 μg/ml), without added inorganic phosphate.*

lactate medium; the cells were then washed and resuspended in chloramphenicol and streptomycin (but without phosphate) as described earlier. Exposure of the cells to inorganic phosphate during the initiation phase had no effect on the loss of viability in the killing phase (Fig. 3). Inorganic phosphate, therefore, has no effect on the chloramphenicol-sensitive, streptomycin-initiated event.

In the second experiment of this series, cells were exposed to phosphate in the killing phase. The results of this experiment showed that killing was markedly reduced (Fig. 4). It is therefore concluded that inorganic phosphate, which has no effect on the chloramphenicol-sensitive initiation phase, prevents or reduces the loss of viability which occurs in the killing phase.

Similar experiments with iodoacetate showed that the presence of this inhibitor in either phase prevents loss of viability, in contrast to phosphate which specifically inhibits only the killing phase.

Proliferating-cell experiments. The preceding

experiments are interpreted to mean that a streptomycin-initiated, chloramphenicol-sensitive event, required for the lethal action of streptomycin, is carried out by nonproliferating cells during the lag period preceding the exponential decline in viability. It seemed desirable to determine whether a like situation exists with growing cells. Since growing cells are more sensitive to streptomycin than are nonproliferating cells, the time of exposure and the concentrations of streptomycin had to be markedly reduced to obtain comparable data.

An *E. coli* B culture in exponential-growth phase in nutrient broth was obtained and concentrated as described under Materials and Methods. Streptomycin sufficient to bring the concentration to 1.0 μg/ml was added and incubation was continued for 20 min, an exposure which results in about 50% loss of viability. Chloramphenicol (30 μg/ml) was then added, and the cells were rapidly centrifuged and washed once in saline-chloramphenicol solution as in the previous experiments with nonproliferating cells.

The cells were then resuspended in nutrient broth containing chloramphenicol (30 μg/ml).
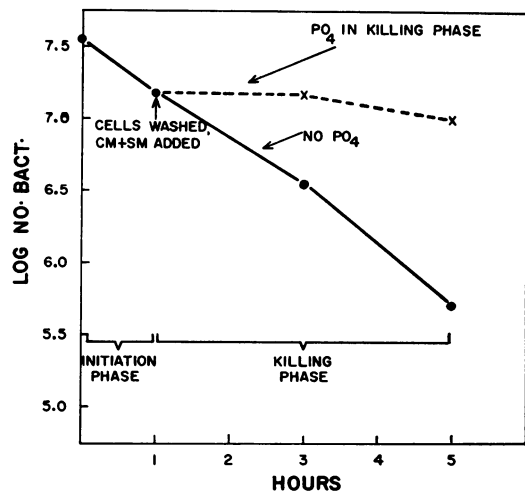

FIG. 4. *Effect of presence of inorganic phosphate during the killing phase on the lethal effect of streptomycin. The experiment was performed in the same manner as reported for Fig. 3, except that inorganic phosphate at 0.067 M was added as indicated at the start of the killing phase, i.e., after the cells were resuspended in the presence of chloramphenicol and streptomycin.*

To one sample was added 1.0 µg/ml of strepto-
mycin, to another were added 2.0 µg/ml, and
to a third no streptomycin was added. A fourth
suspension was treated as above with 1.0 µg/ml
of streptomycin but received no chloramphenicol.
Viable counts were made as described before.

Addition of chloramphenicol without strepto-
mycin after resuspension resulted in an imme-
diate cessation of loss of viability (Fig. 5).
Addition of streptomycin in the presence of
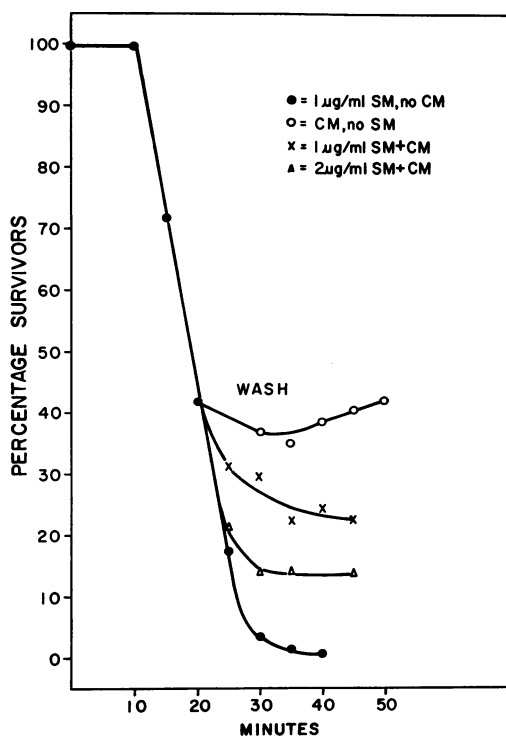chloramphenicol, however, resulted in further

FIG. 5. Effect of streptomycin concentration on
viability of pretreated, growing Escherichia coli
B cells in the presence of chloramphenicol. Cells
growing in nutrient broth were exposed to strepto-
mycin (1.0 µg/ml) for 20 min. The cells were then
washed with saline and resuspended in nutrient
broth containing chloramphenicol (30 µg/ml) plus
the indicated concentrations of streptomycin. One
portion received streptomycin without chloramphen-
icol as indicated. The results are expressed as per
cent survivors capable of producing macrocolonies
on nutrient agar after being diluted in saline. The
cell concentration at the start of the experiment
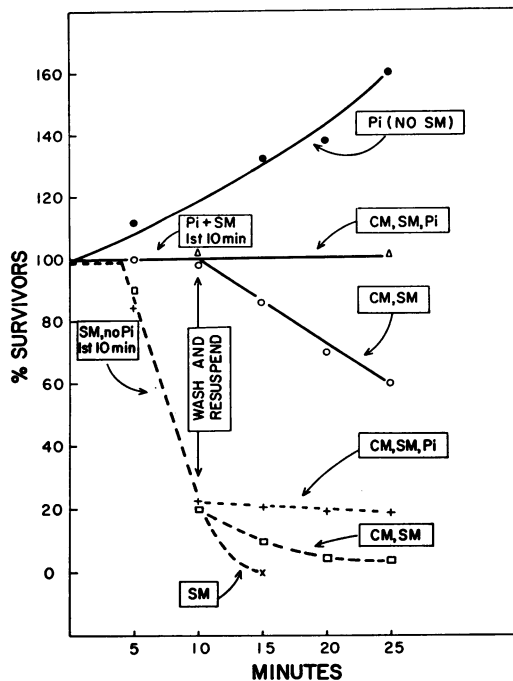was 6×10^8/ml. SM = streptomycin; CM =
chloramphenicol.

FIG. 6. Influence of inorganic phosphate on the
lethal effect of streptomycin on growing Escherichia
coli B. Cells growing exponentially in nutrient
broth were concentrated and divided into two por-
tions. To one was added 2.5 µg streptomycin/ml,
and to the other streptomycin plus inorganic phos-
phate (0.067 M). After 10 min of incubation on a
rotary shaker, each suspension was washed by
centrifugation and resuspended in nutrient broth
containing chloramphenicol (30 µg/ml) and strepto-
mycin (2.5 µg/ml), with or without added inor-
ganic phosphate (0.067 M) as indicated.

loss of viability, and the rate of loss and total
amount of loss increased as the streptomycin
concentration increased. The results closely
parallel the findings in comparable experiments
with nonproliferating cells.

The presence of chloramphenicol in the kill-
ing phase, however, markedly reduced the rate
and extent of loss of viability, as seen from
the fact that omission of chloramphenicol re-
sulted in greater loss of viability from exposure to
1.0 µg/ml streptomycin then resulted from ex-
posure to 2.0 µg/ml streptomycin in the presence
of chloramphenicol. The reasons for this effect
of chloramphenicol are not readily apparent,
but do not alter the fact that cells which have
been pre-exposed to streptomycin in the ab-

sence of chloramphenicol are then subject to the lethal effect of streptomycin in the presence of chloramphenicol. The experiment nevertheless indicates that protein synthesis may play some potentiating role on the lethal effects caused by streptomycin in the killing phase. Another possibility is that the postulated streptomycin-initiated event had not been sufficiently completed in the remaining viable cells to make them sensitive to the lethal action of streptomycin.

Effect of inorganic phosphate in growing-cell experiments. As shown earlier, inorganic phosphate blocks the lethal effect of streptomycin on nonproliferating cells when present in the killing phase. It was therefore desirable to see whether a similar effect could be shown with growing cells.

E. coli B cells growing exponentially in nutrient broth were concentrated as before and were then divided into two portions (A and B). To A were added 2.5 μg streptomycin/ml and to B were added streptomycin and inorganic phosphate (0.067 M). After 10 min of incubation, each suspension was washed by centrifugation and resuspended in nutrient broth containing chloramphenicol (30 μg/ml) and streptomycin (2.5 μg/ml) with or without added inorganic phosphate (0.067 M) as indicated in Fig. 6.

The loss of viability occurring in the presence of chloramphenicol and streptomycin is unaffected by the presence of inorganic phosphate in the initiation phase, but is affected in the killing phase. The loss of viability which occurs during the first 10 min of incubation (in the absence of chloramphenicol) is also prevented by the presence of inorganic phosphate.

It can also be seen that chloramphenicol reduces the rate and extent of the lethal action of streptomycin, since omission of chloramphenicol and inorganic phosphate in the killing phase resulted in greater loss of viability than did omission of inorganic phosphate alone. This last finding is in agreement with a similar effect noted in Fig. 5 and in Fig. 1.

The presence of inorganic phosphate in the killing phase therefore blocks the lethal effect of streptomycin on growing as well as on nonproliferating bacteria. This phosphate effect also explains the far greater sensitivity of *E. coli* to streptomycin when grown in nutrient broth than when grown in phosphate-buffered media such as Difco Penassay broth or synthetic media. Addition of inorganic phosphate to

nutrient broth greatly increases the resistance of *E. coli* to streptomycin, whereas reduction of the phosphate concentration of synthetic media increases the sensitivity of the bacteria to the antibiotic.

DISCUSSION

Chloramphenicol prevents killing of both nonproliferating and growing cells by streptomycin. A prior exposure of the cells to streptomycin, however, infers a sensitivity of the cells to the antibiotic in the presence of chloramphenicol.

Since chloramphenicol at the concentrations used effectively inhibits protein synthesis, it would appear that the chloramphenicol-sensitive, streptomycin-initiated event itself involves protein synthesis. Chloramphenicol presumably may block protein synthesis initiated by streptomycin, or may prevent creation of a protein imbalance caused by streptomycin inhibition of specific protein synthesis.

The latter possibility appears to be the less likely of the two. That a protein imbalance itself is the cause of death is ruled out by the fact that further loss of viability, after a prior exposure to streptomycin, ceases on removal of the streptomycin. Addition of chloramphenicol at this point should prevent any further imbalance from developing, yet progressive loss of viability occurs only in the presence of streptomycin.

The first possibility, that chloramphenicol blocks the synthesis of a streptomycin-initiated protein, results in a more direct and a simpler hypothesis and is favored by the authors. Such a streptomycin-initiated protein could conceivably be an active transport system enabling the rapid entry of streptomycin into the cell. Some presumptive evidence for this hypothesis will be presented in the two following reports.

If synthesis of some protein, initiated by the presence of streptomycin, is required for its lethal action, the experiments with nonproliferating cells indicate that the rate of the streptomycin-initiated protein synthesis is a function of the external streptomycin concentration. Furthermore, once a given amount of this protein is synthesized, the rate of killing should also be a function of the external streptomycin concentration. The experiments with growing cells confirm these results. In both instances, sensitive bacteria can be killed by

streptomycin in the presence of chloramphenicol, provided the cells have received a prior exposure to streptomycin. Accumulation of the postulated streptomycin-initiated protein does not in itself result in loss of viability, since further exposure of cells in the absence of streptomycin does not result in loss of viability.

That protein synthesis may play an additional role in the killing phase is not completely ruled out, since both the rate and extent of loss of viability due to exposure to streptomycin are markedly reduced in the presence of chloramphenicol.

It has therefore been shown that the lethal effect of streptomycin can be divided into two phases: an "initiation phase" during which a streptomycin-initiated protein is apparently synthesized, and a "killing phase" during which loss of viability can occur in the presence of chloramphenicol. The events occurring in the initiation phase are insensitive to inorganic phosphate but are blocked by chloramphenicol. The events in the killing phase leading to loss of viability occur in the presence of chloramphenicol, but at a reduced rate and to a lesser extent, and can be completely blocked by the presence of inorganic phosphate.

Anand et al. (1960) proposed that streptomycin causes the formation of a leaky cellular membrane which permits the loss of low molecular weight intermediates from the cell or the enhanced entry of toxic levels of streptomycin into the cell. According to these authors, chloramphenicol prevents the synthesis of faulty protein components of the cellular membrane resulting from exposure to streptomycin. The possible similarity between this postulated faulty protein and the presently postulated streptomycin-initiated protein is readily apparent.

However, the data in this report would appear to rule out the possibility that streptomycin kills sensitive bacteria solely by the formation of a leaky cellular membrane through which low molecular weight intermediates are lost from the cell. According to this suggestion, a given exposure of the cells to streptomycin would result in the formation of a given amount of faulty cellular membrane. Subsequent addition of chloramphenicol would stabilize this degree of leakiness, since no further membrane protein would be formed. The rate of leakage of metabolic intermediates should then be deter-

mined by the extent of the lesion in the permeability membrane, and the rate of loss of viability of the cells should be determined by the rate of leakage. The rate of loss of viability of these streptomycin-treated cells in the presence of chloramphenicol should not have been affected by the external streptomycin concentration, since no further synthesis of faulty cellular membrane would be possible. It would appear from the present data that streptomycin does not kill sensitive bacteria solely by the formation of a leaky cellular membrane, since the rate of loss of viability in the presence of chloramphenicol was markedly affected by the external streptomycin concentration, provided the cells had received a prior exposure to streptomycin. Killing by streptomycin appears to require a prior streptomycin-initiated protein synthesis followed by subsequent intracellular events also dependent on the external streptomycin concentration.

The possibility that streptomycin alters the permeability barrier in such a way as to enhance nonspecifically the rate of entry of toxic levels of streptomycin into the cell is being investigated. Preliminary results with a β-galactosidase-constitutive, but permease-positive, mutant indicate that no change in permeability to o-nitrophenyl-β-D-galactoside occurs after exposure to streptomycin sufficient to render the population less than 0.1% viable. To test this point more critically, studies are underway with a cryptic, i.e., permease-negative, constitutive mutant.

If the postulated streptomycin-initiated protein does not alter permeability in a nonselective manner, the possibility must be considered that the protein may have specific characteristics and functions. This implies that the streptomycin-initiated protein may have a unique function and structure, and that its synthesis is specifically elicited by streptomycin or closely related compounds. Such a protein might, for example, have the functions of an active transport system for streptomycin itself or an enzymatic potential for converting streptomycin to some toxic form. This of course implies that the streptomycin-initiated protein may indeed be streptomycin-induced.

Medically important antibiotics are useful because they can destroy invading bacteria without at the same time damaging host tissue.

Since the unitary hypothesis of biochemistry has demonstrated that many essential biochemical reactions and pathways are identical in organisms as diverse as bacteria and humans, it is difficult to understand how an antibiotic can selectively kill or prevent growth of invading bacteria in the same milieu as the host cells without damaging the latter. The requirement of a streptomycin-initiated protein synthesis poses a mechanism whereby an antibiotic may selectively kill a sensitive bacterial cell without damaging the invaded host cell even though the sites of action of the antibiotic, i.e., the sensitive biochemical reactions, may be present in both. If the host cell is incapable of carrying out the protein synthesis required for the later lethal action of the drug, it cannot be killed by streptomycin.

The requirement for a streptomycin-initiated protein synthesis might explain the difference between the two patterns of development of resistance to streptomycin: the one step and the multiple step. The one-step pattern could presumably result from a mutation at a single locus controlling the ability of the cells to respond to streptomycin by protein synthesis. The multiple-step process might involve changes in the killing phase of streptomycin activity, which may be controlled by many different functional hereditary loci (Bryson and Demerec, 1955).

The requirement for a streptomycin-initiated protein synthesis, which could presumably be controlled by activity of a single mutable gene, would also explain the observation that, in a diploid strain of *E. coli*, streptomycin sensitivity is dominant over resistance (Lederberg, 1951). Since mutations are commonly recessive to the normal gene, the presence of both sensitive and resistant genes in a heterozygote would result in a cell having the ability to synthesize the protein, resulting in sensitivity of the heterozygote to streptomycin.

Another interesting finding which may be explained by the requirement for the induced protein synthesis is contained in the report by Witkin and Theil (1960). Post-treatment with chloramphenicol of ultraviolet-induced *E. coli* markedly reduced the yield of mutants from *lac⁻* to *lac⁺* but had no appreciable effect on mutations to *str-r* and *str-i*. These workers concluded that the ability to ferment lactose (i.e.,

lac⁻ to *lac⁺*) could be established only if conditions are favorable for synthesis of proteins during the postirradiation "sensitive period." Mutations from *strep-s* to *str-r* and *str-i* presumably have no such requirement.

According to the present hypothesis, mutation to *str-r* would be chloramphenicol-insensitive during the postirradiation period, because mutation to resistance would involve loss, not gain, of an ability to synthesize a protein.

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