Production of Volatile Nitrogenous Compounds from the Degradation of Streptomycin by *Pseudomonas maltophilia*[1]

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Ammonia, methylamine, and pyridine were detected in broth filtrates of a streptomycin-degrading strain of *Pseudomonas maltophilia* during growth on streptomycin as a sole carbon and nitrogen source. Ammonia and methylamine, quantitatively measured by conversion to chromophores with picryl sulfonic acid, were found to accumulate in broth, whereas pyridine concentration increased in the early stages of streptomycin degradation and then decreased as the degradation of the antibiotic neared completion. Exogenous pyridine was metabolized by washed-cell suspensions. Use of *N*-streptomycin-*methyl*-^{14}C showed that the methylamine arose from the *N*-L-glucosamine-methyl moiety of streptomycin. Methylamine was an end product and was not further metabolized by cells.

Streptomycin is attacked by bacteria in a number of ways. Bacteria posessing plasmid R factor are able to phosphorylate streptomycin (11) or to adenylate the antibiotic (18). Nonspecific degradations have also been characterized. An enzyme system was reported in *Staphylococcus aureus* which causes the complete degradation of streptomycin (16). *Streptomyces griseus*, the organism which produces streptomycin in nature, possesses a "deguanidase" which catalytically splits off urea from the guanido group of streptomycin (14).

Pramer and Starkey (12) isolated a soil organism, identified as a *Pseudomonas* species, which grew on streptomycin as a sole source of organic carbon and nitrogen. Klein and Pramer (8, 9) found that this organism attacked all three moieties of streptomycin and was capable of degrading dihydrostreptomycin or hydroxystreptomycin as well. Urea was identified as a metabolic end product and was postulated to arise by a deamidination of the streptidine part of the molecule. The presence of certain unidentified volatile bases which appeared in the broth during growth on streptomycin was recognized.

The present study identifies three of the bases formed during bacterial degradation of streptomycin and the part of the antibiotic from which each base originated. Methylamine was suspected as a potential product of the *N*-methyl group of streptomycin. The addition of methionine-*methyl*-^{14}C to cultures of *S. griseus* permitted the selective labeling of the *N*-methyl carbon of streptomycin (3). Pyridine-like materials were tested for their possible presence in the broth, and pyridine, commonly a toxic agent, was found to be a product of streptomycin metabolism.

MATERIALS AND METHODS

Organisms. A streptomycin-degrading strain isolated from soil by enrichment and selective culture techniques (12) was used in this study. It was originally classified as a strain of the *Pseudomonas fluorescens* group, but was recently reclassified (Table 1) as a strain of *Pseudomonas maltophilia* on the basis of numerous taxonomic criteria (4, 17).

S. griseus K-12, employed in the biosynthesis of radioactive streptomycin, was the kind gift of Shiroh Shirato of Kaken Chemicals, Ltd., Tokyo.

Chemicals. Streptomycin sulfate was a gift of the Squibb Institute for Medical Research, New Brunswick, N. Y. ^{14}C-methylamine hydrochloride (54 mCi/mmol) was obtained from Amersham/Searle Corp. L-methionine-*methyl*-^{14}C (9.1 mCi/mmol) was obtained from New England Nuclear Corp. Picryl sulfonic acid was a product of Sigma Chemical Co., St. Louis, Mo. Chromosorb 103 was purchased from Applied Science Laboratories, Inc., State College, Pa.

TABLE 1. *Characterization of Pseudomonas maltophilia*

Test	Result[a]
Triple sugar iron (slant/butt)	Neg/neg
Phenylalanine deaminase	Neg
Urease	Neg
Oxidase	Neg
DNase	Pos
Lactose (MacConkey's)	Neg
o-Nitrophenylgalactoside hydrolysis	Pos
Dextrose oxidation	Neg
Maltose oxidation	Pos
Nitrate reduction	Pos
Esculin hydrolysis	Weakly pos
Tech agar	Brown pigment
Gelatinase	Pos
Motility	Pos
Ammoniacal odor	Pos
Methionine requirement	Pos

[a] Neg, Negative; pos, positive.

Culture conditions. The growth medium had the following percent composition: $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.003; $CaCl_2 \cdot 2H_2O$, 0.005; KH_2PO_4, 0.18; Na_2HPO_4, 0.29; and streptomycin sulfate, 0.133. The antibiotic was sterilized by membrane filtration and added aseptically to the autoclaved basal salts solution. The final reaction of the medium was pH 6.8. Flasks containing 100 ml of the medium were inoculated with 2 ml of a culture in the logarithmic phase of growth in streptomycin broth and incubated at 28 C on a rotary shaker. Aseptic methods were employed during cell transfer, and the cultures were periodically checked for purity and species identification. For experimental purposes, concentrated, washed-cell suspensions were prepared by harvesting the cells from 2 liters of streptomycin broth after 4 days of growth at 28 C. After one washing with 0.03 M phosphate buffer, pH 6.8, cells were recentrifuged and then suspended in 40 ml of fresh buffer.

Procedures. Streptomycin was assayed by the method of St. John et al. (15). Cell-free broth from cultures in which streptomycin was partly or completely degraded was adjusted to pH 11.5 with N NaOH and distilled into 0.1 N HCl. Ammonia and methylamine in the distillate were derivatized by reaction with picryl sulfonic acid at pH 8.0 with 5% $NaHCO_3$. After 2 h of incubation at 37 C, 6 N HCl was added to stop the reaction, and the products were extracted with ether. Ether extracts were applied quantitatively to a thin-layer plate (Brinkmann, Sil-plate 52) for development in chloroform-cyclohexane (2:1) and were subsequently eluted from the plate with ethyl acetate. Optical densities were determined at the wavelengths of maximum absorption, 317 nm for picramide and 338 nm for N-methyl-picramide, the derivatives of ammonia and of methylamine, respectively.

Pyridine in the distilled broth was detected directly by ultraviolet absorption spectroscopy at pH 1.0 and 11.0. Gas chromatography was used to estimate

pyridine quantitatively on a column of Chromosorb 103 in a Hewlett-Packard model 5750 gas chromatograph at 154 C.

Radioactive analyses. Radioactive samples were counted in Bray's solution (1) in a Packard Tri-Carb model 3320 liquid scintillation counter. Radioactive paper strips from chromatography or electrophoresis were scanned in a Packard radiochromatogram scanner model 7201. Where necessary, strips corresponding to labeled spots were excised and transferred to vials for counting in Bray's solution.

Preparation and characterization of N-streptomycin-methyl-[14]C. *S. griseus* K-12 was grown at 27 C on the proline, mannitol, and salts medium of Bruton et al. (2). Streptomycin concentration was monitored during growth of *S. griseus* and was found to equal 0.1 mg/ml after 4 days of growth. (240 µCi) Methionine-*methyl*-^{14}C was aseptically added at this time, and incubation was continued an additional 2 days. The radioactive streptomycin produced was collected by the procedure of Hunter et al. (6) which involved elution from a column of Amberlite IRC-50 (Rohm and Haas Co.) and crystallization of the antibiotic as the reineckate prior to conversion to the sulfate. Streptomycin sulfate was added as carrier during the purification process.

At the time of streptomycin harvest from the broth the concentration of streptomycin was found to equal 0.32 mg/ml. A sample of the ^{14}C-streptomycin was tested for radiochemical purity by paper electrophoresis for 22 min at 3,000 V in 0.2 M N-ethyl morpholinium acetate. Electropherograms were dried, scanned, and sprayed with the diacetyl reagent (5) which reacts with guanidino groups. A single radioactive peak was observed with mobility toward the cathode of 8.9 cm. This coincided with a diacetyl positive spot and with authentic streptomycin. The specific activity of ^{14}C-streptomycin was found to equal 19 µCi/mmol.

The location of the label within streptomycin was determined by mild acid hydrolysis (N H_2SO_4, 37 C, 48 h) of a sample of ^{14}C-streptomycin to streptidine and ^{14}C-streptobiosamine. Streptidine was removed by filtration, and the ^{14}C-streptobiosamine was refluxed for 2 h in 20% KOH. Volatile reaction products were distilled into 0.1 N HCl. ^{14}C-methylamine, formed in this manner from the N-methyl group of streptomycin, was tested for its specific activity which was found to equal 16.1 µCi/mmol. It was calculated from this that 85% of the label in ^{14}C-streptomycin was located in the N-methyl carbon.

RESULTS

Identification of ammonia and methylamine. The identity of ammonia and methylamine from the alkaline distillate of cell-free broth was established by several methods, and the results listed in Table 2 revealed the presence of ammonia and a compound whose retention time of 50.4 min in the amino acid analyzer coincided with that of authentic methylamine. Reaction with picryl sulfonic acid resulted in the formation of N-methyl picramide and picra-

TABLE 2. *Identification of methylamine from culture broths*

Test	Products from culture broth	Authentic methylamine	Authentic ammonia
Amino acid analyzer retention time (min)	50.4, 75	50.4	75 ± 1
Thin-layer chromatography trinitrophenyl derivatives-chloroform-benzene, 2:1 (R_f)	0.25, 0.37	0.37	0.25
Melting point, trinitrophenyl derivatives (C)	109–110	108 ± 1	

mide, the derivatives of methylamine and ammonia, respectively. These two derivatives, although very similar in structure and differing only by one methyl group, separated well on commercially prepared thin-layer chromatographic plates with chloroform-cyclohexane as the developing solvent. A sample of the yellow compound whose retardation factor (R_f) coincided with that of trinitromethylaniline was eluted from the thin-layer plate and dried. The melting point for this sample was found to equal 109 C, in close agreement with that of the authentic derivative of methylamine (Table 2).

Appearance and fate of the volatile bases. The time course of production of ammonia and methylamine during incubation with streptomycin was monitored by assaying various time samples by using picryl sulfonic acid as a colorimetric reagent. The metabolism of 1.5 µmol of streptomycin per ml coincided with the accumulation in the broth of 0.4 µmol of methylamine and 0.2 µmol of ammonia per ml (Fig. 1). Repeated experiments failed to show a larger total yield of either base. Furthermore, the formation of ammonia and methylamine in this same molar relationship to streptomycin was observed whether growing cultures or washed-cell suspensions were monitored.

^{14}C-streptomycin was incubated with washed cells, and time samples were assayed for residual streptomycin, for methylamine, and for radioactivity. No label became associated with the cells, and 10% of the substrate label was lost as volatile material. The remainder of the radioactivity was vested in methylamine and two other products in the broth (Fig. 2). The largest amount was found in an unidentified product observed, by paper electrophoresis, to have a slight mobility toward the cathode at pH 4.4. The specific activity of the ^{14}C-methylamine formed by metabolism of ^{14}C-streptomycin was determined to equal 15 µCi/mmol, in close agreement with the specific activity

calculated for the N-methyl carbon of ^{14}C-streptomycin.

To determine the fate of methylamine as a possible metabolite, ^{14}C-methylamine was added to two washed-cell suspensions, one of which contained streptomycin at a concentration of 1.0 mg/ml. At periodic intervals, culture samples were withdrawn, and broth was separated from cells to allow separate counting of each. The total label originally added to the broth was found to remain constant over the course of incubation, and no detectable radioac-
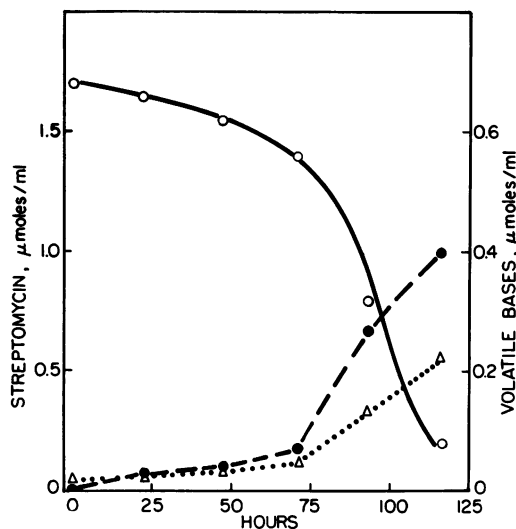

FIG. 1. *Appearance of methylamine (●) and ammonia (△) during streptomycin (○) metabolism by a growing culture of P. maltophilia.*
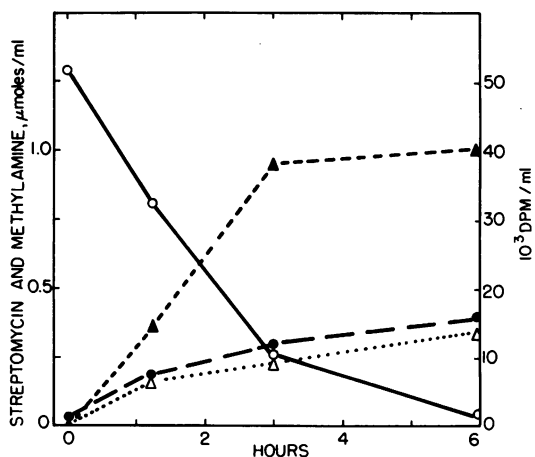

FIG. 2. *Appearance of methylamine (by chemical [●] and isotopic [△] assays) and radioactive (▲) products during metabolism of N-streptomycin-methyl-^{14}C (○) by a washed-cell suspension of P. maltophilia.*

tivity was found in the cells. Paper chromatography of acidified broth samples in isopropanol-ethanol-N HCl (75:75:50) followed by quantitative radiochromatogram scanning indicated that the ^{14}C-methylamine level remained constant and was the only radioactive material in the broth.

Broth from the streptomycin-degrading cultures was distilled, and ultraviolet spectra were taken of the distillate in order to identify the materials responsible for the malt-pyridine-like odor (12). Ultraviolet absorption spectra of the broth distillate made in acid or in alkali resembled those of authentic pyridine (Fig. 3). The product under investigation also behaved exactly like authentic pyridine in paper and gas chromatography. An acidified broth sample chromatographed on paper, by using 1-butanol-12 N HCl-H$_2$O (7:2:1), yielded a spot that absorbed ultraviolet light and had an R_f of 0.44, coincident with the R_f value of authentic pyridine under identical conditions. The broth spot was eluted from the paper and scanned in
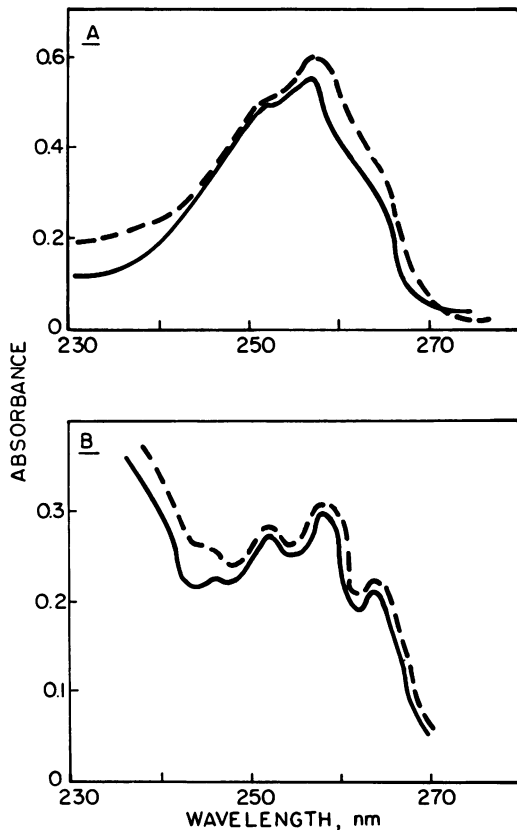
the ultraviolet region. Scanning gave the characteristic pyridine spectrum. A second sample, eluted from paper chromatograms, was subjected to gas chromatography at 154 C on a column of Chromosorb 103. Retention times found on the Chromosorb column were 10.4 min for the broth component and 10.3 min for authentic pyridine.

The potentially extraneous origin of pyridine was examined by assay of broth samples for pyridine at various times during streptomycin metabolism. The pyridine level in broth initially increased and then underwent a decline (Fig. 4). Its maximum concentration coincided approximately with the point of half substrate consumption, suggesting that pyridine is an intermediate in streptomycin metabolism.

To confirm the metabolism of pyridine by these cells, exogenous pyridine was added to a washed-cell suspension. It was observed that pyridine was completely lost from a cell suspension given 5 nmol of pyridine per ml, 10 times the amount of pyridine found during streptomycin metabolism (Fig. 5). Controls indicated that pyridine is not lost through volatilization under the conditions employed nor is pyridine adsorbed in significant amounts by non-metabolizing cells held at 4 C. Because pyridine is both formed and consumed during the process of streptomycin degradation, the product is apparently an intermediate and is initially formed at a rate that exceeds the rate of its consumption.
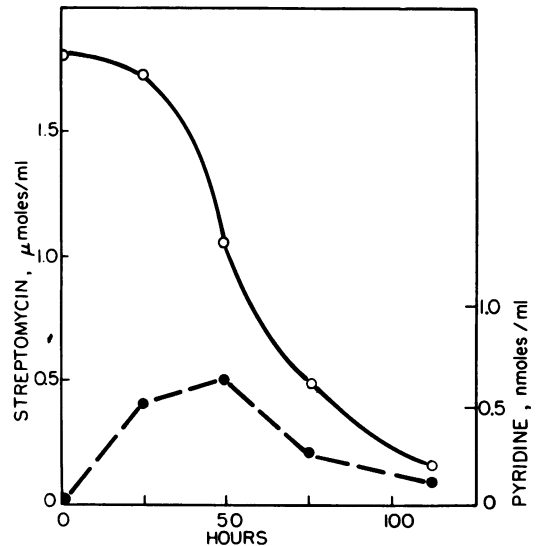

FIG. 3. *Ultraviolet spectra of authentic pyridine* (——) *and of a broth constituent* (-----) *A, pH 1; B, pH 11.*

FIG. 4. *Transient appearance of pyridine* (●) *in broth during growth of P. maltophilia on streptomycin* (○).

DISCUSSION

Urea, streptamine, and other unidentified nitrogenous compounds were reported earlier (9) to be products of the metabolism of streptomycin by *P. maltophilia*. The presence of a relatively large number of nitrogenous by-products from streptomycin metabolism might be expected, because the total nitrogen content provided by the amount of antibiotic in the culture broth is in excess of that amount actually assimilated by the microorganisms for cell growth. Klein and Pramer (9) identified streptamine (1,3-diamino-2,4,5,6-tetrahydroxycyclohexane) as a product of bacterial attack on the streptidine moiety of streptomycin. Streptamine is apparently the source of the ammonia found in broth filtrates of the streptomycin-degrading cultures, because whole cells or cell extracts incubated with streptamine produce ammonia in amounts (0.15 μmol/μmol of substrate) equal to that found in cultures incubated with streptomycin (unpublished data). The addition of ammonium salts to the medium neither enhances nor depresses the rate of streptomycin degradation and thereby suggests that the formation of ammonia in the degradation process is not rate limiting for cell growth.

Methylamine arises from the *N*-methyl-L-glucosamine moiety of streptomycin as shown by the labeling of methylamine during incubation of cells with ^{14}C-streptomycin. A deaminomethylation reaction presumably is involved such as that catalyzed by glycine oxidase (12) which converts *N*-methyl glycine to methylamine plus glyoxylate. The by-product of methylamine formation from streptomycin is likely a keto compound. However, attempts to identify this compound were not successful, and it is possible that it is further metabolized by the streptomycin-degrading cells.

It was of interest that methylamine was not formed stoichiometrically from streptomycin by bacterial action. This may indicate that the reaction giving rise to methylamine occurred after a metabolic branch point with only about 25% of the products going to methylamine. Alternatively, it may be that methylamine arises from the activity of a nonspecific deaminase whose primary function is the deamination of streptamine. The production of ammonia from streptamine and the similarity in structure of *N*-methyl-L-glucosamine and streptamine support this suggestion. In any case, it is clear that the non-stoichiometric amount of methylamine present in broth is not due to further metabolism of methylamine. The addition of ^{14}C-methylamine to whole cells did not result in the uptake or transformation of this material.
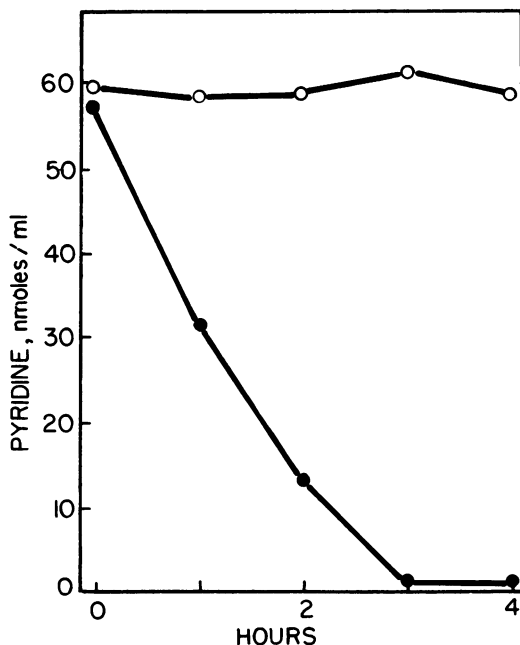

FIG. 5. *Uptake of pyridine in the presence* (●) *and absence* (○) *of washed-cell suspensions.*

Pyridine, an unusual, although not totally unique, bacterial metabolite, has been reported as a constituent of a lipoprotein hydrolysate from *Mycobacteria* (19). It also was shown to serve as a sole source of carbon and nitrogen for the growth of *Proactinomyces* (10). In neither case was the origin or fate of pyridine elucidated. Nothing is known about the pathway of pyridine synthesis from streptomycin by *P. maltophilia*. Streptamine is a possible precursor of pyridine. The pathway from streptamine would involve a ring cleavage with subsequent closure to form the heterocyclic pyridine. After this, dehydrogenation reactions would be suspected in order to achieve the degree of unsaturation present in pyridine.

Generally speaking, the strain of *P. maltophilia* isolated from soil was found to be capable of producing at least three identifiable volatile nitrogenous compounds in the course of streptomycin degradation. These compounds, ammonia, methylamine, and pyridine, are no doubt the basis for the characteristic odor that is associated with actively growing cultures in which streptomycin serves as the sole organic carbon and nitrogen source. Because of the technical difficulty in synthesizing streptomycin that contains isotopic nitrogen atoms at specific sites, the question of which nitrogen atoms are assimilated and which are not, cannot be answered at the present time.

Unlike other members of the genus *Pseudomonas*, which are generally proto-trophic, *P. maltophilia* requires methionine for growth (4, 7, 17). The strain used in these studies also required methionine, but only in a basal salts medium containing a substrate such as maltose as the principal organic carbon source (Table 1). Methionine was not essential for growth when the organism was cultured in the streptomycin-inorganic salts medium, nor was the growth rate stimulated or the final growth yield increased in the presence of methionine or yeast extract. Traces of methionine in the medium were undetectable. Iizuka and Komagata (7) reported that fresh isolates of *P. maltophilia* utilized hydrocarbons as a sole source of carbon, but subsequently found that their strains required methionine in the hydrocarbon-basal salts medium. The abolishment of the amino acid as an essential growth factor in the presence of streptomycin is an intriguing observation that will be the basis for further investigation. (This paper was presented in part at the 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa. 23 to 28 April 1972.)

ACKNOWLEDGMENTS

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