KINETICS OF LOSS OF VIABILITY OF *ESCHERICHIA COLI* EXPOSED TO STREPTOMYCIN

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ABSTRACT

HURWITZ, C. (Veterans Administration Hospital, Albany, N.Y.), C. L. ROSANO, AND J. V. LANDAU. Kinetics of loss of viability of *Escherichia coli* exposed to streptomycin. J. Bacteriol. **83**:1210–1216. 1962.—The first effect of streptomycin on *Escherichia coli* B cells growing in nutrient broth is a decrease or cessation (depending on the concentration of antibiotic) of the rate of increase of viable cells. After this apparent bacteriostasis, a rapid decline in viable count begins. The length of time before onset of decline of viability is a first order function of the concentration of streptomycin between 0.1 and 2.0 μg/ml. Above 2.0 μg/ml, the lag in onset of loss of viability approaches asymptotically a value greater than 2 min. The length of the lag in onset of loss of viability is interpreted to be a measure of the rate of synthesis of the postulated streptomycin-initiated protein required for the later lethal action of the drug. Measurements of the effect of streptomycin on the rate of synthesis of protein by *E. coli* B cells show an immediate effect on the rate of protein synthesis, corresponding to the apparent bacteriostasis; this is followed by a later decrease in the rate of synthesis, which corresponds in time with the decline in viability. Washing exponentially growing *E. coli* B cells by centrifugation and resuspension in fresh nutrient broth makes the cells more resistant to streptomycin. The lag before the onset of decline in viability is prolonged, and the inhibition of protein synthesis is likewise affected. The washing procedure has no effect on total protein synthesis (as measured by incorporation of C^{14}-leucine). Since synthesis of β-galactosidase is also delayed by the washing procedure, this effect is interpreted to mean that washing delays induced protein synthesis, but has no effect on the rate of total protein synthesis.

In an accompanying communication (Hurwitz and Rosano, 1962*a*), evidence was presented in support of the thesis that a chloramphenicol-sensitive, streptomycin-initiated protein synthesis is a necessary precursor for the lethal action of streptomycin. If a streptomycin-initiated protein synthesis is a necessary precursor of the lethal action of streptomycin, the kinetics of loss of viability of growing cells should show delineated phases corresponding to both the initiation and killing phases described in the preceding report. Studies of the kinetics of loss of viability and of the kinetics of inhibition of protein synthesis resulting from exposure of *Escherichia coli* to streptomycin were therefore undertaken to examine further the validity of the above thesis. In another accompanying report, the kinetics of accumulation of radioisotopic label from C^{14}-streptomycin will be discussed in a similar context (Hurwitz and Rosano, 1962*b*).

MATERIALS AND METHODS

Suspensions of *E. coli* B were prepared by inoculating fresh nutrient broth with an overnight aerated culture, and by continuing aeration until the exponential growth phase was reached (about 2.5 to 3.0 $\times$ 10^8 cells/ml). The suspension was then rapidly concentrated by centrifugation and resuspension in one-half the original volume of fresh nutrient broth. This was done to conform with the procedures required for the isotope-incorporation studies reported in the following communication (Hurwitz and Rosano, 1962*b*). After this treatment, the cells showed only a 5 to 10 min lag before re-entering exponential growth phase, which was then maintained for at least 60 min. Viable counts were made in quadruplicate by surface plating on nutrient agar.

Protein synthesis was measured by the rate of incorporation of L-C^{14}-leucine into the hot trichloroacetic acid (TCA)-insoluble fraction of

the bacteria. The bacteria were extracted and washed twice with cold 5% TCA, then extracted with 5% TCA at 90 C for 30 min. After a further wash with 5% TCA, the insoluble residue was dissolved in 1.0 ml of 0.1 N NaOH. Samples (0.25 ml) were plated on 1¼ in. diam stainless-steel planchets, and the radioactivity was measured with a Nuclear Chicago Automatic Gas Flow Counter having an efficiency of counting of 31%. Counting error was reduced to less than 4% by recording appropriate numbers of disintegrations.

RESULTS

E. coli B cells, growing in nutrient broth, were exposed momentarily to various concentrations of streptomycin to see whether the bacteria could be killed on contact, or whether killing required prior exposure for some finite time, as indicated by the streptomycin-initiation hypothesis.

In these first experiments, dilution of the cellular suspension preparatory to plating was performed in distilled water. Under these conditions, at concentrations above about 2 μg streptomycin/ml, contact killing appeared to occur, and the amount of apparent contact killing was proportional to the streptomycin concentration (Fig. 1). Contact killing in these experiments is defined as the loss of viability resulting from exposure to streptomycin for a period just long enough to add the drug to the cell suspension, mix, and withdraw a sample for dilution prior to plating. This operation took less than 10 sec.

The appearance of contact killing at concentrations of streptomycin above 2 μg/ml seemed to be contrary to the thesis that a streptomycin-initiated protein synthesis was a necessary precursor for killing by the antibiotic. However, streptomycin is adsorbed on bacteria and can be removed by washing with sodium chloride solution, but not with water (Berkman et al., 1948). From this it was deduced that the apparent contact killing may have resulted from adsorption of streptomycin on the cells. Such adsorbed streptomycin would not be removed by washing (or dilution) in distilled water and might therefore still be sufficiently high in concentration to kill the cells after plating. Since killing was measured as loss of the ability to produce macrocolonies, adsorbed streptomycin carried over on the plated bacteria might result in subsequent death of the cells and consequent inability to form macrocolonies. Such cells could not be differentiated from cells rendered nonviable before dilution.

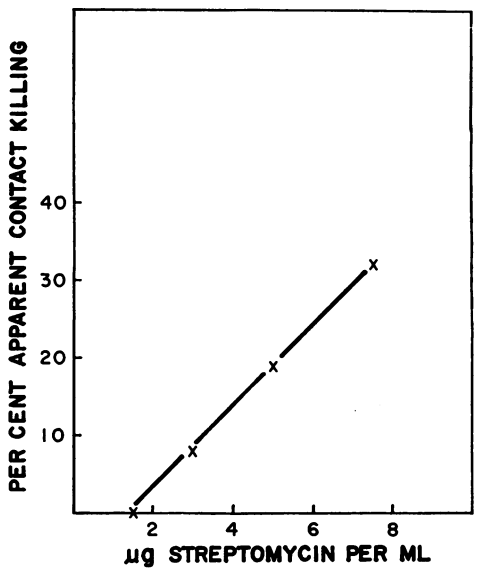

FIG. 1. *Apparent contact killing of Escherichia coli B by streptomycin. Twofold concentrated suspensions of exponentially growing E. coli B* 6×10^8*/ml in nutrient broth were exposed to various concentrations of streptomycin. Samples were removed for dilution in distilled water as rapidly as possible (within 10 sec) and plated in quadruplicate on nutrient agar. The results are expressed as per cent survivors.*

To test the deduction that the apparent contact killing was caused by adsorption of streptomycin on the cells, use was made of the ability of salt solution to displace adsorbed streptomycin.

When the above experiment was repeated with 0.85% saline as the diluent instead of distilled water, no contact killing was found even at 10 μg of streptomycin/ml. Further support for the conclusion that apparent contact killing resulted from adsorbed streptomycin is derived from the finding that initial accumulation of label from C^{14}-streptomycin conforms with the Langmuir adsorption isotherm equation (Hurwitz and Rosano, 1962*b*). In all of the following experiments, the cells were therefore diluted for viable counts in 0.85% saline.

The effect of exposure of the bacteria to

various concentrations of streptomycin on the kinetics of loss of viability was then studied. *E. coli* growing in nutrient broth were exposed to streptomycin at concentrations from 0.1 to 5.0 μg/ml. Samples were removed for dilution in saline and plating on nutrient agar as indicated. The results are shown in Fig. 2.

Change in viable count is expressed as per cent of the count at zero time. Below 0.5 μg/ml of streptomycin, some increase in viable count frequently occurs before the decline begins. When this occurred, the percentage of survivors

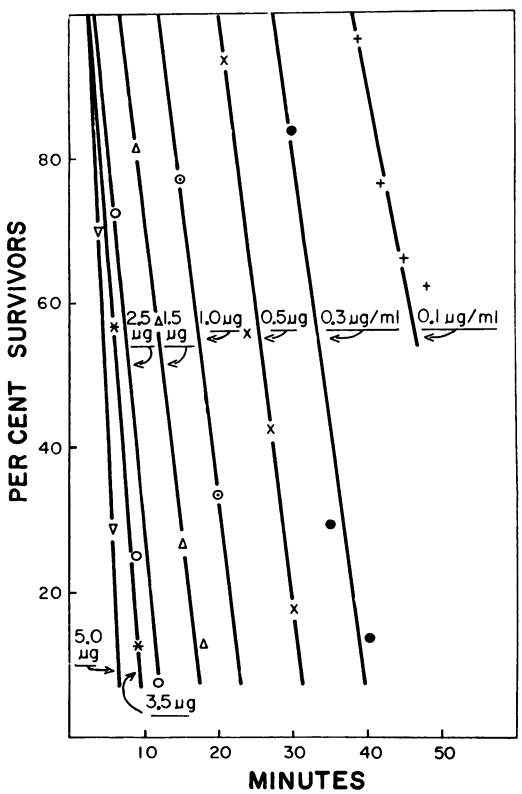

FIG. 2. *Effect of concentration of streptomycin on the kinetics of loss of viability of Escherichia coli B. A twofold concentrated suspension of E. coli B (6 × 10⁸/ml) growing exponentially in nutrient broth was exposed to various concentrations of streptomycin. Samples were removed for dilution in 0.85% saline and plating in quadruplicate on nutrient agar as indicated. The results are expressed as per cent survivors. Where the viable count increased before onset of the decline in viability (at streptomycin concentrations of 0.5, 0.3, and 0.1 μg/ml), the percentages were calculated from the highest viable count achieved.*

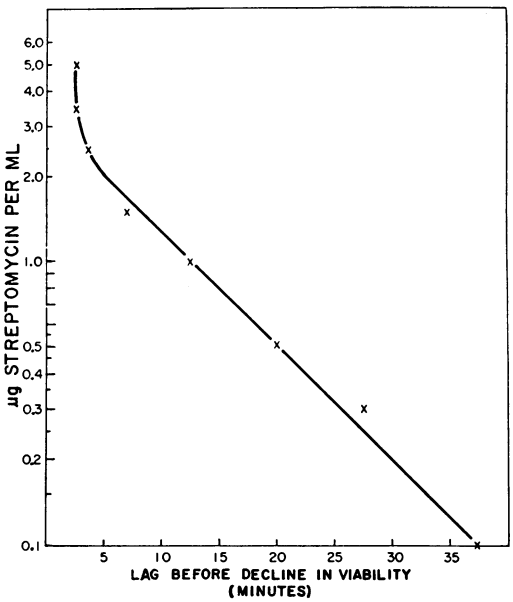

FIG. 3. *Effect of concentration of streptomycin on the length of the lag period before onset of the decline in viability of Escherichia coli B. The data are extracted from the results in Fig. 2.*

was calculated from the highest number of viable cells achieved before the decline began.

As streptomycin concentration increases, the length of time before loss of viability starts decreases. If one assumes that killing starts only after a given amount of streptomycin-initiated protein is synthesized, the length of the lag period before loss of viability starts becomes a measure of the rate of synthesis of the protein. If the length of the lag before onset of loss of viability is plotted as a function of the logarithm of streptomycin concentrations, the points fit a straight line between 2 and 0.1 μg/ml (Fig. 3). The equation of such a function can be expressed as $Y = Ae^{-kt}$; where Y is the concentration of streptomycin and t is the length of the lag in min before the linear decline in viability begins. A and k are constants which can be derived from the data. A is the ordinate intercept of the linear part of the semilog plot and k is the slope of the line.

From these results, A is found to be 2.7 μg of streptomycin/ml and k is found to be 0.02/min. These values are reasonably reproducible, although considerable variation in rate of loss of viability is found from one experiment to another. The greatest variability among the

numerous experiments we have performed is found in the rate of loss of viability after killing starts. This parameter has little effect on the values of A or k.

The reasons for the variations among experiments could not be entirely resolved. Population selection was ruled out as a factor by repeating the experiments with six substrains obtained from selected colonies. Successive experiments with each of the selected substrains showed the same variations as found in the original population. Careful attention to the concentration of the bacterial population and to its physiological state was of considerable importance in controlling variability.

The value of A (2.7 μg of streptomycin/ml) is the concentration at which killing would have occurred on contact if there were no lag period (such as would be required for a streptomycin-initiated protein synthesis). As seen from the data, the length of lag before onset of loss of viability never becomes zero, regardless of the extent to which the streptomycin concentration is raised. At values above about 2 μg/ml, the plotted data depart from the linearity and appear to become asymptotic to some value greater than 2 min.

As seen from Figs. 2 and 3, t (the length of the lag time) decreases exponentially as streptomycin concentration increases. If killing starts only after a given amount of streptomycin-initiated protein per cell is synthesized, then t is an inverse measurement of the rate of synthesis of this protein; and the rate of synthesis (R) increases exponentially with streptomycin concentration. From Fig. 3, this relationship can be expressed as $R = Be^{k_2 v}$, where k_2 is found to be 0.2 per μg of streptomycin/ml.

The rate of synthesis, then, appears to be a first-order function of the streptomycin concentration, reaching saturation levels at about 2 μg/ml of streptomycin. One would therefore expect that the maximal rate of lethal action of streptomycin would be reached within defined limits of the minimal lethal doses if the streptomycin-initiated protein synthesis is the rate-limiting reaction in the killing process.

Experiments were then undertaken to determine the lower limits of the lethal action of streptomycin. On addition of streptomycin to exponentially growing *E. coli* cells, the first effect seems to be a bacteriostasis which prevents or slows the increase in viable count, depending on the concentration of the antibiotic. At concentrations above 0.5 μg/ml, no increase in viable count occurs; at concentrations of 0.5 μg/ml or less, some cell division is found before the viable count declines. More growth occurs before net loss of viability begins at 0.1 than at 0.5 μg/ml (Fig. 4).

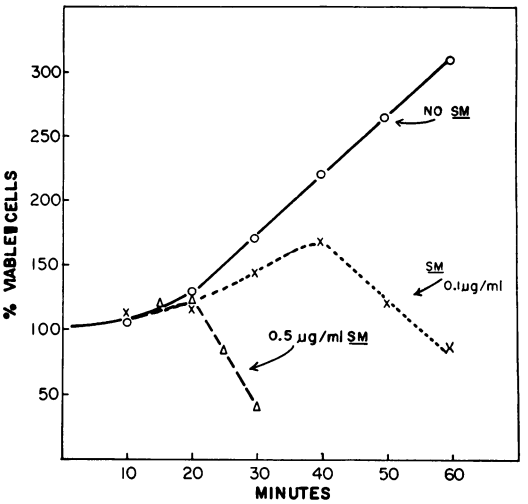

FIG. 4. *Effect of low concentrations of streptomycin on the viability of Escherichia coli B. Two-fold concentrated suspensions (6 × 10⁸/ml) of exponentially growing E. coli in nutrient broth were exposed to 0.5 and 0.1 µg of streptomycin/ml. Samples were removed for dilution in 0.85% NaCl and plating in quadruplicate on nutrient agar. The viable counts are expressed as percentages of the initial viable count.*

Presumably, decreasing the streptomycin concentration below 0.1 μg/ml would decrease still further the growth-inhibiting effect of the antibiotic until, at some limiting concentration, no effect would be found and no loss of viability would result. The lower limit of the lethal action of streptomycin, therefore, appears to be slightly below 0.1 μg/ml under the conditions investigated.

It is interesting to note that once it begins, the rate of loss of viability appears to be the same regardless of the streptomycin concentration between 0.1 and 2.5 μg/ml. The observed proportionality between the rate of decrease in viable count and streptomycin concentration therefore appears to be related to the length of the lag period before onset of loss of viability at concentrations below about 2.5 μg/ml.

At streptomycin concentrations above 2.5 μg/ml, killing starts in less than 3 min (Fig. 2). According to the present hypothesis, the streptomycin-initiated protein synthesis must have occurred before loss of viability begins. Since chloramphenicol blocks the lethal effect of streptomycin, presumably by preventing a streptomycin-initiated protein synthesis, it was necessary to show that the chloramphenicol block of protein synthesis was rapid enough to account for the prevention of killing by streptomycin at concentrations of the latter where the rate of synthesis of the postulated protein was maximal.

Experiments were therefore performed in which the effect of chloramphenicol and of streptomycin on protein synthesis was determined. Incorporation of C^{14}-leucine into the protein fraction was used as the measure of protein synthesis. To *E. coli* at 6×10^8 cells/ml in nutrient broth were added 100 μg/ml of C^{14}-leucine (specific activity 6.7×10^4 counts per min per μmole) and chloramphenicol (30 μg/ml) or streptomycin at the indicated concentrations. Synthesis was stopped by acidifying 1.0-ml samples in 5% TCA and chilling rapidly in an ice bath.

Chloramphenicol appears to block protein synthesis almost on contact (Fig. 5). The earliest sample taken (2 min after addition) shows a marked decrease in incorporation of C^{14}-leucine into the protein fraction. The block is not complete, since a small but significant linear increase in incorporation of label continued for the duration of the experiment.

The effects of streptomycin on protein synthesis are more complex. The first effect to be noticed is that the rate of protein synthesis is reduced but not completely blocked. The reduction in rate of protein synthesis increases with the streptomycin concentration. After some period of contact, the length of which decreases with increasing streptomycin concentration, a further decrease in the rate of protein synthesis begins.

The results again are consistent with the hypothesis that a streptomycin-initiated protein synthesis must precede the lethal action of the antibiotic. The blocking effect of chloramphenicol on protein synthesis is rapid and complete enough to account for its protective action against the lethal action of streptomycin, even

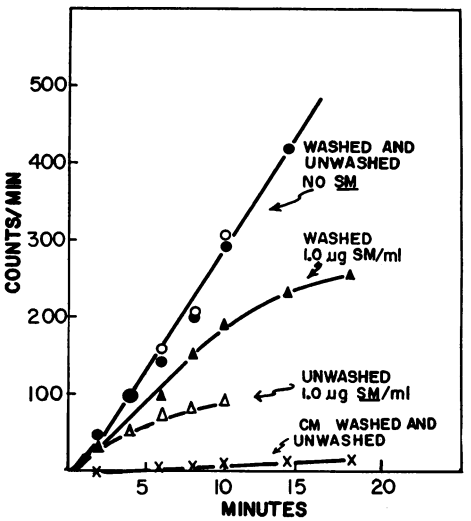

FIG. 5. *Effect of streptomycin and of chloramphenicol on protein synthesis by Escherichia coli B. Twofold concentrated suspensions (6×10^8/ml) of exponentially growing E. coli in nutrient broth were exposed to streptomycin or to chloramphenicol after the addition of 100 μg/ml of L-C^{14}-leucine (specific activity 6.7×10^4 counts per min per μmole). Protein synthesis was measured as the incorporation of C^{14}-leucine into the protein fraction. For the significance of the terms washed and unwashed see the legend under Fig. 6.*

at the high concentration of 5 μg/ml of the latter. It is interesting to note that the accelerated decline in rate of protein synthesis caused by streptomycin, but not the initial decrease of the rate of synthesis, is apparently closely related in time at least to the onset of loss of viability (compare Fig. 2 and Fig. 5).

One further effect should be noted. As seen from Fig. 2 and Fig. 4, the earliest observed effect of streptomycin on cell population is the bacteriostasis which precedes the decline in viable count. The decrease in linear rate of protein synthesis caused by streptomycin might explain the bacteriostatic effect, since this effect coincides well with the initial effect on the rate of protein synthesis. The postulated streptomycin-initiated protein synthesis must also occur during this period of reduced rate of protein synthesis. One can therefore conjecture that both the streptomycin-initiated protein synthesis and the initial effect of streptomycin on total protein synthesis may occur at the same sites, possibly in or on the cellular membrane.

It then becomes necessary to postulate that protein-synthesizing sites exist in or on the cellular membrane itself and that these sites are affected by streptomycin before other internal sites are contacted. Presumably, only effects at the internal sites are associated with death of the cells.

As described under Materials and Methods, all determinations on the effects of streptomycin on kinetics of killing and on kinetics of protein synthesis were carried out on cells which had been concentrated by centrifugation and resuspension in one-half the volume of fresh medium. Experiments were therefore undertaken to determine whether this procedure altered the sensitivity of the cells to streptomycin. The effect of streptomycin and of chloramphenicol on protein synthesis by resuspended and untreated exponentially growing cells was compared. Protein synthesis was measured as before by determining the rate of incorporation of C^{14}-leucine into the protein fraction. The results are reported in Fig. 5 and 6.

Total protein synthesis and the chloramphenicol-inhibition of protein synthesis are unaffected by the resuspension procedure. The effects of streptomycin on the kinetics of protein synthesis, however, are markedly delayed by resuspension of the cells in fresh medium. These experiments have been repeated many times, and in each instance the delay in the effect of streptomycin on protein synthesis caused by centrifugation and resuspension in fresh medium is closely paralleled by a delay in the onset of loss of viability.

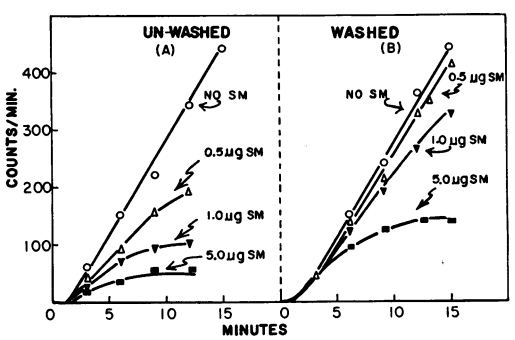

FIG. 6. *Effect of the concentration procedure on the kinetics of inhibition of protein synthesis by streptomycin. Exponentially growing Escherichia coli B cells in nutrient broth were divided into two portions. One portion was centrifuged and resuspended in the same volume of fresh medium. C^{14}-leucine and streptomycin or chloramphenicol, as indicated, were added and incorporation of label into the protein fraction was determined.* (A) *Effect of the antibiotic on protein synthesis by the noncentrifuged, exponentially growing population.* (B) *Effect of centrifugation and resuspension in fresh medium (washing) on these kinetics.*

If the lethal effect of streptomycin must be preceded by a streptomycin-initiated protein synthesis, as presently postulated, it would appear that centrifugation and resuspension in fresh medium delays this protein synthesis, but not total protein synthesis. Preliminary experiments have shown that synthesis of β-galactosidase is also delayed under parallel conditions. Further work is being carried out on this interesting phenomenon.

DISCUSSION

The thesis that a streptomycin-initiated protein synthesis must precede the actual lethal action of streptomycin is compatible with the kinetics of killing by streptomycin. Killing by streptomycin never occurs on contact, but is always preceded by a lag. The higher the concentration of streptomycin, the shorter is the length of the lag before killing begins. The lag period therefore corresponds to the chloramphenicol-sensitive initiation phase described in the previous report (Hurwitz and Rosano, 1962*a*), in which it was postulated that the amount of streptomycin-initiated protein synthesis by nonproliferating cells was also a function of the streptomycin concentration.

The evidence indicates that streptomycin, in addition to initiating protein synthesis and killing the bacteria, also has a bacteriostatic effect during the initiation phase. The first noticeable effect of streptomycin is a decrease in the rate of cell reproduction. At concentrations above 0.5 μg/ml, no increase in viable cell count is found before loss of viability begins. As the streptomycin concentration decreases below 0.5 μg/ml, the bacteriostatic effect also decreases. The bacteriostatic effect may be related to the inhibition of rate of protein synthesis by the antibiotic, which occurs before the onset of loss of viability.

The decrease in the length of the lag before onset of loss of viability as streptomycin concentration increases is interpreted to mean that the

rate of streptomycin-initiated protein synthesis is a first-order function of the streptomycin concentration. If one assumes that killing by streptomycin can occur only after a critical amount of streptomycin-initiated protein is synthesized, and that the rate of synthesis decreases exponentially as streptomycin concentration decreases, at low enough levels of streptomycin no killing should occur since the rate of synthesis of the protein would be less than the rate of cell proliferation.

Accordingly, at some low concentration of streptomycin, at which synthesis of the streptomycin-initiated protein becomes slower than cell proliferation, killing should abruptly cease because the cells could never attain a full complement of the protein. This conclusion is in accord with the increase in the amount of growth before killing begins as streptomycin concentration decreases and with the finding that, below 0.05 μg/ml streptomycin, there is a sharp cessation of killing by the antibiotic, whereas at 0.06 μg/ml, an 80 to 90% decrease in viable count can be detected even at so-called subinhibitory levels (Hurwitz et al., 1955).

The finding that apparent contact killing of streptomycin-exposed cells can be prevented by washing or dilution in saline is reminiscent of the report by Wasserman, Lessner, and West (1954) that actively dividing cells can be rendered incapable of growing by as little as 90 sec of exposure to 25 μg of streptomycin/ml. Suspension of the cells in ammonium salt solution after 5 min, but not after 15 min exposure to the antibiotic, largely prevented loss of viability. This finding was interpreted by these authors to mean that cells were "reactivated" by contact with NH_4^+ salts. In view of the finding (Berkman et al., 1948) that adsorption of streptomycin by bacterial cells is greatly reduced in the presence of sodium chloride, and in view of the presently reported experiments on the effect of the dilution medium on apparent viability of streptomycin-exposed cells, it would appear more likely that apparent "reactivation" of streptomycin-killed cells by NH_4^+ salts reflects displacement of adsorbed streptomycin from the cell surface.

Pardee and Prestidge (1961) found that a lag of 3 min preceded synthesis of the four different induced enzymes studied. It is interesting to note that a similar lag period in the onset of killing by streptomycin is found to be the irreducible minimum of time required. Sufficient time for synthesis of the postulated streptomycin-initiated protein is therefore available to the cells.

ACKNOWLEDGMENTS

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LITERATURE CITED

BERKMAN, S., R. J. HENRY, R. D. HOUSEWRIGHT, AND J. HENRY. 1948. Streptomycin. IV. Adsorption of streptomycin by susceptible and resistant bacteria. Proc. Soc. Exptl. Biol. Med. **68**:65–70.

HURWITZ, C., AND C. L. ROSANO. 1962*a*. Chloramphenicol-sensitive and -insensitive phases of the lethal action of streptomycin. J. Bacteriol. **84**:1202–1209.

HURWITZ, C., AND C. L. ROSANO. 1962*b*. Accumulation of label from C^{14}-streptomycin by *Escherichia coli*. J. Bacteriol. **84**:1193–1201.

HURWITZ, C., C. L. ROSANO, B. BLATTBERG, AND J. ROSENFELD. 1955. Effect of subinhibitory doses of dihydrostreptomycin on culturability of *Escherichia coli*. Antibiotics & Chemotherapy **5**:474–479.

PARDEE, A. B., AND L. S. PRESTIDGE. 1961. The initial kinetics of enzyme induction. Biochim. et Biophys. Acta **49**:77–88.

WASSERMAN, A. E., J. M. LESSNER, AND M. K. WEST. 1954. Reversal of the streptomycin injury of *Escherichia coli*. J. Gen. Physiol. **38**:213–223.