Tropine dehydrogenase: purification, some properties and an evaluation of its role in the bacterial metabolism of tropine

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Tropine dehydrogenase was induced by growth of *Pseudomonas* AT3 on atropine, tropine or tropinone. It was NADP⁺-dependent and gave no activity with NAD⁺. The enzyme was very unstable but a rapid purification procedure using affinity chromatography that gave highly purified enzyme was developed. The enzyme gave a single band on isoelectric focusing with an isoelectric point at approximately pH 4. The native enzyme had an M_r of 58 000 by gel filtration and 28 000 by SDS/PAGE and therefore consists of two subunits of equal size. The enzyme displayed a narrow range of specificity and was active with tropine and nortropine but not with pseudotropine, pseudonortropine, or a

number of related compounds. The apparent K_m s were 6.06 μ M for tropine and 73.4 μ M for nortropine with the specificity constant (V_{max}/K_m) for tropine 7.8 times that for pseudotropine. The apparent K_m for NADP⁺ was 48 μ M. The deuterium of [3-²H]tropine and [3-²H]pseudotropine was retained when these compounds were converted into 6-hydroxycyclohepta-1,4-dione, an intermediate in tropine catabolism, showing that the tropine dehydrogenase, although induced by growth on tropine, is not involved in the catabolic pathway for this compound. 6-Hydroxycyclohepta-1,4-dione was also implicated as an intermediate in the pathways for pseudotropine and tropinone catabolism.

INTRODUCTION

Tropine is an N-heterocyclic compound and one of the constituents of the alkaloid, atropine, in which it occurs esterified to tropic acid (Scheme 1). The first step in the bacterial metabolism of atropine is the hydrolysis of its ester linkage to give free tropine and tropic acid [1,2] and in *Pseudomonas* AT3 growth on atropine is diauxic with the tropic acid being utilized in the first phase of growth and tropine in the second [3]. Tropic acid appears to be metabolized via phenylacetic acid [4] but less is known about the breakdown of the tropine. It is a secondary alcohol and the alcohol group presents a prime target for enzymic oxidation, which would yield the corresponding ketone, tropinone. Such a step has been suggested by Niemer and Bucherer [5] who demonstrated the oxidation of tropine to tropinone by an NAD⁺-linked dehydrogenase in *Corynebacterium belladonna*. Tropinone can be regarded as a substituted cyclic ketone and the metabolism of several cyclic ketones has been shown to involve attack by monooxygenases in biological Baeyer–Villiger reactions to give the corresponding lactones [6]. Ring cleavage is then achieved either spontaneously or by the action of a lactonase (esterase). This sequence of catabolic reactions represents a feasible route for the metabolism of tropine via tropinone and would yield the N-containing compound, tropinic acid, a metabolite reportedly accumulated by *C. belladonna* [5]. *Pseudomonas* AT3 too contains a tropine dehydrogenase induced by growth on tropine [7] but the identification of 6-hydroxycyclohepta-1,4-dione as an intermediate of tropine breakdown in this organism and an induced NAD⁺-linked dehydrogenase that oxidizes this compound to cyclohepta 1,3,5-trione (Scheme 1) both suggest that the initial attack is at the nitrogen atom, not the alcohol group [8]. The 6-hydroxycyclohepta-1,4-dione retains the alcohol group of tropine on the equivalent carbon, which calls into question the involvement of tropine dehydrogenase and tropinone in the pathway for tropine

catabolism. Of course, the observed tropine dehydrogenase activity could be due to the presence of a dehydrogenase of broad specificity that was fortuitously active with tropine. However, in this paper we demonstrate, by purification of the enzyme and examination of its specificity, that in *Pseudomonas* AT3 the enzyme is a true tropine dehydrogenase and we assess its role in the pathway for tropine catabolism.

MATERIALS AND METHODS

Organisms, maintenance and growth

The organism, *Pseudomonas* AT3, was maintained and grown as described by Long et al. [3]. For growth on atropine or tropine, 1 g/l was added. This concentration of tropinone initially proved toxic but after several transfers in medium containing 0.2 g/l the organism was able to grow at the higher concentration. Mutant MS2 was obtained by treatment of *Pseudomonas* AT3 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selection for organisms capable of growth on atropine but not on tropine as their sole carbon source. This particular mutant was able to use tropine as its sole nitrogen source when provided with an alternative carbon source and, under these conditions, accumulated equivalent amounts of 6-hydroxycyclohepta-1,4-dione in the medium. Unlike the wild-type, mutant MS2 did not contain any 6-hydroxycyclohepta-1,4-dione dehydrogenase activity when grown on atropine (Scheme 1).

Preparation of cell extracts

Cell extracts were prepared by sonic disruption as described by Bartholomew et al. [8]. For the enzyme purification, cell paste was resuspended in an equal volume of 42 mM potassium/sodium phosphate buffer, pH 7.1, containing 10 % (v/v) ethanol.

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Scheme 1 The relationship of tropine dehydrogenase and tropinone to the catabolic pathway for tropine in *Pseudomonas* AT3

The positions where hydrogen atoms were replaced by deuterium in some experiments are shown as (^2H).

Purification of enzyme

All buffers used in the purification contained 10% (v/v) ethanol and all procedures were performed at 4 °C.

Cell extract from 5 g wet weight of *Pseudomonas* AT3 grown on tropine was loaded on to a Mimetic Orange 1 A6XL column (5 cm long $\times$ 2.5 cm diam.) equilibrated with 20 mM potassium/sodium phosphate buffer, pH 6.0. The column was washed with buffer until no protein could be detected in the eluate (20 vols.) and then with eight column volumes of this buffer adjusted to pH 7.0. The enzyme was then eluted with the pH 7.0 buffer containing 1 mM NADP^+ . The enzyme-containing fractions were pooled and loaded immediately on to a Reactive Red 120-agarose column (1 cm long $\times$ 2.5 cm diam.) equilibrated with 20 mM potassium/sodium phosphate buffer, pH 6.0. The column was washed with eight column volumes of this buffer and then with six volumes of buffer at pH 7.0. The enzyme was then eluted with pH 7.0 buffer containing 5 mM NADP^+ and 100 mM KCl.

Chromatography of enzyme by FPLC

The purified enzyme solution was concentrated to about 0.5 ml using Millipore Centrifugal Ultrafree units and a portion (0.2 ml) was used for FPLC on a calibrated Superose 12 column. The enzyme was eluted with 42 mM sodium/potassium phosphate buffer, pH 7.0, containing 10% (v/v) ethanol and 50 mM KCl at a flow rate of 0.5 ml/min and fractions of 0.25 ml were collected.

PAGE

Electrophoresis of purified enzyme was performed on non-denaturing 10% (w/v) and 5% (w/v) polyacrylamide gels. Enzyme activity was detected by incubating gels in 0.1 M glycine/NaOH buffer, pH 10, containing 1 mM tropine, 1 mM NADP^+ and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride hydrate (2 mg/ml). This gave a precipitate of a red formazan in the presence of enzyme.

SDS/PAGE was performed on Biorad Mini Protean II Ready Gels (4–20% gradient). Gels were calibrated with M_r standards (Sigma 4000–70 000 and 30 000–200 000 molecular mass markers).

Isoelectric focusing was performed in 5% polyacrylamide slab gels over the pH range 3.5–10 using an LKB Multiphore 2117 flat bed electrophoresis apparatus. A gel of 110 $\times$ 125 $\times$ 2 mm was prefocused at 200 V for 2 h and then run for 3 h at this voltage after addition of the protein sample. The pH gradient was determined by cutting strips from the edges of the gel into 1 cm lengths and measuring the pH of the solutions after these had each been soaked in 1 ml of distilled water for 4 h.

Proteins in all gels were detected by staining with Coomassie Brilliant Blue R-250.

Protein assays

Protein was determined by the tannic acid turbidimetric method of Mejbaum-Katzenellenbogen and Dobryszczyka [9] with BSA as standard.

Enzyme assays

Tropine dehydrogenase was assayed in the forward direction by following the reduction of NADP^+ spectrophotometrically at 340 nm and 30 °C. The reaction mixture contained, in 1 ml of 80 mM glycine/NaOH buffer, pH 10, 1 μmol tropine, 1 μmol NADP^+ and enzyme. This assay, with 0.5 μg of enzyme, was used for the kinetic experiments, with the tropine concentration varied within the range 0.01–0.5 mM (1 mM NADP^+) or the tropine replaced by nortropine in the range 0.05–0.5 mM. The NADP^+ concentration was varied within the range 0.1–1 mM (1 mM tropine). Assays were performed in triplicate using freshly purified enzyme and the kinetic constants and their standard errors were calculated by using the ENZFITTER non-linear-regression analysis computer program [10]. In the reverse direction the oxidation of NADPH was monitored at 340 nm in a

reaction mixture containing in 3 ml of 0.1 M potassium phosphate buffer, pH 6.0, 0.6 μ mol of NADPH, 3 μ mol of tropinone and enzyme. Enzyme units are μ mol of substrate transformed/min.

In the search for tropinone monooxygenase activity, oxygen consumption was measured using an oxygen electrode. The 3 ml reaction volume contained, in buffers of various pH values (0.1 M potassium/sodium phosphate buffer, pH 6–8; 0.1 M Tris/HCl buffer, pH 8–9) 1 μ mol of NADH or NADPH, 3 μ mol of tropinone and cell extract.

Conversion of [3-²H]tropine and [3-²H]pseudotropine into 6-hydroxycyclohepta-1,4-dione

A starter culture of mutant MS2 was grown in medium containing atropine (1 g/l) as the sole carbon and nitrogen source. When growth ceased, 5 ml portions of this culture were harvested; the cells were washed with 42 mM potassium/sodium phosphate buffer, pH 7.1, to avoid carry-over of any accumulated 6-hydroxycyclohepta-1,4-dione, and then portions were resuspended in 100 ml of medium containing 50 mg of tropic acid as the carbon source and 50 mg of either [3-²H]tropine or [3-²H]pseudotropine as the sole nitrogen source. The cultures were grown at 30 °C on a gyrotary shaker and when growth was complete the cells were removed by centrifuging. In each case the supernatant was acidified to pH 5.5 with 5 M HCl and extracted three times with an equal volume of ethyl acetate. The ethyl acetate was dried over anhydrous Na₂SO₄ and then evaporated to dryness. The product, 6-hydroxycyclohepta-1,4-dione, was examined by GC–MS and then purified by flash chromatography as described by Bartholomew et al. [8] and crystallized from diethyl ether.

NMR spectra

NMR spectra were performed by Dr. O. W. Howarth at the SERC NMR Service, The University of Warwick, U.K. using a Bruker WH 400 spectrometer with the sample dissolved in [2H]-chloroform and with tetramethylsilane included as the reference compound for calculation of chemical shifts.

GC–MS

GC–MS was performed on a Hewlett-Packard 5890 instrument with a 5971 mass-selective detector. An HP5 (cross-linked 5% phenylmethylsilicone) column (25 m $\times$ 0.2 mm $\times$ 0.33 μ m film) was used with helium as the carrier gas, and a temperature programme of 5 min at 70 °C rising at 20 °C/min to 275 °C.

Chromatography

TLC was performed using precoated silica-gel GHLF plates (Analtech, Newark, NJ, U.S.A.) with solvent A [ethyl acetate/methanol/880' ammonia (85:10:5, by vol.)], solvent B [toluene/1,4-dioxane/acetic acid (90:20:4, by vol.)] and solvent C [toluene/ethyl formate/formic acid (50:20:4, by vol.)]. Alkaloids were detected with Dragendorff's KBil₄ reagent [11].

Chemicals

Atropine, ecgonine, ecgonine methyl ester, Reactive Red 120, scopine, tropine, tropinone and tropic acid were from Sigma Chemical Co. (Poole, Dorset, U.K.). Mimetic Orange 1 A6XL was from Affinity Chromatography Ltd. (Freeport, Ballasalla, Isle of Man, U.K.). 4-Hydroxypiperidine and 4-hydroxytropinone were from Aldrich Chemical Co. (Gillingham, Dorset,

U.K.). Pseudotropine, [3-²H]pseudotropine and [3-²H]tropine were prepared as described by Bartholomew et al. [12]. Nortropine was prepared from tropine by the method of Kraiss and Nádor [13]. This procedure was also used to prepare pseudonortropine from pseudotropine. 6-Hydroxycyclohepta-1,4-dione was produced biologically from tropine as described by Bartholomew et al. [8].

RESULTS AND DISCUSSION

Tropine dehydrogenase in cell extracts

NADP⁺-linked tropine dehydrogenase activity was detected in extracts of *Pseudomonas* AT3 grown on atropine, tropine or tropinone (Table 1). No activity was found in extracts of cells grown on succinate or tropic acid, indicating the inducible nature of the activity associated with growth on these alkaloids. The activity could be assayed in either direction, with pH optima of 10 in the forward direction in 0.1 M glycine/NaOH buffer and 6.5 in the reverse direction in 0.1 M potassium/sodium phosphate buffer.

Other dehydrogenases, including 6-hydroxycyclohepta-1,4-dione dehydrogenase, tropic acid dehydrogenase and phenylacetaldehyde dehydrogenase, are involved in the pathway of tropine or atropine metabolism [7,8] and there was the possibility that one of these was giving rise to the observed tropine dehydrogenase activity. This was resolved by purification of the enzyme and examination of its substrate specificity.

Purification and properties of tropine dehydrogenase

The enzyme was unstable in crude extracts and lost 65% of its activity in 60 h at 4 °C but could be stabilized by addition of 10% (v/v) ethanol which reduced the loss to 15%. Ethanol was added to all buffers used in the purification, but, even in the presence of 10% ethanol, purified enzyme lost 90% of its activity in 10 h. Rapid purification of the enzyme was therefore essential, and a procedure that was complete in one day was developed.

The enzyme was purified by affinity chromatography on Mimetic Orange 1 and then on Reactive Red 120–agarose (Table 2). Attempts to assess purity by non-denaturing PAGE were unsuccessful as the enzyme did not appear to penetrate the gel and, even with a 5% gel, activity staining detected activity only on the top of the gel in the sample wells. However, only one protein band was detected after isoelectric focusing of the purified enzyme (Figure 1) with an isoelectric point at approx. pH 4, although no activity could be detected using the activity stain. Also, SDS/PAGE gave one major band with only one other very minor band running ahead of it (Figure 1). Thus the enzyme appears to be highly purified.

Table 1 Specific activity of tropine dehydrogenase after growth on various substrates

Tropine dehydrogenase was assayed in the direction of tropine oxidation and specific activity is expressed as units/min.

Growth substrate	Specific activity
Atropine	0.05
Tropine	0.3
Tropinone	0.15
Tropic acid	< 0.01
Succinate	< 0.01

Table 2 Purification of tropine dehydrogenase

Purification step	Volume (ml)	Total protein (mg)	Enzyme activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	11.4	522	165	0.316	100	—
Mimetic Orange 1	36.0	7.16	135	18.85	81.8	59.7
Reactive Red	21.5	1.36	86.8	63.82	52.6	202

Figure 1 Isoelectric focusing and SDS/PAGE of purified tropine dehydrogenase

A gel stained for protein after isoelectric focusing of the concentrated purified enzyme is shown in (a). The peak fraction from gel filtration of the purified enzyme after SDS/PAGE is shown in (b) with the positions of M_r markers indicated.

When examined by FPLC on a gel filtration column the enzyme gave a single protein peak, corresponding to an M_r of 58 000, which coincided with the peak of enzyme activity. SDS/PAGE of the peak fraction gave a protein band corresponding to an M_r of 28 000 (Figure 1), indicating that the enzyme is a dimer of equal sized subunits.

The dehydrogenase was specific for NADP⁺ as acceptor and no activity was detected when this was replaced with NAD⁺ at the same concentration (1.0 mM) in the assay. The enzyme was active with tropine as substrate but no activity was detected with pseudotropine, the epimer of tropine, or with a number of closely related compounds including 6-hydroxytropinone, atropine, scopolamine, ecgonine, ecgonine methyl ester, 4-hydroxypiperidine, cycloheptanol, cyclooctanol, cyclopentanol, tropic acid or 6-hydroxycyclohepta-1,4-dione. The only other substrate was the tropine homologue, nortropine, which lacks the methyl group on the nitrogen, and again there was no activity with its epimer, pseudonortropine. The ratio of the rate with nortropine to that with tropine was about 1.5 and this was maintained throughout the purification, indicating that the same enzyme was active with both substrates. The higher rate with nortropine might suggest

that this is the true substrate for the enzyme but kinetic studies gave a much lower apparent K_m for tropine ($6.06 \pm 0.46 \mu\text{M}$) than for nortropine ($73.4 \pm 3.9 \mu\text{M}$) and although the V_{max} values from these studies were 0.0341 ± 0.0005 for nortropine compared with $0.022 \pm 0.0005 \mu\text{mol/min}$ for tropine, the specificity constant (V_{max}/K_m) for tropine was 7.8-fold higher than that for nortropine. The apparent K_m for NADP⁺ was $48 \pm 0.8 \mu\text{M}$.

To identify the product of tropine dehydrogenase, 3 μmol of tropine was incubated in 3 ml of 0.1 M glycine/NaOH buffer, pH 10, with 3 μmol of NADP⁺ and 0.14 units of enzyme at 30 °C for 30 min. The mixture was then made more alkaline (> pH 12) and extracted with diethyl ether. The ether extract was dried over anhydrous Na₂SO₄ and the solvent evaporated under a stream of nitrogen. The product was identified as tropinone by TLC and GC-MS. In the reverse direction tropinone and NADPH were used as substrates in 0.1 M phosphate buffer, pH 6.5. In this case tropine was identified as the product and there was no pseudotropine produced. No conversion occurred in controls using enzyme that had been boiled for 5 min.

It is known that the organism also contains a dehydrogenase for 6-hydroxyhepta-1,4-dione [8] and also tropic acid and phenylacetaldehyde dehydrogenases [7], but these are all NAD⁺-dependent and the purified dehydrogenase was not active with these or a number of analogues of tropine. This narrow specificity and the low K_m for tropine show that the enzyme is indeed a tropine dehydrogenase rather than a dehydrogenase of broad specificity that happens to be active with tropine.

The role of tropine dehydrogenase in tropine metabolism

The induction of an enzyme by growth of a bacterium on a single compound is usually interpreted as preliminary evidence that the step catalysed is part of the catabolic pathway. Thus the induction of tropine dehydrogenase by growth of *Pseudomonas* AT3 on tropine suggests that the enzyme has a role in tropine metabolism and consequently that tropinone is an intermediate in the catabolic pathway. However, the discovery that 6-hydroxycyclohepta-1,4-dione is an intermediate in that pathway raised doubts about this interpretation because the alcohol group of tropine is retained intact in this compound. One possible explanation was that the alcohol group is restored at a later stage in the formation of the 6-hydroxycyclohepta-1,4-dione, either by reduction of the keto group of tropinone or of a later intermediate. Reduction of tropinone could form pseudotropine with the tropine dehydrogenase acting as an epimerase, either alone or in conjunction with a separate tropinone reductase. Pseudotropine was oxidized rapidly by whole cells of *Pseudomonas* AT3 grown on tropine and it would also serve as a growth substrate for the organism. However, it is unlikely that the tropine dehydrogenase, acting alone as an epimerase, was responsible for this metabolic flexibility because only tropine was formed as the product in the reduction of tropinone by purified

enzyme. In addition, no other tropinone reductase or pseudotropine dehydrogenase activity was found in crude cell extracts.

The question of whether or not oxidation of the alcohol group of tropine occurs during its conversion into 6-hydroxycyclohepta-1,4-dione and the possibility of its epimerization to pseudotropine, as part of the process, by successive oxidation and reduction was resolved by the use of deuterium-substituted tropine and pseudotropine. The compounds were prepared substituted with deuterium in the 3-position (Scheme 1) [12]. Oxidation of the alcohol group (3-position) would eliminate the deuterium from these compounds and this can be determined by MS analysis of the 6-hydroxycyclohepta-1,4-dione that is formed. If epimerization was occurring by dehydrogenation and rehydrogenation, with pseudotropine as an intermediate in the tropine pathway, then the deuterium would be retained in the product from $[3\text{-}^2\text{H}]$ pseudotropine but not from $[3\text{-}^2\text{H}]$ tropine (see Scheme 1).

In order to ensure high yields of the 6-hydroxycyclohepta-1,4-dione MS2, a mutant of *Pseudomonas* AT3, blocked at the dehydrogenase step for this compound, was used and, to achieve complete conversion, this was grown with labelled tropine or pseudotropine as the sole nitrogen source. Cultures (100 ml) of mutant MS2 were grown with the two components of atropine: tropic acid as the carbon source and labelled tropine or pseudotropine as the nitrogen source. It is important to note that when grown on atropine as its sole carbon and nitrogen sources the mutant contained tropine dehydrogenase in amounts (specific activity 0.38 units/mg) comparable to those in wild type cells. When growth with the labelled compounds had ceased the cells were removed by centrifuging and the 6-hydroxycyclohepta-1,4-dione was extracted with ethyl acetate. The extracted products were examined by GC-MS and each gave one major peak with the retention time previously established for 6-hydroxycyclohepta-1,4-dione. The product from $[3\text{-}^2\text{H}]$ pseudotropine gave a mass spectrum identical in pattern with that of 6-hydroxycyclohepta-1,4-dione but with some of the peaks at 1 m/z higher than for the material accumulated from unlabelled atropine [8]. The molecular ion was at m/z 143 instead of 142 and peaks were seen at m/z 125 ($\text{M}^+ - \text{H}_2\text{O}$) and 115 ($\text{M}^+ - \text{CO}$) rather than 124 and 114. Thus the product from $[3\text{-}^2\text{H}]$ pseudotropine still contained deuterium and the lack of any peak at m/z 142 showed that very little of the deuterium had been lost during the conversion. Similarly the mass spectrum of the 6-hydroxycyclohepta-1,4-dione from $[3\text{-}^2\text{H}]$ tropine showed a high degree of deuterium labelling with the molecular ion at m/z 143. This time there was a peak at 142 but only one third the abundance of that at 143. Since unlabelled 6-hydroxycyclohepta-1,4-dione gave no peak at $\text{M}^+ - 1$ this indicated that a small proportion of the deuterium had been lost during the incubation.

The identities of the products and evidence of labelling were further confirmed from their ^1H - and ^{13}C -NMR spectra. For this they were purified by flash chromatography and crystallized. In each case about 25 mg of crystalline material was recovered from an initial 50 mg of starting material in the growth medium. The proton noise decoupled ^{13}C -NMR spectra gave the following δ values (from trimethylsilane), 209 (C-1 and C-4 carbonyl), 63.6 (t , 23 Hz, C-6- ^2H), 51 (C-5 and C-7), 38 (C-2 and C-3). These were similar to those reported for the undeuterated derivative [8] except for a characteristic triplet centred at 63.6 p.p.m. due to C-6 bonded to deuterium and hydroxyl ($^1J_{\text{CD}} = 23$ Hz). The peak due to C-5 and C-7 was shifted upfield by 0.9 p.p.m. compared with the undeuterated compound due to the presence of deuterium on C-6. The ^1H -NMR spectrum for each product was as follows: (δ values from tetramethylsilane) 3.35 (1H, broad s, OH), 2.91 (4H, s, 6 and 7 methylenes), 2.58–2.75 (4H, m, 2- and

3-methylenes). The percentage deuterium incorporation was calculated by measurement of the integrals of peaks at 2.91 and at 4.43 (septet, residual 6-hydrogen). For the product from pseudotropine this was 93 % and from tropine, 71 %, results that are in general agreement with the mass spectral data. These results confirm the relationship between the carbons of the tropine and the 6-hydroxycyclohepta-1,4-dione and also show that most of the deuterium from labelled tropine was retained. The loss from tropine was probably due to some cycling of the substrate with tropinone, catalysed by the tropine dehydrogenase, during the period of growth. Nortropine is also a substrate for this enzyme and there is evidence that it is an intermediate in tropine metabolism (D. J. Hopper and P. W. Trudgill, unpublished work). Thus, some further loss of deuterium could also have occurred by cycling with tropine dehydrogenase at this stage of the conversion of tropine into 6-hydroxycyclohepta-1,4-dione. This explanation for the loss of a small amount of the deuterium from tropine is supported by the results for pseudotropine. In the conversion of pseudotropine into 6-hydroxycyclohepta-1,4-dione, presumably by the same pathway as for tropine, there is almost complete retention of the deuterium and it is significant that neither pseudotropine nor pseudonortropine is a substrate for the tropine dehydrogenase. The evidence shows that the alcohol group of tropine remains largely intact during its metabolism past the point of nitrogen removal and that tropinone, therefore, is not an intermediate in the pathway. Epimerization of tropine to pseudotropine is not a catabolic prerequisite. The tropine dehydrogenase, although induced by growth on atropine or tropine, does not appear to have a role in the degradative pathway for these compounds (see Scheme 1). This finding underlines the caution that must be applied in the interpretation of enzyme induction patterns for the elucidation of metabolic pathways.

Although apparently not an intermediate in tropine metabolism, tropinone will serve as a growth substrate for *Pseudomonas* AT3 [3]. It is likely to be encountered in nature along with tropine and atropine and, as a precursor in the biosynthesis of tropane alkaloids, has been found in several plant species [14]. A possible role for tropine dehydrogenase is in tropinone and nortropinone metabolism where it could act physiologically as a reductase for these compounds rather than for the oxidation of tropine. Tropinone-grown cells, which have a high specific activity for tropine dehydrogenase (Table 1), rapidly oxidized tropine without lag and no Baeyer-Villiger type of monooxygenation of tropinone by cell extracts could be detected. These results suggest a common metabolic fate for tropinone and tropine. Evidence to support this came from growth of mutant MS2 with tropic acid as its carbon source and tropinone as its sole nitrogen source. After growth, the cells were removed from the spent medium and the products extracted. The only major product from tropinone was identified by TLC and GC-MS as 6-hydroxycyclohepta-1,4-dione, showing that tropinone is metabolized through the same intermediate as tropine and that its keto group is reduced in the process. This would explain the co-induction of a single enzyme that links tropinone metabolism with the pathway for tropine.

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