Bacteriostatic Action of Streptomycin on Ribosomally Resistant Mutants (\textit{rpsL}) of \textit{Salmonella typhimurium}

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Incubation of streptomycin-resistant (\textit{rpsL}) mutants of \textit{Salmonella typhimurium} in alkaline nutrient medium containing streptomycin brought about an inhibition of cell growth that was readily reversed by removing the antibiotic or neutralizing the medium. Growth inhibition was maximal at pH 8.2 and a streptomycin concentration of 800 \textmu{g}/ml. A similar amount of dihydrostreptomycin had a negligible effect, and 10-times higher concentrations of this antibiotic were required to reproduce the streptomycin action. Addition of streptomycin (400 \textmu{g}/ml) to \textit{rpsL} cells in alkaline (pH 8.2) nutrient medium caused inhibition of protein and DNA synthesis and also, but to a lower degree, of RNA synthesis. This effect on macromolecular synthesis was not due to ATP deprivation, since ATP content rose after addition of the antibiotic. At pH 8.2, the rate of entrance of streptomycin increased fourfold with respect to the rate at pH 7.0, leading to a large accumulation of streptomycin into \textit{rpsL} cells. Uptake of the antibiotic was halted by addition of KCN or chloramphenicol. Equal uptake was obtained with 800 \textmu{g} of dihydrostreptomycin or 400 \textmu{g} of streptomycin per ml, yet the former did not affect cell growth at that concentration. It is concluded that high pH stimulates streptomycin and dihydrostreptomycin uptake by \textit{rpsL} strains but only streptomycin accumulation causes growth inhibition in cells lacking the high-affinity ribosomal site.

Streptomycin and dihydrostreptomycin are closely related antibiotics differing only by the presence in the latter of a hydroxyl group instead of the aldehyde group carried by the former. This difference is not important as far as antibacterial effect is concerned, and for a long time the two substances were considered about identical and were used indiscriminately in extensive research aimed at disclosing the mode of action of streptomycin and the mechanism allowing its accumulation in the cell.

In 1963, Moskowitz (15) discovered that streptomycin was much more effective than dihydrostreptomycin in precipitating nucleic acids. Shortly afterwards, Brock (4) found that streptomycin but not dihydrostreptomycin inhibited plaque formation when certain bacteriophages were plated on streptomycin-resistant host cells. He also reported that streptomycin produced that effect by interacting with the proteins of phage and suggested that a complex formed between the aldehyde and the N-methyl group of streptomycin was responsible for those actions not shared by dihydrostreptomycin (4).

Although Moskowitz (15) proposed some conditions that could help to reveal differences in the action of streptomycin and dihydrostreptomycin on bacteria, no reports on such a difference manifested in vivo have appeared up to now.

While performing genetic studies on a group of cell envelope-defective mutants of \textit{Salmonella typhimurium} which are unable to grow on alkaline (pH 9.5) nutrient agar (2, 3), it was observed that addition of streptomycin to alkaline medium resulted in growth failure of ribosomally resistant \textit{rpsL} strains that in neutral medium endured large amounts of streptomycin.

This paper reports a preliminary study of this phenomenon. The results obtained indicate that alkaline pH promotes massive entrance of streptomycin into the cell, resulting in an almost simultaneous inhibition of protein, DNA, and RNA synthesis. Similar cellular concentrations of dihydrostreptomycin are not effective in impairing cell growth.

**MATERIALS AND METHODS**

**Bacterial strains.** \textit{S. typhimurium} DA1030 (\textit{rpsL125}) was used in all the experiments reported. This strain was obtained by transducing into wild-type strain LT2 the mutation \textit{rpsL125} present in strain SB558 (\textit{purF145 rpsL125}), received from P. E. Hartman. Transduction was performed with phage P22 HT1051/1 \textit{int-201} grown on SB558, and transductants carrying the \textit{rpsL125} allele were selected directly on nutrient agar containing 500 \mu{g} of streptomycin per ml after 4 h of incubation in nutrient broth to allow phenotypic expression of the transduced mutation. Strain DA1030 was obtained free of phage by three successive single-colony isolations. The strain was constructed by transduction to avoid treatment of LT2 with a mutagenic agent.

**Media.** Routine liquid medium was nutrient broth (NB; Difco Laboratories) containing 5 g of NaCl per liter. Viable counts were performed by plating in nutrient agar obtained by adding 15 g of agar per liter to NB. All the experiments related to the effect of streptomycin or dihydrostreptomycin in alkaline medium were performed in NB containing 2.5 g of NaCl per liter and 50 mM MOPS (3-(N-morpholino)propanesulfonic acid) buffer, and the pH was adjusted to the required value with NaOH. This medium, named NBM, was preferred to plain NB because cultures in NBM maintained the original pH even after prolonged incubation, whereas the pH of NB cultures changed during incubation.

All of the drugs used were obtained from commercial sources. Streptomycin sulfate was bought from Lepetit S.A. (Buenos Aires, Argentina), and dihydrostreptomycin sulfate was a gift of Laboratorios Bagó (Buenos Aires, Argentina). \text{[2,14C]}Juracil (50 mCi/mmol), \text{[6-3H]}thymidine (29 Ci/mmol), and \text{[L-\text{U-14C}]}leucine (300 mCi/mmol) were purchased from the Commissariat à l’Energie Atomique (Saclay, France); NaB\textsubscript{3}H\textsubscript{4} (8.0 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

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The OD$_{650}$ (TCA)-precipitable tures by the required measured. centrifugation. of 0.850 in water. and then eluate the time and BaCl$_2$ and pH 8.2 without drug. (Inset) Effect of high pH and streptomycin on the viability of strain DA1030.

Preparation of $[^3]$H)dihydrostreptomycin. $[^3]$H)dihydrostreptomycin (2 Ci/mmol) was prepared in this laboratory by reduction of streptomycin with NaB$_4$H$_4$ following, with some modifications, a procedure patented by Pfizer and Co. (Chem. Abstr. 68:2155, abstr. no. 22212b, 1964). To 100 mg of wet Dowex 50, 1 ml of streptomycin sulfate (5 mg/ml) was added, and the mixture was adjusted to pH 9.0 with NaOH. After sedimentation, the supernatant was discarded, and 50 mCi of NaB$_4$H$_4$ was added to the resin, which was maintained at room temperature for 1 h with permanent stirring and then washed five times with 4-ml portions of distilled water. To elute the $[^3]$H)dihydrostreptomycin synthesized, the resin was treated three times with 2-ml portions of 0.5 M BaCl$_2$ and was stirred for 45 min between each treatment. All the eluate fractions were pooled, and enough Na$_2$SO$_4$ was added to precipitate all Ba$^{2+}$ as BaSO$_4$, which was removed by centrifugation.

Effect of streptomycin on cell growth. Experimental cultures were started by diluting exponential cells to an optical density at 650 nm (OD$_{650}$) of 0.1 in the appropriate medium. The cultures were incubated with shaking at 37°C, and at regular intervals, samples were withdrawn and the OD was measured. OD values were plotted against time, and the time required by the control cultures (without streptomycin) to reach an OD$_{650}$ of 0.850 was determined (usually 150 min). The OD of the experimental cultures at that time was compared with the OD of the control culture, taking an OD$_{650}$ of 0.850 as 0% inhibition.

Synthesis of DNA, RNA, and protein. Incorporation of $[^3]$H)thymidine (5 μCi/ml) and either $[^14]$C)uracil (0.63 μCi/ml) or $[^14]$C)leucine (0.75 μCi/ml) into cold trichloroacetic acid (TCA)-precipitable material was measured. Exponential cultures in NBM broth containing the corresponding radioac-tive compounds and 400 μg of adenosine per ml were grown to an OD$_{650}$ of 0.1, and then streptomycin was added to a final concentration of 400 μg/ml (time zero). At different times, 0.1-ml samples were withdrawn and mixed with 3 ml of cold 10% TCA. After 5 min, the samples were filtered through membranes (pore size, 0.45 μm; Millipore Corp.) and washed three times with 3-ml portions of cold 10% TCA. The membranes were dried and counted with a toluene-based fluid in a liquid scintillation counter (Packard Tri-Carb 4530) set for double-label counting.

Oxygen consumption. Oxygen consumption was measured with a Clark electrode in conjunction with a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio).

ATP determination. ATP content was measured in Tris hydrochloride extracts by the luciferase-luciferin method described by Holm-Hansen and Karl (14).

Accumulation of streptomycin and dihydrostreptomycin. Uptake of streptomycin and dihydrostreptomycin was measured by the procedure of Bryan and Van Den Elzen (5), with $[^3]$H)dihydrostreptomycin (2 Ci/mmol) used as the radioactive tracer. Cold streptomycin or cold dihydrostreptomycin was added to $[^3]$H)dihydrostreptomycin to obtain a specific activity of 22.7 μCi/ml in all the experiments.

RESULTS
Effect of streptomycin on growth of rpsL strains in alkaline medium. Addition of streptomycin to exponential cultures of rpsL mutants of S. typhimurium growing in alkaline (pH 8.2) nutrient medium had an inhibitory effect on the growth rate (Fig. 1). That alteration was dependent both on the presence of the antibiotic and on the pH, since the OD increment was normal in alkaline medium without antibiotic and in neutral medium with streptomycin (Fig. 1).

Growth inhibition was not accompanied by cell death (Fig. 1, inset) and could be readily reversed by removing the antibiotic or by modifying the pH (Fig. 1). The response of strain DA1030 to streptomycin plus high pH appeared to be general for rpsL mutants of S. typhimurium and was also shown by two rpsL strains of Escherichia coli K-12 tested.

The inhibitory action of streptomycin plus high pH depended on the streptomycin concentration. The effect was negligible at concentrations under 200 μg/ml but increased sharply at higher concentrations and reached its maximal expression at about 800 μg of streptomycin per ml (Fig. 2). Moreover, the extent of inhibition was proportional to the pH between 7.0 and 8.2. At higher pHs, inhibition remained constant or even decreased slightly (Fig. 2, inset).

Dihydrostreptomycin is closely related to streptomycin, from which it can be obtained by removing the aldehyde group in the streptose moiety to an alcohol group. It shares with streptomycin the mechanism of entrance into the cell and the high-affinity binding site in the ribosome of sensitive bacteria (7). It was found that dihydrostreptomycin at pH 8.2 and at concentrations equivalent to those used with streptomycin did not inhibit growth. It was necessary to increase the dihydrostreptomycin concentration about 10 times to reproduce the streptomycin effect (Fig. 2). In fact, the behavior of dihydrostreptomycin at pH 8.2 was very similar to that displayed by streptomycin at pH 7.0 (Fig. 2).

In experiments with mixtures of streptomycin and dihydrosreptomycin, no evidence of competition between them was found, and according to the extent of growth inhibition observed they appeared to act additively.

Effect of streptomycin on macromolecular synthesis. Incorporation of labeled leucine, thymidine, and uracil into mac-

FIG. 1. Effect of high pH and streptomycin (STR) on growth of strain DA1030. Streptomycin (400 μg/ml) was added to samples of exponential cultures of strain DA1030 growing at pH 7.0 or 8.2 at time zero. After 85 min (arrow), samples from the culture growing at pH 8.2 with streptomycin were centrifuged, and the cells were suspended in medium at pH 7.0 with streptomycin or pH 8.2 without drug. (Inset) Effect of high pH and streptomycin on the viability of strain DA1030.
romolecules was curtailed by addition of streptomycin to rpsL cells growing in medium at pH 8.2 (Fig. 3). Inhibition of protein synthesis appeared 9 min after addition of the antibiotic, and inhibition of DNA and RNA synthesis appeared 6 min later. There were also differences in the magnitude of the effect; whereas the rate of incorporation of leucine and thymidine suffered a reduction of about 40 to 50%, incorporation of uracil was inhibited by only 17%.

Such a generalized and almost simultaneous inhibition of macromolecular synthesis could be caused by a shortage of ATP; therefore, ATP content was also investigated. Not only was there no reduction in the level of ATP but, in fact, it even increased in the culture containing streptomycin. Thus, the ATP content, which was 0.57 nmol/mg (dry weight) of cells in the culture at pH 8.2, reached 0.75 nmol/mg 1 h after addition of the antibiotic.

On the other hand, it was observed that oxygen consumption decreased progressively after streptomycin addition, and 50 min later it amounted only to half of the initial level (data not shown).

**Effect of high pH on uptake of streptomycin and dihydrostreptomycin.** The effect of high pH on the accumulation of streptomycin (400 μg/ml) is shown in Fig. 4. The rate of entrance was fourfold higher at pH 8.2 than at pH 7.0. At variance with a previous work reporting no difference whether streptomycin or dihydrostreptomycin was used as the unlabeled component (5), the rate fell by half when dihydrostreptomycin (400 μg/ml) instead of streptomycin was used at the same pH. Therefore, it was necessary to increase the dihydrostreptomycin concentration to 800 μg/ml to obtain an accumulation similar to that produced by 400 μg of streptomycin per ml (Fig. 4). As dihydrostreptomycin was not able to alter cell growth at that concentration, it is evident that dihydrostreptomycin inactivity was not due to an inability to enter the cell.

In spite of the pronounced increase caused by high pH, streptomycin accumulation still showed known characteristics of the first, slow, energy-dependent phase of entrance (EDP-I) of aminoglycosides (5). Thus, addition of 2 mM KCN or 25 μg of chloramphenicol per ml together with streptomycin produced a drastic arrest of streptomycin uptake after a short delay (Fig. 4). The same effect was noticed when any of those inhibitors was added 25 min after streptomycin addition (data not shown).

**FIG. 2.** Effect of antibiotic concentration on the bacteriostatic action of streptomycin (STR) and dihydrostreptomycin (DHS) at different pHs. The procedure was as described in the legend to Fig. 1; growth inhibition was calculated as described in the text. (Inset) Effect of pH on the bacteriostatic action of streptomycin.

**FIG. 3.** Effect of streptomycin (STR) on the incorporation of leucine, thymidine, and uracil into cold TCA-precipitable material. The procedure was done as described in the text.

**FIG. 4.** Uptake of streptomycin (STR) and dihydrostreptomycin (DHS) at different pHs, and effect of inhibitors on uptake of streptomycin. Uptake was measured as described by Bryan and Van Den Elzen (5). In all cases, the specific activity of [1H]dihydrostreptomycin was 22.7 μCi/mg. Chloramphenicol (CM, 25 μg/ml) and KCN (2 mM) were added together with streptomycin at time zero.
DISCUSSION

It has been established that uptake of aminoglycoside antibiotics by susceptible cells is driven by the transmembrane electrical potential (8, 10) and is also strongly influenced by other factors, such as electron transport (6, 11), growth rate (16, 17), chloramphenicol (1), high-affinity binding to the ribosome (5), etc., whose roles are not yet well defined.

Three phases have been described by Bryan and Van Den Elzen (5) in the complex process allowing uptake of streptomycin by susceptible cells. The first one, which does not require energy, is practically instantaneous and represents the ionic binding of streptomycin to the cell surface. The second phase (EDP-I), which is characterized by a slow, energy-dependent entrance of streptomycin, is followed by the third phase (EDP-II), which is also dependent on energy but displays a highly accelerated uptake of the antibiotic. The last phase, which is coincidental with cell death, develops only in susceptible strains, and in ribosomally resistant mutants uptake of streptomycin shows the slow rate characteristic of EDP-I.

It is also known that the susceptibility of rpsL+ strains to aminoglycoside antibiotics increases as the pH of the medium rises (8, 9). This effect of pH has been assigned not only to the corresponding increase brought about in membrane potential (8), but also to changes in the ionization of polar groups from the antibiotic molecule and the cell envelope (9, 12).

The data reported in this paper demonstrate that the rate of entrance of streptomycin into ribosomally resistant strains greatly increases at high pH and results in the accumulation of amounts of streptomycin comparable to those reported for susceptible cells after development of the rapid phase of uptake. Thus, the rpsL mutant of S. typhimurium accumulated, after 10 min, 1.7 µg of streptomycin per mg (dry weight) (Fig. 4), while susceptible cells of E. coli K-12 contained, 10 min after onset of EDP-II, 1.0 µg/mg in experiments performed with a concentration of 50 µg of streptomycin per ml (5).

Conditions resulting in high intracellular concentrations of streptomycin and the absence of the ribosomal site of high affinity allowed demonstration of a bacteriostatic effect of streptomycin not detected until now. Bacteriostasis was accompanied by inhibition of macromolecular synthesis and depression of respiratory activity. As the inhibitory effect of streptomycin was manifested almost simultaneously by protein, DNA, and RNA synthesis, no primary target could be discerned. In fact, the evidence suggests that streptomycin exerts its action on several targets which probably have different sensitivities to the antibiotic and for that reason are inhibited at slightly different times, that is, at slightly different intracellular streptomycin concentrations.

Taking into account the well-known ability of streptomycin to bind to nucleic acids and other macromolecules (4, 15), it is tempting to postulate that the action observed is the result of disturbance produced in the functioning of nucleic acids by the binding of high amounts of streptomycin. Support for this idea is lent by the inability of dihydrostreptomycin to accomplish the same effects, since it was demonstrated long ago that streptomycin and dihydrostreptomycin differ markedly in their ability to precipitate nucleic acids (15).

Some conclusions bearing on the mode of action of streptomycin can be drawn from the experiments reported here. The fact that streptomycin and dihydrostreptomycin were equally effective in producing cell death of susceptible bacteria, whereas only streptomycin caused the bacteriostatic effect described in this paper, indicates that the latter phenomenon is not involved in the bactericidal activity of those antibiotics. On the other hand, according to the "two-hits" model proposed by Hancock (13), cell death would be due to the inactivation by aminoglycosides of two different targets, neither of which, by itself, would be able to cause death. Considering the apparent innocuousness of such high intracellular concentrations of dihydrostreptomycin as those observed in this work, it would appear that the second target, if it does exist, is reached only through the action of the antibiotic on the sensitive ribosomal site. It seems, therefore, that the ribosomal site of high affinity is still the key factor for understanding the mode of action of aminoglycoside antibiotics.

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LITERATURE CITED

