Atropine Metabolism by *Pseudomonas* sp. Strain AT3: Evidence for Nortropine as an Intermediate in Tropine Breakdown and Reactions Leading to Succinate

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***Pseudomonas* strain AT3, isolated by elective culture with atropine, hydrolyzed atropine and grew diauxically, first on the tropic acid and then on the tropine. Tropine was also used as a sole carbon and energy source. The methyl group of tropine was eliminated as formaldehyde, and the nortropine thus formed was a precursor of 6-hydroxycyclohepta-1,4-dione. Ammonia was detected as a product of nitrogen elimination. 6-Hydroxycyclohepta-1,4-dione was oxidized to cyclohepta-1,3,5-trione by an induced NAD⁺-specific dehydrogenase. Although cyclohepta-1,3,5-trione is a β -diketone with two potential hydrolytic cleavage sites, an induced hydrolase was specific for one of these sites, with 4,6-dioxoheptanoate as the only hydrolysis product. Unlike the alternative cleavage product (3,6-dioxoheptanoate), this compound is also a β -diketone, and a second hydrolytic cleavage formed succinate and acetone. Although *Pseudomonas* strain AT3 was not capable of growth with acetone, the compound was not detected in the culture medium and may have been lost to the atmosphere. Exhaustive experimentation with a wide range of conditions did not result in detection of the enzymes required for cleavage of the carbon-nitrogen bonds leading to the formation of nortropine and 6-hydroxycyclohepta-1,4-dione.**

Atropine, one of the solenaceous alkaloids (7), contains two cyclic structures, the alicyclic nitrogen-containing alcohol tropine and the aromatic tropic acid, joined by an ester linkage (Fig. 1). Its degradation by microorganisms has been reported by several groups (5, 11, 12, 18, 19), and, in cases for which the mechanism has been studied, hydrolysis of the ester linkage to give the two separate cyclic components is the initial step. Until recently, published studies of the pathway of tropine degradation have been limited to those of Niemer and Bucher (17), who reported the oxidation of the alcohol group of tropine by an NAD⁺-linked dehydrogenase in *Corynebacterium belladonna* and the accumulation of small amounts of methylamine and tropinic acid in the culture medium. The proposed metabolism of tropine through tropinone and tropinic acid to 2,6-dioxopimelic acid implied cleavage of tropine at the carbon bearing the hydroxyl group before release of the nitrogen. Although plausible enzymatic steps can be postulated for this catabolic sequence, the evidence presented was of a preliminary nature; tropinic acid was identified by its melting point, and 2,6-dioxopimelic acid was proposed, not identified. In our studies, *Pseudomonas* strain AT3, isolated by elective culture on atropine, has been found to cleave the ester linkage with an induced esterase and grow diauxically on the products, with tropic acid being used in the first phase of growth and then slower growth on the accumulated tropine moiety taking place (14). During growth in a nitrogen-limited medium, tropic acid was still used preferentially, with nitrogen being provided from the limited metabolism of tropine, and a novel keto compound that accumulated in the culture medium was identified as 6-hydroxycyclohepta-1,4-dione (2). This compound, which served as a growth substrate for *Pseudomonas* strain AT3, was shown to be an intermediate in the catabolism of tropine. An NAD⁺-linked dehydrogenase that used 6-hydroxycyclohepta-1,4-dione

as a substrate was assayed, and the oxidation product was tentatively identified as cyclohepta-1,3,5-trione (2). A mutant (MS2) of *Pseudomonas* strain AT3, blocked at the 6-hydroxycyclohepta-1,4-dione dehydrogenase, in contrast to the parent strain would not grow with tropine as the sole carbon and energy source but when grown on atropine accumulated near-stoichiometric amounts of 6-hydroxycyclohepta-1,4-dione in the medium (3). Although an NADP⁺-dependent tropine dehydrogenase was induced in *Pseudomonas* strain AT3 by growth on atropine, tropine, or tropinone, it was shown with [3-²H]tropine, [3-²H]pseudotropine (1), and mutant MS2 that tropine dehydrogenase is not involved in the catabolic pathway leading from tropine to central metabolites and that tropinone is not a pathway intermediate (3). Tropine dehydrogenase links tropinone into the pathway of tropine catabolism (3). The pathway of tropine metabolism by *Pseudomonas* strain AT3 is therefore quite different from that proposed for *C. belladonna* by Niemer and Bucher (17), in which tropinone is a key intermediate. In this paper we present evidence for a sequential cleavage of carbon-nitrogen bonds of tropine with nortropine as an intermediate and precursor of 6-hydroxycyclohepta-1,4-dione, confirm the identity of the product of 6-hydroxycyclohepta-1,4-dione dehydrogenase as cyclohepta-1,3,5-trione, and identify the reactions that lead from it to succinate and acetone.

MATERIALS AND METHODS

Organisms, maintenance, and growth. *Pseudomonas* strain AT3 was maintained and grown as previously described (14). Mutant MS2 was obtained by treatment of *Pseudomonas* strain AT3 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selection for organisms capable of limited growth on atropine but unable to grow on tropine as the sole carbon source. This mutant was able to use tropine as the sole nitrogen source when provided with an alternative carbon source and, under these conditions, was used to accumulate stoichiometric amounts of 6-hydroxycyclohepta-1,4-dione in the medium. *Corynebacterium* strain A1 was grown on isopropanol as the sole carbon source as previously described (20). *Pseudomonas putida* ATCC 17453 was grown on (+)-camphor (10).

Preparation of cell extracts. Cell extracts were prepared by ultrasonic disrupt-

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FIG. 1. Atropine.

tion as previously described (2) followed by centrifugation at $28,000 \times g$ for 30 min at 4°C.

Protein assays. Protein was assayed either by the method of Lowry et al. (15) or by the tannic acid turbidimetric method (16). Bovine serum albumin was used as the standard.

Respirometry. Oxygen consumption by whole bacteria was measured manometrically in a Warburg apparatus as previously described (14). Oxygen consumption in coupled enzyme assays was measured polarographically with an oxygen monitor.

Extraction of metabolites. Metabolites were routinely extracted from neutral or acidified culture supernatants or reaction mixtures by two extractions, each with the same volume of redistilled diethyl ether. The ether extracts were pooled and dried over anhydrous Na₂SO₄, and the solvent was removed under a stream of nitrogen. Occasionally ethyl acetate or methylene chloride was used as an alternative solvent.

Derivatization. Polar compounds were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide prior to gas chromatography-mass spectrometry (GC-MS) analysis. Neutral ketones were converted into their 2,4-dinitrophenylhydrazones by incubation with 0.1% (wt/vol) 2,4-dinitrophenylhydrazine (2,4-DNPH) in 2 M HCl at 30°C for 30 min. The products were extracted into ethyl acetate, which was dried over anhydrous Na₂SO₄ and analyzed directly by thin-layer chromatography (TLC).

GC-MS. GC-MS was performed on a Hewlett-Packard 5890 instrument with a 5971 mass-selective detector. Compounds were separated on an HP5 (cross-linked 5% phenylmethylsilicone) column (25 m by 0.2 mm; 0.33- μ m film thickness) with helium as the carrier gas. The column temperature was held at 70°C for 5 min and then increased to 275°C at a rate of 20°C/min. The mass spectrometer was operated at an electron ionization energy of 70 eV. The injector and analyzer temperatures were set at 250 and 280°C, respectively. Instrument library searches and comparison with authentic compounds were used for identification of metabolites as appropriate. Relative amounts of metabolites were calculated from peak areas of GC-MS total ion current chromatograms. Methane was used for chemical ionization.

¹H NMR. Proton nuclear magnetic resonance (NMR) spectra were obtained by O. W. Howarth at the Science and Engineering Research Council high-field NMR service at the University of Warwick, Coventry, United Kingdom, using a Bruker WH 400 spectrophotometer with the sample dissolved in [²H]chloroform and with tetramethylsilane included as the reference compound for calculation of chemical shifts.

Chromatography. Flash chromatography was performed on a column (15 by 2 cm) of silica gel (230-400 mesh; Merck 9385) with ethanol as the solvent for purification of 4-hydroxycyclohepta-1,4-dione or with ethanol-ethyl acetate (8:1, vol/vol) as the solvent for purification of cyclohepta-1,3,5-trione. Keto compounds were located by testing a small portion of each fraction with 2,4-DNPH reagent. TLC analysis of the products of 6-hydroxycyclohepta-1,4-dione dehydrogenation was done on 0.25-mm-thick precoated silica gel GHFL plates (Analtch, Newark, N.J.) developed with ethanol-ethyl acetate (8:1 or 1:1 [vol/vol]) as the solvent. Ketonic compounds were located as yellow spots after the plates were sprayed with 2,4-DNPH reagent. The 2,4-DNPH derivative of acetone was visually observed on plates developed with petroleum ether (bp 40 to 60°C)-diethyl ether (7:3, vol/vol) or heptane-ethyl acetate (4:1, vol/vol).

Enzyme assays. 6-Hydroxycyclohepta-1,4-dione dehydrogenase was assayed spectrophotometrically by monitoring the reduction of NAD⁺ at 340 nm at 30°C in a 1-cm-path-length cuvette containing, in 3 ml of 0.1 M sodium pyrophosphate buffer (pH 8.8), 2 μ mol of NAD⁺, 0.2 μ mol of substrate, and enzyme. Formaldehyde dehydrogenase was similarly assayed in a cuvette containing, in 3 ml of 42 mM sodium-potassium phosphate buffer (pH 7.1), 1.5 μ mol of NAD⁺, 5 μ mol of formaldehyde, and cell extract. Isopropanol dehydrogenase was assayed in a cuvette that contained, in 1 ml of 0.1 M sodium-potassium phosphate buffer (pH 7.1), 0.25 μ mol of NADH, 1 μ mol of acetone, and extract of isopropanol-grown *Corynebacterium* strain A1. NADH oxidase from *P. putida* ATCC 17453 was assayed by monitoring NADH oxidation at 340 nm in a cuvette that contained, in 1 ml of 42 mM sodium-potassium phosphate buffer (pH 7.1), 0.15 μ mol of NADH, 25 nmol of flavin mononucleotide (FMN), and 0.02 to 0.2 U of enzyme. Hydrolytic cleavage of 4,6-dioxoheptanoate in 42 mM sodium-potassium phosphate buffer (pH 7.1) was monitored by measuring the decrease in A₃₀₀. The

enzyme unit used throughout is defined as the consumption of 1 μ mol of substrate or the formation of 1 μ mol of product per min.

4,6-Dioxoheptanoate hydrolysis was also monitored by measuring carboxyl group production in an autotitrator (Pye Unicam). A typical assay mixture contained 5 mg of extract protein in 12 ml of distilled water containing just sufficient buffer to maintain the initial pH. The reaction was started by the addition of 10 μ mol of 4,6-dioxoheptanoate, adjusted to the initial pH, and acid production was monitored by the controlled addition of 1 mM NaOH.

Partial purification of 6-hydroxycyclohepta-1,4-dione dehydrogenase. (i) **Chromatography on Reactive Red 120 agarose.** An extract of atropine-grown *Pseudomonas* strain AT3 containing 5 mg of protein was loaded onto a 0.5-ml column of Reactive Red 120, type 3000CL, contained in a Pasteur pipette and equilibrated with 21 mM potassium-sodium phosphate buffer, pH 7.1. The column was washed with 5 ml of buffer, and the enzyme was eluted with 2 ml of buffer containing 5 mM NAD⁺. This gave a 10.7-fold purification with 36% recovery of activity.

(ii) **Chromatography on Q-Sepharose.** An extract of atropine-grown *Pseudomonas* strain AT3 containing 10 mg of protein was loaded onto a 2-ml column of Q-Sepharose (Pharmacia Biotech, St. Albans, United Kingdom) contained in a Pasteur pipette and equilibrated with 42 mM potassium-sodium phosphate buffer, pH 7.1. The column was washed with 5 ml of buffer, and then the enzyme was eluted by passage of 1 M NaCl in the same buffer and 1-ml fractions were collected. Fractions containing enzyme were pooled. This gave threefold purification and 100% recovery of activity.

Purification of NADH oxidase from *P. putida* ATCC 17453. The NADH oxidase, which catalyzes NADH oxidation in the presence of excess FMN, was purified from camphor-grown *P. putida* ATCC 17453 as previously described (21).

Enzymatic preparation of cyclohepta-1,3,5-trione. Cyclohepta-1,3,5-trione was prepared enzymatically from 6-hydroxycyclohepta-1,4-dione with the dehydrogenase partially purified by Q-Sepharose chromatography. A typical reaction mixture contained, in a volume of 30 ml, 2.3 mmol of sodium pyrophosphate buffer (pH 8.8), 30 μ mol of NAD⁺, 14 μ mol of 6-hydroxycyclohepta-1,4-dione, and 60 μ mol of EDTA to inhibit further metabolism of the dehydrogenation product. Reactions, at 30°C, were started by the addition of 1.2 U of partially purified dehydrogenase, and the progress of the reaction was monitored at 340 nm until the reaction ceased. Continuous diethyl ether extraction, removal of the solvent, and flash chromatography of the product yielded material that was 93% pure by GC-MS analysis.

Detection and assay of metabolites. 6-Hydroxycyclohepta-1,4-dione was assayed by formation of 2,4-dinitrophenylhydrazones and measurement of the A₄₂₇ in alkaline solution as previously described (2). The assay was calibrated with a pure sample of the compound. Ammonia production by washed bacterial suspensions, incubated with tropine or nortropine, was measured spectrophotometrically with Nessler's reagent at 460 nm. Ammonium chloride was used to prepare a standard curve. The 2,4-dinitrofluorobenzene spectrophotometric method (9) was used to search for primary and secondary amines in culture supernatants.

Chemicals. Atropine, 4,6-dioxoheptanoic acid, Reactive Red 120 type 3000CL, and tropine were from Sigma Chemical Company (Poole, United Kingdom). *N,O*-Bis(trimethylsilyl)trifluoroacetamide was from Fluka Chemicals (Gillingham, United Kingdom). Nortropine was prepared from tropine (13). 6-Hydroxycyclohepta-1,4-dione was produced biologically from tropine by using mutant MS2, extracted from the culture medium with redistilled ethyl acetate, and purified by flash chromatography (2). The compound was recrystallized from diethyl ether, and its purity was checked by GC-MS.

RESULTS

Evidence for nortropine as a catabolic intermediate. Although we have clearly established that the catabolism of tropine by *Pseudomonas* strain AT3, in contrast to the proposed pathway for *C. belladonna*, occurs with nitrogen elimination preceding carbocyclic ring cleavage (2), our evidence suggests that cleavage of the methyl carbon-nitrogen bond, to yield nortropine, occurs before excision of the nitrogen from the ring. *Pseudomonas* strain AT3 grew rapidly with nortropine as the sole C, or sole C and N, source, with a stationary-phase attenuation similar to that obtained with tropine (1 g/liter). Bacteria grown on tropine oxidized nortropine immediately and rapidly (4.5 μ mol of O₂ per μ mol of nortropine), even in the presence of 50 μ g of chloramphenicol per ml to inhibit enzyme induction (this concentration was shown to inhibit induction of enzymes for *p*-hydroxybenzoate oxidation in succinate-grown bacteria). *Pseudomonas* strain AT3, harvested in the tropine phase of growth with atropine, oxidized formalde-

TABLE 1. Enzymes of 6-hydroxycyclohepta-1,4-dione metabolism in tropine- and succinate-grown *Pseudomonas* strain AT3

Enzyme	Activity (U/mg of protein) in extracts of cells grown on:	
	Tropine	Succinate
6-Hydroxycyclohepta-1,4-dione dehydrogenase	0.21 ^a	0.008
Formaldehyde dehydrogenase	0.064	<0.005
Formate dehydrogenase	Present	Absent
Cyclohepta-1,3,5-trione hydrolase	0.08	<0.005
Succinylacetone hydrolase	0.011	<0.001

^a The enzyme was not detectable in extracts of mutant MS2 grown on succinate with tropine as a nitrogen source.

hyde and formate rapidly and immediately. Methylamine and methanol were not oxidized, and methylamine did not appear in the culture medium during growth on tropine. Formaldehyde oxidation was biphasic, with the second, slower phase corresponding to the rate of formate oxidation. Total oxygen consumption was approximately equal to the theoretical value for each substrate. Succinate-grown bacteria showed no significant oxidation of formaldehyde or formate. An induced NAD⁺-linked formaldehyde dehydrogenase was detected in extracts of tropine-grown bacteria, with a specific activity of 0.064 U/mg of protein (Table 1), while the specific activities in extracts of nortropine- or tropic acid-grown bacteria were approximately 10-fold lower (0.005 to 0.006). Although an NAD⁺-linked formate dehydrogenase was detected in extracts of tropine-grown bacteria, it was so unstable as to make quantitative measurement unreliable. Analysis of reaction mixtures compared with controls showed increased liberation of ammonia during tropine and nortropine oxidation by washed bacteria. When mutant MS2, grown on atropine as the sole carbon and nitrogen source, was incubated with nortropine, 6-hydroxycyclohepta-1,4-dione accumulated quantitatively in the incubation medium in a manner identical to that observed when tropine was used as a substrate. Proof of the intermediate formation of nortropine during tropine oxidation by *Pseudomonas* strain AT3 was obtained from experiments in which mutant MS2, grown on atropine as the sole C and N source, was incubated with tropine and the reaction mixture was sampled at timed intervals. Metabolites identified by GC-MS were tropinone, nortropine, nortropinone, and 6-hydroxycyclohepta-1,4-dione. Although these results suggested that different enzymes may be involved in cleaving the carbon-nitrogen bond that releases the methyl group as formaldehyde and in excising the N atom from the ring, exhaustive experimentation with different disruption regimens, tropine and nortropine as substrates, a range of buffers and assay conditions, and the inclusion of a variety of additional components failed to demonstrate subcellular cleavage of carbon-nitrogen bonds. Although we have therefore been unable to characterize the enzymology of the established conversion of tropine and nortropine into 6-hydroxycyclohepta-1,4-dione, the further metabolism of 6-hydroxycyclohepta-1,4-dione, including ring cleavage and the subsequent formation of succinate, has been elucidated.

Induction of 6-hydroxycyclohepta-1,4-dione dehydrogenase. Extracts of *Pseudomonas* strain AT3 grown on atropine in the presence of NH₄⁺ and harvested in the late phase of growth on tropine contained an NAD⁺-specific dehydrogenase activity towards 6-hydroxycyclohepta-1,4-dione with an alkaline pH optimum and a specific activity of 0.083 U/mg of protein. A higher specific activity of 0.21 was obtained with extracts of

tropine-grown bacteria, while in contrast, extracts of bacteria grown on succinate or harvested during the tropic acid phase of growth on atropine had specific activities of 0.008 and <0.0008, respectively (Table 1), clearly showing that the enzyme is induced by growth on tropine. Its key role in the metabolism of tropine by *Pseudomonas* strain AT3 was confirmed with mutant MS2. This mutant did not grow with tropine as the sole C source. It grew on atropine as the sole carbon and nitrogen source but lacked any detectable level of the enzyme when thus grown and accumulated stoichiometric amounts of 6-hydroxycyclohepta-1,4-dione, which also accumulated stoichiometrically when tropine was used as the N source for growth on succinate.

Accumulation and identification of the product of 6-hydroxycyclohepta-1,4-dione dehydrogenation. The logical product of the dehydrogenation of 6-hydroxycyclohepta-1,4-dione would be the triketone cyclohepta-1,3,5-trione. Initial experiments, in which a crude extract was incubated with 6-hydroxycyclohepta-1,4-dione in alkaline buffer in the presence of NAD⁺ and then subjected to diethyl ether extraction and GC-MS, yielded a somewhat confused picture in comparison with controls. The substrate was no longer detectable, and a number of small product peaks included one with M⁺ at *m/z* 140, the expected molecular ion for cyclopenta-1,3,5-trione. Because of the possibility that cyclohepta-1,3,5-trione was either unstable or being further metabolized, possibly to more-polar compounds that were not detected, additional experiments were carried out with the dehydrogenase partially purified by affinity chromatography as described in Materials and Methods.

A 6-ml reaction mixture contained 450 μmol of sodium pyrophosphate buffer (pH 8.8), 6 μmol of NAD⁺, and 0.74 U of partially purified 6-hydroxycyclohepta-1,4-dione dehydrogenase. The substrate (2 μmol) was added, and the reaction, at 30°C, was monitored at 340 nm until it ceased. The observed absorbance increase corresponded to the reduction of 1.9 μmol of NAD. The reaction mixture was acidified and extracted with an equal volume of diethyl ether, which was dried and analyzed directly by GC-MS. A single major peak (retention time, 13.27 min) represented 90% of the material detected and was absent from a boiled control. The MS gave an M⁺ at *m/z* 140, which was confirmed by the chemical ionization spectrum. Other fragmentation peaks at *m/z* 112 (M⁺ - C=O) and 98 (M⁺ - CH₂CO) were compatible with the structure cyclohepta-1,3,5-trione (Fig. 2A). TLC analysis showed a minor residual substrate spot (*R_f*, 0.6) and a single additional major 2,4-DNPH-positive spot (*R_f*, 0.38) for the reaction product.

Larger quantities of the compound were also obtained in reactions in which NADH formed during catalysis was reoxidized and thus recycled by NADH oxidase in the presence of FMN. A typical reaction mixture contained, in 20 ml of 0.1 M sodium pyrophosphate buffer (pH 8), 4.5 μmol of NAD⁺, 75 μmol of FMN, 2 U of catalase, 7 U of NADH oxidase, and 1 U of 6-hydroxycyclohepta-1,4-dione dehydrogenase. The reaction, at 30°C, was started by the addition of 12 μmol of substrate. A parallel small-scale reaction was monitored in the oxygen monitor, and in the presence of 1.2 μmol of substrate, the stimulated consumption of oxygen was 0.64 μmol, which is compatible with a single oxidation step in which two H atoms are removed from the substrate.

The results of proton NMR analysis of the putative triketone, prepared in a bulk reaction and purified by flash chromatography as described in Materials and Methods, were inconclusive as an aid to identification because of extensive tautomerization of the compound.

FIG. 2. Mass spectra of cyclohepta-1,3,5-trione formed from 6-hydroxycyclohepta-1,4-dione (A), the 2TMS derivative of authentic 4,6-dioxoheptanoic acid (B), and the 2TMS derivative of 4,6-dioxoheptanoic acid formed by enzymatic hydrolysis of the triketone (C).

Further metabolism of cyclohepta-1,3,5-trione. Cyclohepta-1,3,5-trione is a β -diketone and thus a potential substrate for a β -diketone hydrolase (EC 3.7.1.-). There are two possible products of hydrolytic cleavage of this triketone: cleavage between carbon atoms 2 and 3 would yield 4,6-dioxoheptanoate (route 1 in Fig. 3), and cleavage between carbon atoms 1 and 2 would yield 3,6-dioxoheptanoate (route 2 in Fig. 3). Only the former product, 4,6-dioxoheptanoate, is a β -diketone and, like its precursor, a potential substrate for further hydrolytic cleavage with the formation of succinate and acetone.

Investigations of triketone cleavage were hampered by the

lack of a commercial source of the compound. All attempts to prepare the compound by the chemical oxidation of 6-hydroxycyclohepta-1,4-dione with mild oxidizing agents that ranged from bleach to periodinane failed. Consequently, experiments with cyclohepta-1,3,5-trione were routinely performed with the compound made by using partially purified 6-hydroxycyclohepta-1,4-dione dehydrogenase as described in Materials and Methods. For routine experiments, the diethyl ether extraction and flash chromatography steps were omitted and the compound was prepared in situ.

When 1.4 μ mol of the triketone preparation was incubated with the crude cell extract (1.1 mg of protein) in sodium pyrophosphate buffer (pH 8.8) and samples were removed at timed intervals, acidified, extracted with diethyl ether, and derivatized for GC-MS analysis, there was a steady time-dependent conversion of the triketone into a product which, when derivatized, had the same retention time (13.65 min) and mass spectrum as the trimethylsilyl (2TMS) derivative of authentic 4,6-dioxoheptanoate, i.e., M^+ at m/z 302 and major fragmentation peaks at m/z 287 ($M^+ - CH_3$), m/z 169, and m/z 157 (Fig. 2B and C). After 20 min of incubation, only a trace of triketone remained. A trace of 2TMS-succinate (11.13 min) was also detected, indicating that although further hydrolysis of 4,6-dioxoheptanoate may occur, under this particular assay regimen it was much slower than the hydrolysis of the triketone. Two parallel controls, one without the substrate and one without the crude extract addition, were also run. Acidification followed by diethyl ether extraction and GC-MS analysis confirmed the presence of only the triketone in the control to which the crude extract had not been added. No significant metabolite peaks were evident in the second control.

Succinate formation from 4,6-dioxoheptanoate by cell extracts. The β -diketone 4,6-dioxoheptanoate has two possible hydrolytic cleavage points leading to two alternative sets of products: either succinate and acetone or 4-oxopentanoate and acetate. Both cleavage reactions would generate an additional carboxyl group. The metabolism of 4,6-dioxoheptanoate by crude extract of tropine-grown *Pseudomonas* strain AT3 was monitored by measuring the decrease in A_{300} . Reactions were performed over a range of pH values, with the maximum rate being observed in sodium-potassium phosphate buffer at pH

FIG. 3. Alternative sites for hydrolytic cleavage of the β -diketone cyclohepta-1,3,5-trione. Cleavage only at site 1 is supported by experimental evidence.

FIG. 4. Proposed pathway for the degradation of tropine by *Pseudomonas* strain AT3. Enzymes: 1, atropine esterase; 2, tropine dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, 6-hydroxycyclohepta-1,4-dione dehydrogenase; 6, cyclohepta-1,3,5-trione hydrolase; 7, 4,6-dioxoheptanoate hydrolase. X and Y are carbon-nitrogen bond cleavage reactions that we have been unable to demonstrate in cell-free systems.

7.1. Although the specific activity of the enzyme was low (0.011 U/mg of protein), it was 16-fold greater than that observed with extracts of succinate-grown bacteria (Table 1). When the products of cleavage of 2.4 μ mol of 4,6-dioxoheptanoate by 1 mg of crude extract protein were extracted, derivatized, and analyzed by GC-MS, the 4,6-dioxoheptanoate was no longer detectable and a large 2TMS-succinate peak was identified (11.13 min). In contrast, analysis of a control with an extract of succinate-grown bacteria revealed only the starting material; succinate was absent. The formation of an additional carboxyl group on cleavage of 4,6-dioxoheptanoate was confirmed in assays when acid production in near-buffer-free reactions triggered the controlled addition of 1 mM NaOH to the reaction vessel to maintain the set pH.

A consequence of the identification of succinate as one product of the cleavage of 4,6-dioxoheptanoate by extracts of

tropine-grown *Pseudomonas* strain AT3 is that acetone should be the other catabolic fragment.

Acetone formation from 4,6-dioxoheptanoate by cell extracts. Because of its volatile nature and the limited range of GC columns available to us for routine analysis, acetone could not be detected by GC-MS. The compound was identified by chromatography of the 2,4-DNPH derivative and enzymatically with extracts of isopropanol-grown *Corynebacterium* strain A1 which contained an induced NAD⁺-linked isopropanol dehydrogenase (20). Crude extract protein (2 mg) from tropine-grown *Pseudomonas* strain AT3 was incubated with 2.3 μ mol of 4,6-dioxoheptanoate in 3 ml of sodium-potassium phosphate buffer (pH 7.1) in spectrophotometer cuvettes at 30°C. To cuvettes at zero time and after 1 and 2 h of incubation were added 0.3 μ mol of NADH and 50 μ l of a crude extract of isopropanol-grown *Corynebacterium* strain A1 containing 0.07 U of isopropanol dehydrogenase. NADH oxidation was then monitored at 340 nm. *Pseudomonas* strain AT3 extract, 4,6-dioxoheptanoate, and *Corynebacterium* strain A1 extract were variously omitted from controls. When NADH and *Corynebacterium* strain A1 extract were added to the complete reaction, immediately following addition of the 4,6-dioxoheptanoate, NADH oxidation occurred at a low rate that was only marginally higher than that observed with the controls. However, when *Pseudomonas* strain AT3 extract was preincubated with 4,6-dioxoheptanoate for 1 h, the addition of NADH and *Corynebacterium* extract resulted in an extended and rapid oxidation of the NADH which was not observed with control incubations. No stimulation of NADH oxidation was observed when an extract of succinate-grown *Pseudomonas* strain AT3 was used.

DISCUSSION

The pathway proposed for the metabolism of tropine by *Pseudomonas* strain AT3 is shown in Fig. 4. The involvement of nortropine as a catabolic intermediate in the breakdown of tropine by *Pseudomonas* strain AT3 has been established. The nortropine accumulation, the oxidation of formaldehyde and formate but not methylamine or methanol, and the detection of ammonia rather than methylamine as the nitrogen-containing metabolite released from tropine by washed bacteria all reinforce this interpretation. In addition to nortropine, nortropinone also accumulated in the culture medium. This observation complemented that of the transient accumulation of tropinone that also occurred during culture growth. It has been shown that tropinone is peripheral to the catabolic pathway leading to central metabolites (3). Tropine and nortropine are the only known substrates for the tropine dehydrogenase that is induced by growth of *Pseudomonas* strain AT3 on tropine and which probably serves to integrate tropinone of plant origin into the microbial degradative pathway for tropine (3). A consequence of the presence of this enzyme in tropine-grown *Pseudomonas* strain AT3 is alcohol-ketone interconversion and the consequent detection of the respective pairs of alcohols and ketones.

6-Hydroxycyclohepta-1,4-dione has been unequivocally identified as a product of nitrogen elimination from tropine that lies on the catabolic pathway leading to central metabolites (2), thus confirming the pathway as distinct from that proposed for *C. belladonna*. The central role played by 6-hydroxycyclohepta-1,4-dione dehydrogenase was established with mutant MS2. It is of interest that although we were able to obtain mutants of the organisms that lacked the ability to synthesize active dehydrogenase with relative ease, and none of these was able to grow with tropine as carbon source, we

were not successful in identifying mutants blocked at other steps in the tropine degradation pathway.

The GC-MS results obtained for the product of 6-hydroxycyclohepta-1,4-dione dehydrogenation are compatible with it being cyclohepta-1,3,5-triketone, and although the H^+ NMR spectra were unhelpful in absolute terms in establishing the structure because of keto-enol tautomerism, this phenomenon is compatible with the proposed structure of the compound. Attempts to synthesize the triketone by oxidation of 6-hydroxycyclohepta-1,4-dione with a variety of oxidizing agents were generally unsuccessful, although periodinane (8) did produce a trace of a compound with GC-MS characteristics identical to those of the product of 6-hydroxycyclohepta-1,4-dione dehydrogenation in addition to succinic acid. Indirect confirmation of the structure of cyclohepta-1,3,5-trione was provided by the unequivocal identification of 4,6-dioxoheptanoate as the product of its further metabolism by cell extracts in the absence of added cofactors. The commercial availability of 4,6-dioxoheptanoate allowed a direct TLC and GC-MS comparison to be made. Bacterial hydrolases that cleave β -diketones are not widely distributed but include those that catalyze the hydrolysis of fumarylpyruvate formed from gentisic acid and of fumarylacetoacetate formed from homogentisic acid (4). Hydrolytic cleavage of the cyclic β -diketone 2,6-diketocamphane, proposed as a step in the catabolism of (+)-camphor by *Mycobacterium rhodochrous* T1 (6), is one example in which a cyclic neutral compound is cleaved. Cyclohepta-1,3,5-trione has two potential β -diketone cleavage sites. It is interesting that cleavage occurs only at the site leading to the formation of 4,6-dioxoheptanoate, which is also a β -diketone and thus a substrate for a second hydrolytic cleavage. It is also of interest that cleavage of the seven-member ring does not take place at the oxidation level of 6-hydroxycyclohepta-1,4-dione, as this molecule would be a potential substrate for nonhydrolytic cleavage by an aldolase. The identified catabolic intermediates, in conjunction with the induced enzymes that have been detected, all support the pathway proposed for the metabolism of tropine by *Pseudomonas* strain AT3 shown in Fig. 4. While one of the products of cleavage of the seven-carbon skeleton is the central metabolite succinate, the other product, acetone, does not easily integrate into central metabolism. For those organisms that are able to use it as a sole source of carbon, an induced pathway, in which acetol and methylglyoxal are intermediates leading to pyruvate, has been reported (20). Although we have not investigated in detail the fate of acetone produced from tropine by *Pseudomonas* strain AT3 we have established that the organism is not capable of growth with acetone as the sole source of carbon and that tropine-grown cells are not capable of significant oxidation of the compound. It is possible that the compound is excreted into the culture medium and that, under the culture conditions used, it is lost to the atmosphere.

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