BACTERIAL DISSIMILATION OF STREPTOMYCIN

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ABSTRACT

KLEIN, DOLPH (Rutgers, The State University, New Brunswick, N. J.), AND DAVID PRAMER. Bacterial dissimilation of streptomycin. J. Bacteriol. 82:505-510. 1961.—The influence of various nutritional and environmental factors on the dissimilation of streptomycin by a pseudomonad isolated from soil was investigated, and conditions most suitable for growth of the bacterium, in a medium that contained streptomycin as a sole source of energy, nitrogen, and organic carbon, were determined. Development of the bacterium as measured by plate counts was correlated with degradation of streptomycin as measured by both biological and spectrophotometric assay procedures.

Periodic analyses of culture filtrates indicated that the three constituent moieties of the streptomycin molecule underwent simultaneous transformation when the antibiotic was degraded microbiologically. Washed cell suspensions were capable of immediate and rapid oxidation of streptomycin, dihydrostreptomycin, and hydroxystreptomycin, but cell-free sonic extracts, in the absence and presence of cofactors, did not oxidize streptomycin or any of a number of derivatives or degradation products of the antibiotic. Evidence was obtained that the bacterial dissimilation of streptomycin involves an oxidation system in which methylene blue can act as hydrogen acceptor.

There are a limited number of publications concerned with the microbiological dissimilation of streptomycin and the information they contain is generally vague and contradictory. Sureau et al. (1948) reported that filtrates of cultures of Bacillus pyocyanus and an enterococcus were capable of inactivating streptomycin. Similar results were obtained by Simkovics and Korosy (1948) and it was suggested that the antibiotic was inactivated and degraded enzymatically. However, no evidence was presented for the presence in the broths of a streptomycinase and no degradation products of the antibiotic were isolated or identified. Moreover, Grumbach, Rybak, and Gros (1949) provided evidence that loss of activity of streptomycin in culture filtrates of the enterococcus of Sureau et al. (1948) was not enzymatic but due to the liberation by the bacterium of nucleoprotein which, in the medium, formed an insoluble complex with streptomycin (Berkman et al., 1947).

Hobby and Dougherty (1948) isolated a pseudomonad from an aqueous solution of streptomycin and described it as capable of utilizing the antibiotic for growth. The sole basis for characterizing the organism as a streptomycin decomposer was the observation that cell numbers increased during storage in antibiotic solution. Espersen (1951) failed to demonstrate that streptomycin-resistant tubercle bacilli produced streptomycinase, but Sakakibara (1951a, b) reported that streptomycin-resistant strains of Staphylococcus aureus produced streptomycinase and were able to develop in a medium that contained streptomycin as the sole organic constituent.

Pramer and Starkey (1951) employed conventional enrichment and selective culture techniques for the isolation from soil of a bacterium capable of utilizing streptomycin. The organism was a gram-negative, nonsporulating, motile rod, identified as a member of the Pseudomonas fluorescens group. It developed in a medium that contained streptomycin as the sole source of energy and organic carbon for growth. The present communication describes environmental and nutritional conditions that are most suitable for the bacterial dissimilation of streptomycin. Evidence is presented that streptomycin is oxidized and each of the constituent moieties

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of the antibiotic molecule transformed by the pseudomonad.

MATERIALS AND METHODS

Changes in streptomycin concentration during growth of the antibiotic-decomposing bacterium were measured by both biological and chemical procedures. The bio-assay employed was described by Loo et al. (1945). *Bacillus subtilis* (ATCC 6633) was used as the test organism. The chemical determinations were made by the method of Boxer, Jelinek, and Leghorn (1947) which is dependent on the production of maltol from streptomycin by alkaline hydrolysis. Maltol concentration was estimated at 274 µg using a Beckman model DU spectrophotometer. Hydroxystreptomycin and mannosidostreptomycin concentrations were determined by both the maltol and oxidized nitroprusside (Welsh, 1949) methods. The latter procedure was also employed to measure dihydrostreptomycin and streptidine concentrations. N-Methyl-L-glucosamine was determined by the Elson-Morgan reagents (Seudi, Boxer, and Jelinek, 1946).

Growth of the pseudomonad was estimated by plate counts. For this purpose, 0.1 ml of culture samples or appropriate dilutions thereof was added to the surface of nutrient agar plates and colonies counted after 3 days of incubation at 28 C.

Washed cell suspensions were prepared by harvesting the cells from 2 liters of streptomycin broth after 4 days of growth at 28 C. The cells were concentrated by centrifugation and washed three times with 0.033 M phosphate buffer at pH 6.8. The washed pellet was suspended in 40 ml of buffer to give a 50-fold concentration of cells. The cellular content of the suspensions was determined by drying an aliquot at 105 C for approximately 18 hr and found to be 10 mg/ml. The packed cell volume of the suspensions was 4% after centrifugation in a Wintrobe hematocrit tube for 30 min at 5,000 × g. For experimental purposes, quantities of 10 to 15 ml of washed cell suspensions were placed in 125-ml Erlenmeyer flasks and streptomycin was added at a final concentration of 1,000 µg/ml. The flasks were placed in angle-clamps (30°) and incubated at 28 C in a model G-76 gyroratory water-bath shaker (New Brunswick Scientific Company, New Brunswick, N. J.) operating at 280 rev/min.

Oxygen uptake was measured by conventional manometric techniques (Umbreit, Burris, and Stauffer, 1957) using a Warburg respirometer at 28 C. The main compartment of each vessel received 2.3 ml of a washed cell suspension in 0.033 M phosphate buffer at pH 6.8, 0.5 ml of substrate (2.41 µmoles) was added to the side arm, and 0.2 ml of 20% KOH was placed in the center well.

Cell-free extracts were prepared by disrupting washed cell suspensions (1.2 to 2.5 g wet weight of cells in 10 ml of 0.033 M phosphate buffer at pH 6.8) for 15 min at 5 C using an MSE-Mullard ultrasonic disintegrator fitted with a 1/4-in. stainless steel probe. Microscopic examination showed that approximately 50% of the cells were disrupted by the procedure. Whole cells and debris were removed by centrifugation at 3,000 × g for 30 min at 5 C. The straw-colored, opalescent, supernatant fluid was diluted to a final volume of 20 ml with 0.033 M phosphate buffer at pH 6.8. Warburg vessels each received 1.8 ml of the cell-free extract.

Dehydrogenase activity was estimated by the Thunberg technique. Each Thunberg tube received 1 ml of a 2.67 × 10⁻⁴ M solution of methylene blue, 2 ml of water containing 5.5 mg of streptomycin, and 2 ml of 0.067 M phosphate buffer at pH 6.8. One-half milliliter of a washed cell suspension was added to the side arm of each tube. Controls were prepared similarly using distilled water in place of the antibiotic solution. All tubes were evacuated for 3 min and then tipped to bring the cells into contact with the substrate and indicator. The time required for 90% reduction of the indicator was recorded.

RESULTS AND DISCUSSION

The influence of various environmental and nutritional factors on the microbial degradation of streptomycin was determined. Included in these studies were the effects of size and age of inoculum, pH, buffer concentration, trace metals, streptomycin concentration, temperature, aeration, yeast extract, and nitrogen source. The results provided a clear definition of conditions optimal for growth of the antibiotic-utilizing pseudomonad. The most suitable medium was a streptomycin-mineral salts broth formulated with 0.033 M phosphate buffer at pH 6.8 and having the following composition: MgSO₄·7H₂O,
0.05%; FeSO₄·7H₂O, 0.003%; CaCl₂·2H₂O, 0.005%; KH₂PO₄, 0.18%; Na₂HPO₄, 0.29%; and streptomycin, 0.10%. In this medium, streptomycin served as the sole source of energy, nitrogen, and organic carbon for growth of the bacterium. The antibiotic was sterilized by filtration and added aseptically to 100-ml quantities of autoclaved basal salt solution in 250-ml Erlenmeyer flasks. Each flask was inoculated with 2 ml of a culture in the logarithmic phase of development in streptomycin broth, and the flasks were incubated at 28°C on a rotary shaker. Under these conditions, the culture degraded approximately 100 mg of streptomycin in 3 to 4 days, whereas 12 to 14 days were required for comparable antibiotic degradation under conditions employed prior to the present studies (Pramer and Starkey, 1951).

Growth of the streptomycin-decomposing pseudomonad was measured by plate counts and correlated with degradation of the antibiotic as measured by both the biological and spectrophotometric procedures. The results, illustrated in Fig. 1, show that the course of streptomycin degradation was reflected similarly by both the bio-assay and the spectrophotometric method. After 5 days of incubation, antibiotic activity was no longer detected by the bio-assay, but the chemical method indicated a residual streptomycin concentration equal to 12.3% of the initial level. This disparity resulted from interference with the spectrophotometric procedure by an ultraviolet-absorbing end product. Growth of the antibiotic-utilizing bacterium was an inverse function of the change in streptomycin concentration as measured by both the bio-assay and the spectrophotometric procedure. Cell counts increased exponentially for 5 days and then approached equilibrium. Growth and antibiotic degradation occurred simultaneously and the number of cells per ml of medium increased from $1.6 \times 10^6$ to $2.3 \times 10^6$ in 7 days. The generation time of the pseudomonad during logarithmic growth on streptomycin was approximately 16 hr. This is considerably more than the 30 to 40 min of generation reported for various Pseudomonas species in nutrient broth (Mason, 1935).

Since similar results were obtained with the bio-assay and the spectrophotometric procedure, the latter method, which is the more convenient, rapid, and precise, was used routinely in all subsequent studies.

An insight into the fate of the constituent moieties of the streptomycin molecule was obtained by analyses of culture filtrates. Since approximately 86% of the nitrogen in the medium employed for growth of the bacterium was contained in the two guanido groups of streptidine and the remaining 14% was present as N-methyl-L-glucosamine, some transformation of the organic nitrogen was expected.

From the results illustrated in Fig. 2, it ap-
peared that each of the three constituent moieties of the streptomycin molecule was modified during growth of the bacterium. The oxidized nitroprusside test showed that the guanido groups of streptidine were altered. The spectrophotometric measurement of maltol was, in effect, a determination of streptose. Failure of streptomycin to undergo rearrangement and yield maltol during alkaline hydrolysis was taken as evidence that the streptose moiety of the streptomycin molecule was modified. However, maltol formation will not take place unless the glycosidic linkage of streptose to streptidine is intact at the time of analysis (Kuehl et al., 1946) and it is therefore possible that the decrease in maltol concentration resulted from cleavage of the streptose-streptidine bond and was not due to direct action by the bacterium on the pentose. Finally, the N-methyl-L-glucosamine moiety of streptomycin was transformed during growth of the pseudomonad but the apparent loss was less than that of streptomycin as measured using oxidized nitroprusside or the maltol method. Approximately 25% of the N-methyl-L-glucosamine remained in the broth after 6 days. Since a great variety of amino sugars react with the Elson-Morgan reagent, further study is required to determine the nature of the residual material. It may be N-methyl-L-glucosamine or other amino sugars produced by the organism during growth on streptomycin.

From the foregoing results it was apparent that each of the three moieties of the streptomycin molecule underwent simultaneous change when the antibiotic served as a sole source of energy, organic carbon, and nitrogen for growth of the pseudomonad. An experiment was performed to determine if the addition to the medium of inorganic nitrogen (NH₄NO₃) would influence the fate of the nitrogen-bearing moieties of streptomycin. Changes in concentration of streptidine and N-methyl-L-glucosamine during growth of the bacterium in media containing streptomycin with and without inorganic nitrogen were measured but the results indicated no sparing action of inorganic nitrogen on the antibiotic nitrogen. The rate and extent of degradation of streptomycin and transformation of the streptidine and N-methyl-L-glucosamine moieties of the antibiotic by the organism were similar in both media.

It was observed that growing cultures required 3 to 4 days to degrade the amount (75 to 100 mg) of streptomycin transformed by washed cell suspensions in 2 hr. Although adapted inocula were employed consistently, rapid degradation of streptomycin by growing cultures was preceded by a lag period of approximately 2 days, whereas transformation of the antibiotic by washed cell suspensions was initially linear and tended to become asymptotic only when 70% or more of the antibiotic was degraded.

Information concerning the uptake of streptomycin by bacteria is conflicting. Aanand, Davis, and Armitage (1960) reported an initial uptake of streptomycin by resistant cells of *Escherichia coli* which was completed 1 min after addition of the antibiotic and which was equal at both 0 and 37 C. On the other hand, Szybalski and Shizuyoshi (1959) observed that there was little if any streptomycin uptake by cells of a streptomycin-resistant strain of *E. coli* growing at 37 C. The extent of uptake of streptomycin by washed cell suspensions of the antibiotic-utilizing pseudomonad was determined by measuring disappearance of the antibiotic from solution at 5 and 28 C. At 28 C, there was uptake of streptomycin in 3 min, but disappearance of the antibiotic from solution at 5 C was negligible compared to that at 28 C (Table 1). Since adsorption varies inversely with temperature (Glassstone, 1946) and loss of streptomycin varied directly with temperature, it is concluded that the decrease in antibiotic level at 28 C was the result of metabolic transformation.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Residual streptomycin at 5 C (%)</th>
<th>Residual streptomycin at 28 C (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
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<tr>
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<tr>
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<td>59</td>
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<td>34</td>
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<tr>
<td>2.5</td>
<td>100</td>
<td>20</td>
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TABLE 1. Uptake of streptomycin by washed cell suspensions at 5 and 28 C
N-methyl-L-glucosamine, and mannosidostreptomycin exceeded that required for endogenous respiration, but the rate and extent of oxidation of these substances were much less than those obtained with streptomycin. Although the results are suggestive regarding the status of streptidine, maltol, and N-methyl-L-glucosamine as intermediates in the dissimilation of streptomycin, no conclusion seems justified since failure of cells to respire on an intermediate supplied exogenously may be due to the cells' impermeability to the compound (Stanier and Tsuchida, 1949).

To circumvent the permeability problem, cell-free extracts of the streptomycin-decomposing pseudomonad were prepared and the oxidative capacity of these preparations was tested in the presence and absence of the following cofactors: diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin adenine dinucleotide, cytochrome c, coenzyme A, lipoic acid, thiamine pyrophosphate, riboflavin 5-phosphate, adenosine diphosphate, adenosine triphosphate, methylene blue, ethyl viologen, and benzyl viologen. However, the cell-free extracts were inactive. They did not oxidize streptomycin or any of the derivatives or degradation products of the antibiotic that were tested. This was true in the absence and presence of cofactors and hydrogen acceptors which were supplied individually and in combination. Therefore, the status of streptidine, N-methyl-L-glucosamine, and maltol as intermediates in the microbial transformation of streptomycin remains uncertain and cannot be resolved until cell-free preparations capable of transforming the antibiotic are obtained.

The ability of streptomycin-grown cells to oxidize dihydrostreptomycin and hydroxystreptomycin suggests that these compounds are intermediates in the microbial transformation of streptomycin. However, it is possible that the oxidative systems employed by the bacterium for streptomycin dissimilation have limited specificity and are capable of also transforming the dihydro and hydroxy derivatives of the antibiotic. The rate and extent of oxygen uptake by washed cell suspensions that received dihydrostreptomycin were consistently greater than those obtained from identical suspensions that received streptomycin. It was calculated from the results of four manometric studies that the oxidation of dihydrostreptomycin required approximately 0.5 mole more of oxygen than did the oxidation of streptomycin. Since the number of hydrogen atoms or electrons necessary to reduce 0.5 mole of oxygen is equivalent to the amount required to reduce 1 mole of streptomycin to dihydrostreptomycin, the results suggest that one of the sites of oxidation of dihydrostreptomycin is the carbonyl group of the streptose moiety of the molecule. The increased oxygen uptake by cells that received dihydrostreptomycin indicates that a dehydrogenase not operative in streptomycin oxidation is activated when the reduced antibiotic is used as substrate by the organism (Stanier, 1948).

Washed cell suspensions of the pseudomonad were capable of dehydrogenating streptomycin when methylene blue was employed as hydrogen acceptor. The time required for 90% reduction of methylene blue by cells supplied with streptomycin was 2.5 min, whereas 15 min were required to obtain equal reduction of the indicator by cells which were not supplied with the antibiotic. Although similar activity could not be demonstrated using culture filtrates or cell-free extracts, it appears that the bacterial dissimilation of streptomycin involves an oxidation system in which methylene blue can act as hydrogen acceptor.
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LITERATURE CITED


