REVERSAL OF THE STREPTOMYCIN INJURY OF ESCHERICHIA COLI*

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(Received for publication, June 30, 1954)

It has been reported that the inhibitory action of streptomycin on bacteria can be counteracted by culture medium components, pH, salts, etc. (1–4). In many instances, however, these results were obtained by seeding a small inoculum of organisms into media containing varying concentrations of the antibiotic and the material tested, and determining the occurrence of growth after a period of incubation of approximately 24 hours. Little cognizance was taken of the metabolic activity of the organisms surviving the antibiotic treatment, although this may differ markedly from that of the parent organisms. Growth may have resulted from (1) selection of a few resistant organisms, (2) adaptation of the majority of cells in the original inoculum to alternate metabolic pathways, or (3) a general stimulatory effect of the added test compound which would increase growth of normal cells as well as that of cells exposed to streptomycin.

To demonstrate the true reversal of the antibiotic action of streptomycin it should be shown that (1) almost all the organisms present (at least 99.9 per cent) are injured after a short exposure to the antibiotic, (2) in the presence of the reversing agent, the viable count of the streptomycin-treated culture returns to approximately that of the control, and (3) the duration of exposure to the reversing agent is short enough to preclude the possibility of selection of resistant organisms or the adaptation of the culture to the new conditions.

To fulfill these requirements it would appear desirable to utilize a culture in the logarithmic growth phase, carried in a chemically defined medium. The presence of an inhibitor, therefore, would interfere with the normal activities of actively metabolizing cells, and the organisms would be injured in such a manner that death would follow quickly unless some interruption of the process were introduced. Reversal of this pathological condition could be accomplished by the removal of the toxic agent and the addition of substances which could be

* This paper was presented in part at a meeting of the Southeastern Pennsylvania Branch of the Society of American Bacteriologists in April, 1953.

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utilized by the cell for the immediate resumption of normal activity. The effects of such an injury, and the activity of various substances in reversing the injury, can be detected in several ways; *e.g.*, changes in viable cell counts, or metabolic activity. The study of the non-lethal injury of bacterial cells by antibacterial agents, and the means whereby these injuries may be reversed may be useful in revealing the mode of action of these agents relatively unobscured by the subsequent degradative reactions that take place in irreversibly injured cells.

This paper reports on the results of an investigation, based on the above principles, of the antibiotic activity of streptomycin on *Escherichia coli*, and the process of the reversal of this inhibition.

Methods and Material

The organism used in these studies was a laboratory strain of *E. coli* (No. 760). It was grown in a casein hydrolysate medium (CH) composed of Difco casamino acids, 1 per cent; K_2HPO_4, 0.05 per cent; $MgCl_2$, 0.01 per cent; KCl, 0.02 per cent; pH 6.8–6.9. 5 ml. of an overnight culture was seeded into 200 ml. of CH medium in a 500 ml. Blake bottle. This inoculum was usually sufficient to start the culture with a turbidity of 4 to 5 units on the Klett-Summerson photometer (No. 54 filter). The culture was incubated at 35°C. on a reciprocal shaker until the turbidity reached approximately 70 Klett units, usually within 2 ½ to 3 hours. To aliquots of the culture was added sufficient streptomycin to give the final desired concentration, using a stock solution of 5000 μg./ml. streptomycin base, as the calcium chloride complex. Plate counts were made prior to the addition of the antibiotic, and subsequently at the time intervals noted. Nutrient agar was used for plating purposes, and the dilutions of the cultures were made in distilled water unless otherwise specified. The final plates never contained more than one-tenth of the bacteriostatic concentration of streptomycin.

The synthetic medium used in certain of these studies was a glucose-inorganic salt solution (GIS) composed of glucose, 1.25 per cent; K_2HPO_4, 0.5 per cent; $MgSO_4$, 0.025 per cent; NH_4NO_3, 0.625 per cent; Na_2SO_4, 0.625 per cent.

RESULTS

Rate of Streptomycin Inhibition.—Streptomycin was added to an aliquot of a culture to give a final concentration of 25 μg./ml. Samples were removed at the time intervals indicated in Fig. 1, and viable cell counts made. It was found that in samples taken 30 seconds after the addition of the antibiotic, the shortest time of sampling possible, and plated within 5 minutes in nutrient agar, the viable count had dropped approximately 99.9 per cent. Further incubation of the organisms in the presence of the antibiotic caused only an additional small decrease in the number of viable cells. An exposure time of 2 ½ to 5 minutes was selected as the treatment that would cause injury to a suitable number of organisms (in the shortest convenient time interval).

The concentration of streptomycin (20 to 200 μg./ml.) does not appear to affect greatly the initial rate of bactericidal effect of the antibiotic. Culture aliquots treated with 20 and 200 μg. streptomycin (final concentration) show very little difference in the viable count within the first 15 minutes of treatment. After 2 hours of exposure the 20 μg. culture exhibited only a small, further decrease in the number of viable organisms, whereas the viable count in the 200 μg. culture had dropped to less than 100 organisms/ml.

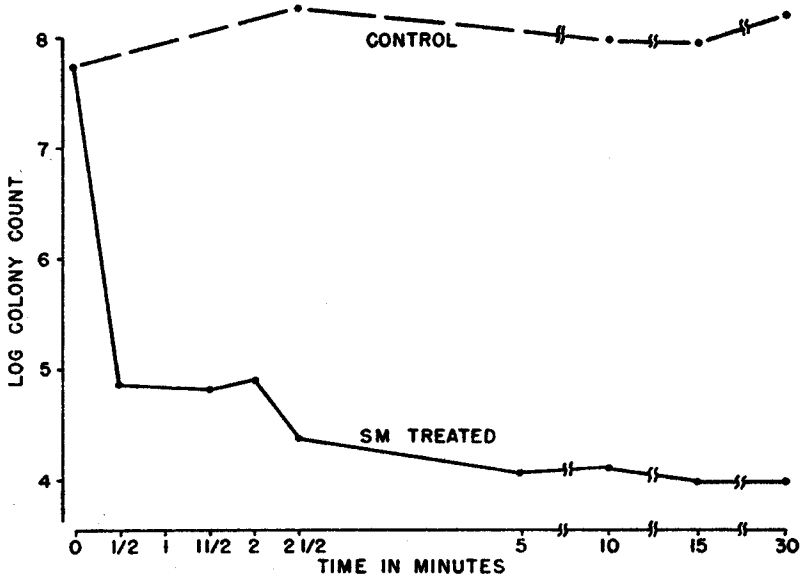

FIG. 1. Rate of survival of an *Escherichia coli* culture exposed to 25 μg. streptomycin/ml.

Reversal of Streptomycin Inhibition.—Streptomycin, at a final concentration of 20 μg/ml., was added to 1 aliquot of a culture. After 5 minutes of continued incubation at 35°C. the viable cell count had dropped from 8×10^7 to 2.6×10^3 organisms/ml. (Fig. 2). Subcultures at a 1:100 dilution of the control and treated cultures were then made into fresh CH medium. At intervals plate counts were made of both the original cultures and the subcultures. 15 minutes after subculturing, the culture exposed to the antibiotic had returned to the original count, 8.6×10^7 viable organisms/ml. (correcting for the 1:100 dilution), and the rate of growth then parallelled that of the control subculture.

Thus, the CH medium appears to have induced an immediate reversal of the streptomycin injury in the cells exposed to the antibiotic. That this is a true reversal and not a regrowth of cells may be inferred from the rapid rate of recovery of the number of viable organisms. In the 15 minute incubation period

there was an increase of approximately 3.5 logs in the viable count. For normal multiplication to account for this increase a generation time of the order of a few minutes would be required.

Although CH used as a broth is capable of reversing the streptomycin injury, this same medium when solidified with agar and used as a plating medium did not cause reversal. It would appear, therefore, that reversal is dependent on the rapid and intimate contact of the culture medium with the cell, as occurs in the liquid state.

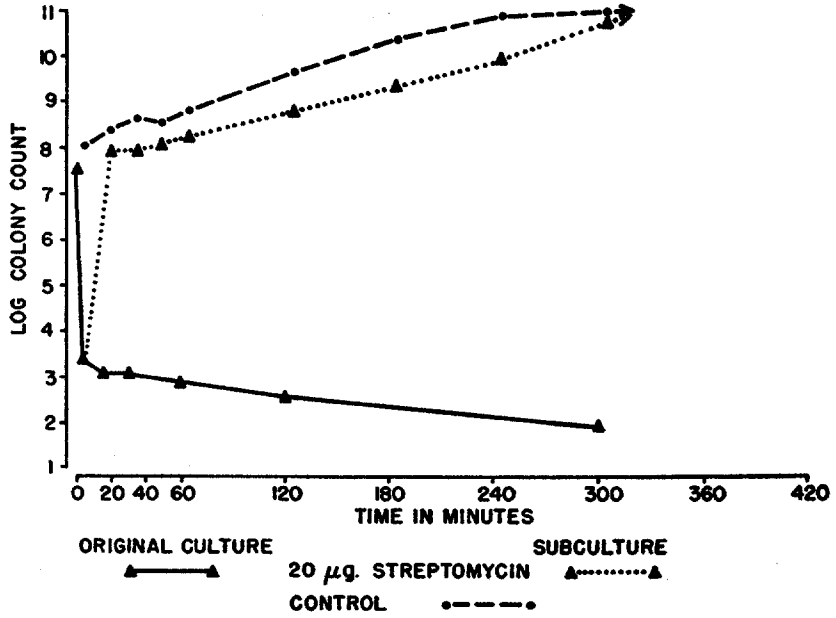

FIG. 2. Reversal of injury of a streptomycin-treated culture of *Escherichia coli* on subculture into fresh medium.

The Effect of Duration of Exposure to Streptomycin upon Reversal of Injury.— To determine the relationship between the duration of exposure to streptomycin and the reversibility of the injury thus induced, the antibiotic was added to an aliquot of a culture to a final concentration of 50 μg./ml. In samples taken after 2½ minutes of exposure the viable count dropped from 1.8×10^8 to 1.7×10^4 organisms/ml. Subcultures were made at intervals into fresh CH medium and incubated at 35°. Plate counts of the subcultures were made beginning 15 minutes after the introduction of the injured cells into the fresh medium. It was found (Fig. 3) that complete reversal occurred if the treated organisms were transferred within 2½ minutes of exposure to the antibiotic. As the duration of exposure increased, the degree of recovery became cor-

respondingly less until at 15 to 30 minutes of exposure to streptomycin, there was practically no noticeable recovery. In addition, a lag period was imposed on the subculture before growth resumed. The lag increased directly with the length of exposure (see Fig. 3). The organisms exposed to the antibiotic for 60 minutes exhibited no signs of recovery in the fresh medium for the 2 hours during which they were under observation. However, at some later time growth occurred, as continued overnight incubation resulted in fully grown cultures.

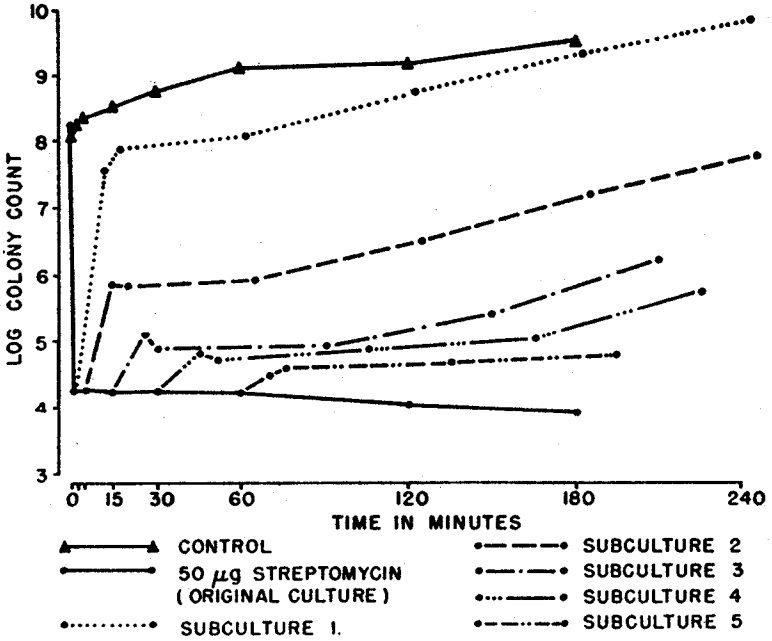

FIG. 3. Reversal of injury of *Escherichia coli* exposed to streptomycin for varying periods of time prior to subculture in fresh medium.

Development of Resistance in Streptomycin-Treated Cultures.—It was noted in the above experiment, as with previous ones, that all the subcultures contained approximately the same number of viable organisms if allowed to incubate at 35° for 20 hours. Since some of the subcultures recovered from the streptomycin injury very rapidly and continued to multiply at a normal rate, it was believed the organisms in these cultures would exhibit no change in their streptomycin sensitivity. However, in some of the subcultures, in which recovery of viability appeared to be slow and a lag period intervened before multiplication resumed, it was possible that selection of streptomycin-resistant organisms could be repopulating these cultures and that the lag period was the prelude to the organization of a streptomycin-resistant culture.

To determine whether resistance was a factor in the apparent recovery of these subcultures the streptomycin sensitivity of the 20 hour cultures in the previous experiment was determined. It was found that the original control culture and all the subcultures, from both the control and streptomycin-treated cultures, were sensitive to <5 μg. of streptomycin/ml. Only the growth in the culture continuously exposed to 50 μg./ml. of the antibiotic agent was found to be resistant to >500 μg. streptomycin/ml. Thus, even after 1 hour of exposure to the antibiotic, and the presence of an injury which did not respond to subculturing in fresh medium for 2 hours, resistance was not established.

Effect of Some Substances on the Reversal of Streptomycin Injury.—It was not feasible to study the effects of the individual components of the CH medium on the reversal of the injury because of the complexity of the casein hydrolysate. The simple synthetic medium, GIS, was suggested as a substitute, since cells grown in the CH medium and treated with streptomycin, exhibited reversal of injury as readily on subculture into GIS as into fresh CH medium. It was ascertained that 50 μg. of streptomycin/ml. did not inhibit the growth of the organism in this medium. This is probably due to the shift in pH that occurs during the growth of the culture. The pH drops to approximately 5.2 after 2 ½ to 3 hours of growth. When the various components of the GIS medium were tested individually for streptomycin injury reversal, it was found (Fig. 4) that complete reversal was obtained when 0.078 M NH_4NO_3 was used as the subculture medium. Transference into water, 0.07 M glucose, or 0.001 M $MgSO_4$, at a 1:100 dilution of the original culture, not only did not reverse the injury but actually appeared to accelerate the bactericidal action of the antibiotic. Anomalous results were obtained with 0.029 M K_2HPO_4 and 0.045 M Na_2SO_4—there was recovery from streptomycin injury in samples plated within 15 minutes of transference to the salt solutions, but on continued incubation (up to 60 minutes) the number of viable cells fell off again very rapidly.

When streptomycin-injured cells were transferred into various 0.05 M salt solutions, it was found that with ammonium citrate reversal was complete in 15 minutes, and the cells maintained their viability over the 2 hour period. Ammonium tartrate and dibasic ammonium phosphate induced a slow reversal which was complete in 60 minutes. Potassium nitrate and sodium nitrate, however, behaved like glucose or water, there was a rapid decrease in the number of viable cells in 15 minutes. Ammonium chloride, like K_2HPO_4 and Na_2SO_4, induced complete recovery within 15 minutes, but the number of viable cells declined again by the end of 2 hours of incubation.

Since in all the previous experiments water had been used as the diluent in preparing the plate counts, it appeared to be of interest to determine the effect of the salts used as diluents for the determination of the viable count

after streptomycin treatment. Accordingly, after exposing a culture to 50 μg. streptomycin/ml. for 2½ minutes, dilutions were made in various concentrations of salt solutions and plated on nutrient agar. The response obtained can be divided into four families of curves, and representatives of each are shown in Fig. 5. Curve I indicates that with from 0.01 to 0.5 M concentrations of NH_4Cl, NH_4NO_3, KNO_3, and $NaNO_3$ complete reversal of streptomycin injury was obtained. $Ca(NO_3)_2$ was used in the range 0.005 to 0.25 M concentrations, also with complete reversal at each level. Curve II

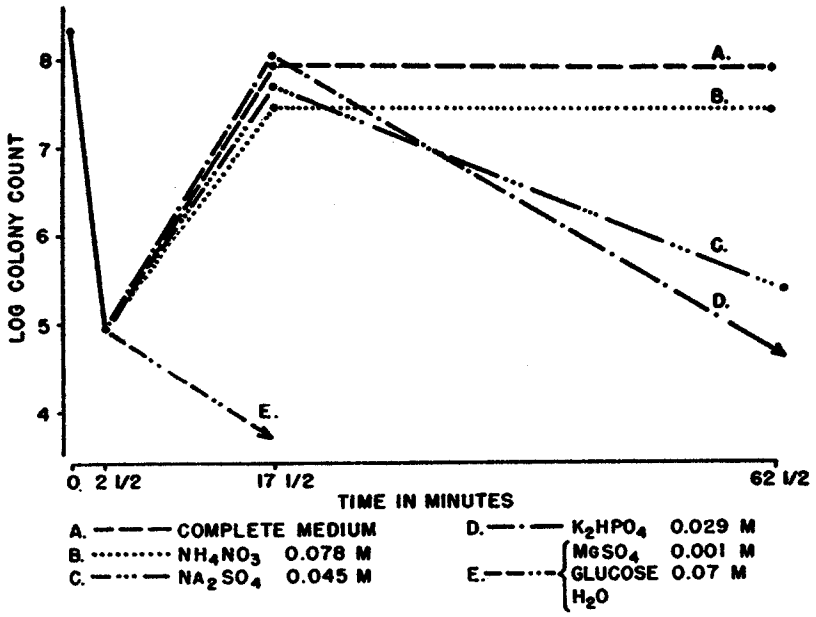

FIG. 4. Reversal of streptomycin injury of *Escherichia coli* on subculture into a chemically defined medium and its component constituents.

shows that increasing recovery occurred with increasing concentration of NaCl, until complete reversal was obtained with 0.1 M concentration of the salt. The same picture was found with K_2HPO_4, with complete reversal occurring at 0.03 M concentration. Glucose and urea caused no reversal of injury from 0.01 to 0.1 M concentrations. Partial recovery occurred at 0.25 M, and complete recovery at 0.5 M concentrations of these compounds (curve III). Glycine and glycerol (curve IV) allowed no reversal of the antibiotic injury over the range of concentrations tested—0.01 to 0.5 M.

The reversal of streptomycin injury by some concentrations of various salts apparently depends on the duration of the contact between the organisms and the salt solutions. For example, KNO_3 and $NaNO_3$, at 0.05 M concentra-

tion, were shown to cause a very rapid decrease in the viable count of streptomycin-treated cultures, if the organisms were transferred into these salt solutions for 15 minutes. However, this concentration of both salts induced complete recovery of the injured cells if they were only diluted through the salt solutions prior to plating for viable cell count determinations.

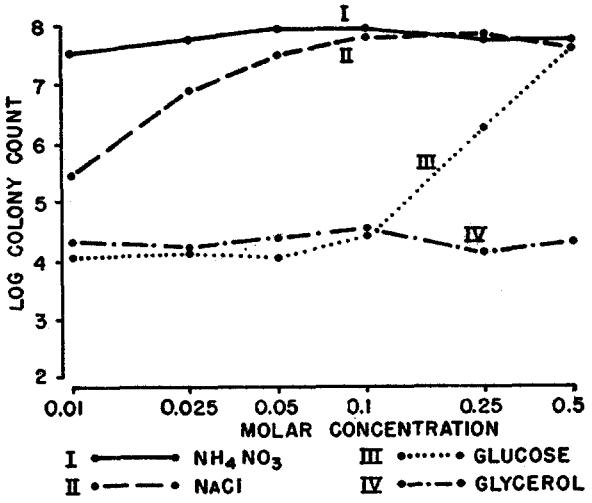

FIG. 5. Reversal of streptomycin injury of *Escherichia coli* following subculture and dilution in varying concentrations of salt solutions. (See text for complete list of salts.)

DISCUSSION

Using a young actively growing culture of *E. coli*, it is possible to study the activity of streptomycin. In samples taken within 30 seconds of adding the antibiotic it was found that the viable count had decreased 99.9 per cent. Cells in such a pathological condition are ideal tools for the study of the reversal of the activity of streptomycin. The organisms, in a state of high metabolic activity, have been injured to such an extent that under certain conditions of further treatment death of the cells is observed. Before death ensues, however, the cells have the potentiality of circumventing the injury and resuming normal reproductive activity. This is in contrast to the state of the cells in a small inoculum from an overnight culture, added directly to medium containing streptomycin. Such organisms are at a low level of metabolic activity and would have a decreased potentiality for overcoming the inhibition by the antibiotic, particularly in the continued presence of the antibiotic. Growth occurring under such circumstances may be due to selection of streptomycin-resistant organisms, or to the development of alternate metabolic pathways.

The inhibition of young cells can be reversed by subculture into fresh culture medium. This is a true reversal of the injury since the original viable count again is attained within 15 minutes of subculturing the injured cells. Dilution of the antibiotic below the active level on subculturing cannot be the explanation of the recovery, since upon similar dilution in water the death rate of the streptomycin-treated cells is accelerated, and the control culture of normal cells maintains its viable count at a constant level.

The physical state of the culture medium is of importance in the reversal of the antibiotic injury. The cell must be bathed by the medium for recovery to occur, since the same medium solidified with agar permits the destruction of injured cells. This suggests that the organisms require a "reversing substance" which is present in the medium in limited concentration. In the liquid state, constant replacement of this substance in close proximity to the cells is possible. This, however, would not occur in the solidified medium since the agar may prevent the "reversing substance" from reaching the cell, either by (1) reducing the rate of replacement in the vicinity of the colony, or (2) formation of a complex.

There is a time limit, however, beyond which reversal as such does not occur. After 15 to 30 minutes of exposure to streptomycin the cells no longer show true reversal of the injury. After a lag period, which increases with the duration of the exposure to streptomycin, the cells begin to multiply again at approximately the normal rate. Selection of streptomycin-resistant organisms has been ruled out, and the presence of the lag period suggests the possibility of adaptation to alternative metabolic pathways as the cause of this regrowth. The length of the lag period depends on the extent of the streptomycin injury and the ability of the cell to supply another route for maintaining metabolic equilibrium.

It has been claimed that the salts, NaCl and K_2HPO_4, reverse the inhibition induced by streptomycin by desorbing, or replacing, the antibiotic on the cell surfaces (4–6). However, the action of various salts, and other substances, on the reversibility of the streptomycin injury, as demonstrated in this paper, leaves this explanation open to question. It is believed that the salts reversing the antibiotic injury are physiologically active rather than acting solely in a physical manner.

The toxic effect of streptomycin on *E. coli* occurs in two stages. Contact with the antibiotic immediately results in an injury to the organism which manifests itself in abrupt cessation of multiplication (7) as determined by turbidimetric measurement, and in disorganization of the cellular membrane. This injury may be reversed completely within a few minutes of exposure to streptomycin. The second state occurs on longer contact with the antibiotic, and appears to be irreversible. The primary injury may be caused by the inhibition of an enzymatic system lying on, or near, the cell surface. This

system may be involved in reactions supplying the energy necessary to maintain the integrity of the cell membrane. Disruption of this vital function by streptomycin results in the loss of selective permeability of the membrane, accompanied by plasmoptysis, and even lysis, of the cells (8–10). It has been postulated that reactions involving oxidative phosphorylation supply the energy necessary for the maintenance of the membrane and for cellular reproduction, and that streptomycin inhibits oxidative phosphorylation (7). The effect of some salts in reversing the primary injury may be ascribed to their ability to reestablish an osmotic equilibrium, thus preventing the loss of vital components from the organism, or inhibiting free passage of water into the cell.

The secondary, or irreversible, phase of the lethal action of streptomycin occurs on exposure of the cells to the antibiotic for a period of 30 minutes or more. In this interval other vital enzymatic systems have been affected irreversibly. At this stage, the action of the salt solutions can no longer cause the recovery of the cells.

After the completion of this manuscript a paper appeared (11) describing the reversal of the killing effects of heat, chlorine, hydrogen peroxide, zephiran, and ethyl alcohol. Various metabolites of the Krebs tricarboxylic acid cycle, individually and in combination, reactivated the "injured" bacterial cells.

SUMMARY

The number of viable *Escherichia coli* in a young, actively growing culture is decreased approximately 99.9 per cent by a 30 second exposure to 25 μg. streptomycin/ml. The injury induced by the antibiotic is only potentially lethal, however, and may be reversed by subculture within 5 minutes into fresh culture medium, NH_4NO_3, NH_4Cl, $(NH_4)_2HPO_4$, NH_4 citrate, and NH_4 tartrate. Subculturing into water, glucose, or $MgSO_4$ results in a more marked decrease in the number of viable organisms. In KNO_3, $NaNO_3$, K_2HPO_4, and Na_2SO_4 solutions reversal occurs first, followed by a rapid decrease in viability.

True reversal of the streptomycin injury takes place, as demonstrated by the rapid rate of recovery to the viable count of the original culture. Development of resistance has been eliminated as the cause of regrowth since the streptomycin sensitivity of recovered cultures remained the same as that of the original culture.

The use of water as diluent for viability determinations potentiates the lethal effect of streptomycin activity. Several compounds, at various dilutions, substituted for water as the diluent gave rise to four types of responses, group I, NH_4NO_3, NH_4Cl, KNO_3, $NaNO_3$, $Ca(NO_3)_2$, showed complete reversal of the streptomycin injury at all levels of the salts tested, from 0.01 to 0.5 M concentrations. Group II, NaCl and K_2HPO_4 showed complete reversal at 0.03

and 0.1 M. Group III, glucose and urea allowed complete reversal at 0.5 M. Group IV, glycerol and glycerine showed no reversal at 0.5 M concentration.

The reversal of the streptomycin injury to young actively growing bacteria is suggested as a tool for studying the pathology of the injury to the cells.

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