SOME PRODUCTS OF THE BACTERIAL DISSIMILATION OF STREPTOMYCIN[1]

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ABSTRACT

KLEIN, DOLPH (Rutgers, The State University, New Brunswick, N. J.) AND DAVID PRAMER. Some products of the bacterial dissimilation of streptomycin. J. Bacteriol. **83**:309–313. 1962.—Bacterial dissimilation of streptomycin resulted in products detectable with Folin-Ciocalteu's phenol reagent, ninhydrin, and Ehrlich's reagent; these products absorbed ultraviolet light, with a maximum at 265 mμ. Evidence was also obtained for the production of a volatile base, possibly methylamine.

Urea and streptamine were formed by growing cultures and washed-cell suspensions supplied with streptomycin. More than 90% of the amidine-nitrogen of the antibiotic was recovered as urea-nitrogen in culture filtrates. It was evident that the bacterial dissimilation of streptomycin involved hydrolysis of the guanido groups of the streptidine moiety of the antibiotic molecule. The site of action was the bond between the amidine group and the secondary amino-nitrogen of each guanido group, and the products of the reaction were urea and streptamine.

A bacterium of the *Pseudomonas fluorescens* group isolated from soil by Pramer and Starkey (1951) developed in a medium that contained streptomycin as the sole source of energy, nitrogen, and organic carbon, and transformed each of the three constituent moieties of the antibiotic molecule (Klein and Pramer, 1961). Perlman (1952) examined the chemical changes that occurred during growth of the bacterium in a streptomycin-NH_4Cl-salts medium and reported that streptomycin oxime and one other substance (giving a positive Sakaguchi test) were produced. Results of the present investigations were not consistent with those of Perlman. No evidence for the formation of free or bound NH_2OH or streptomycin oxime was obtained from analyses of culture filtrates and washed-cell suspensions. However, bacterial deamidination of streptomycin was demonstrated, and urea and streptidine were identified as products of the reaction.

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MATERIALS AND METHODS

The medium and conditions employed for growth of the streptomycin-utilizing pseudomonad, as well as the procedure for preparation and use of washed-cell suspensions, were described previously (Klein and Pramer, 1961).

Bound and free NH_2OH were estimated by the method of Csaky (1948), but rigid control of the digestion procedure was necessary when the method was used for the determination of streptomycin oxime. A paper chromatographic procedure for streptomycin oxime was provided by Perlman (*personal communication*). An ascending system (65% pyridine), using Whatman no. 1 paper, was employed; after 15 hr, papers were removed from solvent-saturated chromatographic tanks, dried, and developed with the reagents of Sakaguchi. This procedure separated and located streptomycin and streptomycin oxime at R_F values of 0.04 and 0.19, respectively.

Urea and volatile bases were measured by the microdiffusion method of Conway (1950). Products reacting with Folin-Ciocalteu's phenol reagent (FCR) were determined by adding 1 ml of the reagent and 2.5 ml of 20% Na_2CO_3 to a tube containing 0.5 ml of test solution. The contents of the tube were mixed and stored for 2 hr at room temperature, and the optical density of the blue color that developed was then measured at 660 mμ. Ninhydrin-reacting products

were determined by adding 1 ml of 0.25% 1,2,3-triketohydrindene in 0.067 M phosphate buffer (pH 6.8) to 0.5 ml of the test samples. The solution was heated at 100 C for 15 min, cooled to room temperature, increased to 10 ml with the buffer, and the optical density of the purple color that developed was measured promptly at 570 mμ. Absorption spectra were determined with a Beckman DU spectrophotometer after diluting 0.5-ml quantities of test solutions to 10 ml with either 0.1 N HCl or 0.1 N NaOH. Changes in concentration of ultraviolet-absorbing products of streptomycin dissimilation were estimated by measuring changes in optical density at the wave length of maximum absorption (265 mμ in acid solution).

In one experiment, the filtrate of a 4-day-old culture was concentrated (50-fold) in vacuo at 50 C, and 15 μliters of the concentrate were chromatographed on paper to separate and identify products of the bacterial dissimilation of streptomycin. Whatman no. 3 MM paper was employed in a descending system, using butanol–acetic acid–water (4:1:5) as the solvent system. Fifty μliters of each of the following standard solutions were spotted and chromatographed simultaneously: 0.1% streptomycin, 0.3% streptidine, 0.6% streptamine, 0.6% strepturea, 0.2% urea, and 0.4% *N*-methyl-L-glucosamine. Four different reagents were employed to locate the standards. Ninhydrin reacted with streptamine and *N*-methyl-L-glucosamine when the sprayed chromatograms were placed in an oven at 105 C for 10 min. *N*-methyl-L-glucosamine was located by the Elson-Morgan reagents. Papers were sprayed with acetylacetone, dried at 105 C for 10 min, sprayed with *p*-dimethylaminobenzaldehyde, and redried. The *p*-dimethylaminobenzaldehyde reagent was also employed to detect urea and strepturea, but the sprayed paper was not heated. Oxidized nitroprusside (Welsh, 1949) was used to locate streptomycin and streptidine. Chemical and spectrophotometric methods for estimating streptomycin concentration were described previously (Klein and Pramer, 1961).

RESULTS

Preliminary experiments. A number of conventional biochemical tests were applied to culture broths and washed-cell suspensions to detect dissimilation products of streptomycin. Included in these studies were ammoniacal $AgNO_3$, biuret, diazotized sulfanilic acid, Fehling's reducing sugar reagent, FCR, indole, ninhydrin, Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), and spectrophotometric examination in the ultraviolet range. It was found that the bacterial dissimilation of streptomycin resulted in products detectable with ninhydrin, FCR, and Ehrlich's reagent and which in acid solution absorbed ultraviolet light, with a maximum at 265 mμ. The intensity of the color produced with Ehrlich's reagent was weak and consequently unsuitable for quantitative analysis. Figure 1 illustrates the appearance with time in a growing culture and washed-cell suspension of streptomycin-dissimilation products, as measured by ninhydrin, FCR, and absorption at 265 mμ. In each case, product formation and streptomycin degradation occurred simultaneously. The ninhydrin reaction tended to intensify throughout the 8-day growth experiment, but the FCR reaction and ultraviolet absorption reached a maximum after 3 to 4 days of incubation and then decreased. In washed-cell suspensions, products were formed rapidly and persisted.

The products were not extracted from culture filtrates by solvents such as ether, butanol, benzene, and ethyl acetate, but they were adsorbed by cationic exchange resins and eluted therefrom with alkaline ethanol. Broth samples subjected to mild alkaline hydrolysis (0.3 N NaOH at 100 C for 3 min) lost their ability to react with FCR and absorb ultraviolet light, but reaction with ninhydrin was only partially reduced by the treatment.

The absorption spectra of a culture filtrate in both acidic and alkaline solutions were compared with those of crystalline maltol and streptomycin hydrolyzed by acid (0.25 N H_2SO_4 at 100 C for 30 min) and alkali (0.3 N NaOH at 100 C for 3 min). It was evident from the results that the absorption maximum of the filtrate in acid (265 mμ) and minimum in alkali (255 mμ) did not coincide with those of maltol (274 and 270 mμ, respectively), but they were similar enough to suggest that the degradation product may originate, as does maltol, from the streptose moiety of the streptomycin molecule. Furthermore, the ability of filtrates to react with FCR and absorb ultraviolet light varied similarly with time (Fig. 1B). The intensity of both

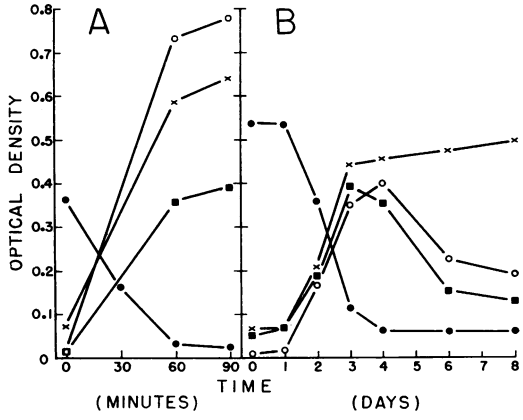

FIG. 1. *Appearance with time in a washed-cell suspension (A) and a growing culture (B) of products of the bacterial dissimilation of streptomycin. Antibiotic concentration was measured by the spectrophotometric method for maltol (●). Products were detected using phenol reagent (○), ninhydrin (×), and by adsorption at 265 mμ (■).*

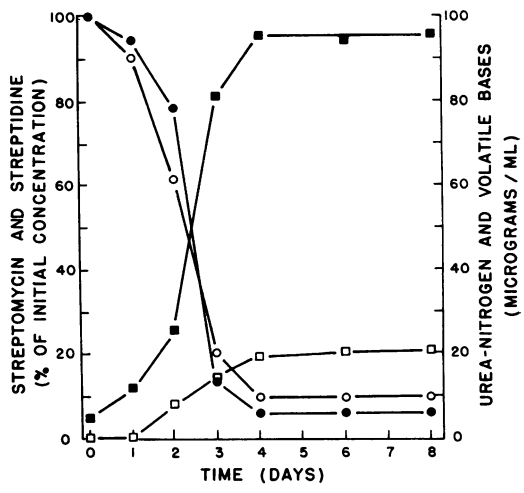

FIG. 2. *Appearance with time of urea (■) and volatile bases (□) during the bacterial dissimilation of streptomycin. Antibiotic concentration was measured by both the spectrophotometric method for maltol (●) and the oxidized nitroprusside test (○).*

reactions first increased and subsequently decreased, and it is possible that they were attributes of one rather than different products of the bacterial dissimilation of streptomycin. Efforts to concentrate and characterize this product, using such methods as that described by Roth, Amos, and Davis (1960), were unsuccessful. Changes in the ultraviolet-absorption spectrum of the preparation during treatment indicated that the compound was unstable and that it was modified by the isolation procedure.

Urea and streptamine. It was noted above that culture filtrates reacted with Ehrlich's reagent to produce a yellow color, which is characteristic of urea and ureido compounds. Further investigation revealed that the addition of the substrate-specific enzyme urease to culture filtrates caused ammonia liberation and that urea was a product of the bacterial dissimilation of streptomycin.

Figure 2 illustrates the results of an experiment in which changes in concentration of streptomycin during growth of the antibiotic-decomposing pseudomonad were measured spectrophotometrically, and the fate of the streptidine moiety of the antibiotic molecule was determined by the oxidized nitroprusside test. Production of urea by the bacterium was determined by microdiffusion analysis and was found to be an inverse function of degradation of streptomycin and streptidine. Analysis showed that 5 μg/ml of urea-nitrogen was present initially in the medium, owing to carry-over with the 4% inoculum. The level of urea-nitrogen in the medium increased rapidly for 4 days and then remained constant at a concentration of 95.4 μg/ml. It was assumed that the origin of the urea was the amidine portion of the guanido groups of the streptidine moiety of the streptomycin molecule. The medium was prepared to contain 1,020 μg/ml of streptomycin having an amidine-nitrogen content of 98.3 μg/ml. When correction was made for urea carried over with the inoculum, approximately 92% of the total amidine-nitrogen added as streptomycin was recovered as urea-nitrogen.

Unidentified volatile bases were detected in the medium on the second day of incubation. Their concentration increased to a maximum equivalent to approximately 20 μg/ml of ammonia-nitrogen after 4 days and did not change significantly thereafter (Fig. 2). Since the microdiffusion method employed does not differentiate between ammonia and volatile amines, the nature of this material is not known.

If, as indicated by the foregoing results, urea was derived by hydrolysis of the guanido groups of streptomycin, the streptidine moiety of the antibiotic molecule would be converted to

streptamine. Evidence that this reaction does occur and that streptidine is hydrolyzed to form urea and streptamine was obtained when a culture filtrate was concentrated and chromatographed on paper by use of butanol–acetic acid–water. Separate sheets were sprayed with ninhydrin, Ehrlich's reagent, the Elson-Morgan reagents, and oxidized nitroprusside. Both urea and streptamine were identified in the culture filtrate, but no free streptidine, strepturea, *N*-methyl-L-glucosamine, or residual streptomycin was detected. The compounds located with ninhydrin had R_F values of 0.12, 0.36, and 0.42. The first of these was streptamine. Although the identity of the other two substances was not established, it is possible that the material at R_F 0.36 was methylamine. The single spot located by Ehrlich's reagent had a value of 0.50 and was urea. The papers contained no substances that reacted with oxidized nitroprusside or the Elson-Morgan reagents.

Washed-cell suspensions liberated urea from both streptidine and streptomycin, but streptomycin was more readily transformed than streptidine. In 2 hr, twice as much urea was liberated from the former as from the latter. Chromatographic analysis of the supernatant liquid obtained after centrifugation of the washed-cell suspensions that had received streptomycin revealed products identical to those obtained by analysis of filtrates from growing cultures.

TABLE 1. *Dissimilation of streptomycin and streptomycin oxime by washed-cell suspensions*

Time	Dissimilation	
	Streptomycin	Streptomycin oxime
hr	*μg/ml*	*μg/ml*
0	0	0
0.5	280	60
1.0	480	120
1.5	500	130

Streptomycin oxime. No free or bound NH_2OH or streptomycin oxime was detected by chemical and chromatographic analyses of culture filtrates or washed-cell suspensions supplied with streptomycin. This was true under conditions identical to those employed by Perlman (1952), as well as under conditions demonstrated by Klein and Pramer (1961) to be optimal for antibiotic dissimilation by the bacterium. Furthermore, oxygen uptake by washed-cell suspensions supplied with streptomycin oxime did not greatly exceed that required for endogenous respiration and was considerably less than that obtained with streptomycin. Table 1 summarizes the results of an experiment in which washed-cell suspensions were provided with 1,000 μg/ml of streptomycin and streptomycin oxime. Periodic analyses demonstrated that streptomycin-grown cells were able to transform streptomycin oxime, but the rate and extent of the transformation were much less than those observed for streptomycin.

DISCUSSION

The results of the present investigations are not consistent with Perlman's (1952) suggestion that the primary transformation of streptomycin was a detoxification reaction between NH_2OH

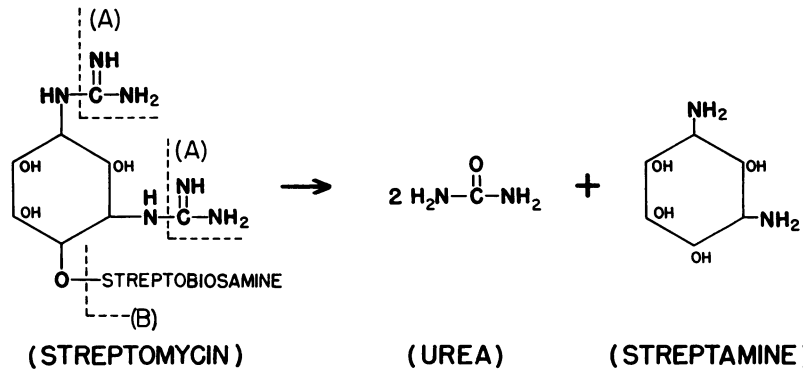

FIG. 3. *Urea and streptamine are products of the bacterial dissimilation of streptomycin, formed by hydrolytic cleavage of the antibiotic molecule at sites A and B.*

and the antibiotic to form streptomycin oxime. No free or bound NH_2OH or streptomycin oxime was detected by analysis of culture filtrates or washed-cell suspensions. Furthermore, washed-cell suspensions provided with streptomycin oxime did not readily transform the compound, and in manometric studies streptomycin oxime was not oxidized at a rate rapid enough to suggest that it is an intermediate in the bacterial dissimilation of streptomycin.

It was evident that the bacterial dissimilation of streptomycin involves hydrolysis of the guanido groups of the streptidine moiety of the antibiotic molecule. The site of action is the bond between the amidine group and the secondary amino-nitrogen of the guanido group, and the products of the reaction are urea and streptamine. The former was identified by its susceptibility to urease and by paper chromatography. The latter was identified by paper chromatography only. The bacterium may deamidinate the intact antibiotic molecule to form diaminostreptomycin, and streptamine may be released subsequently by hydrolysis of the glycosidic bond by which it is linked to streptobiosamine (site A and then B in Fig. 3). Alternatively, the glycosidic linkage may be first hydrolyzed to liberate streptidine and this diacidic base then hydrolyzed to urea and streptamine (site B and then A in Fig. 3).

The bacterial and chemical degradations of streptomycin proceed differently. Hydrolysis of streptomycin with aqueous acid yields the diacidic base streptidine, and stepwise alkaline degradation of streptidine gives first the urea derivative, strepturea, and then the diamine, streptamine (Peck et al., 1946). Urea is a product of the bacterial but not of the chemical degradation of streptidine. It is not known at this time whether streptamine persists in the medium or is further transformed by the bacterium. Pyrolysis of streptamine yields diaminophenol, which absorbs ultraviolet light with a maximum at 273 mμ (Peck et al., 1946). Since the absorption spectrum of culture filtrates had no maximum at 273 mμ, diaminophenol is not a product of the bacterial dissimilation of streptomycin.

Although the ninhydrin-reacting product obtained on paper chromatograms at an R_F of 0.36 was not identified, it is of interest that Block, Durrum, and Zweig (1955), using a similar chromatographic system, reported the same value for methylamine. It is reasonable to assume that the bacterium would hydrolyze the *N*-methyl-L-glucosamine moiety of the antibiotic to liberate methylamine and the free sugar. The latter compound may serve as the primary source of energy and carbon for growth of the bacterium. The former is a volatile base, as was detected by the microdiffusion method (Fig. 2), and may be responsible for the characteristic odor of growing cultures (Pramer and Starkey, 1951).

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