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Enzymology of Oxidation of Tropic Acid to Phenylacetic Acid in Metabolism of Atropine by *Pseudomonas* sp. Strain AT3

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***Pseudomonas* sp. strain AT3 grew with *dl*-tropic acid, the aromatic component of the alkaloid atropine, as the sole source of carbon and energy. Tropic acid-grown cells rapidly oxidized the growth substrate, phenylacetaldehyde, and phenylacetic acid. Crude cell extracts, prepared from *dl*-tropic acid-grown cells, contained two NAD^+-linked dehydrogenases which were separated by ion-exchange chromatography and shown to be specific for their respective substrates, *dl*-tropic acid and phenylacetaldehyde. Phenylacetaldehyde dehydrogenase was relatively unstable. The stable tropic acid dehydrogenase was purified to homogeneity by a combination of ion-exchange, molecular-sieve, and affinity chromatography. It had a pH optimum of 9.5 and was equally active with both enantiomers of tropic acid, and at this pH, phenylacetaldehyde was the only detectable product of tropic acid oxidation. The formation of phenylacetaldehyde from tropic acid requires, in addition to dehydrogenation, a decarboxylation step. By analogy with NAD^+-specific isocitrate and malate dehydrogenases, phenylmalonic semialdehyde, a 3-oxoacid, would be expected to be the precursor of phenylacetaldehyde. Other workers have established that isocitrate and malate dehydrogenases catalyze the decarboxylation of enzyme-bound or added 3-oxoacid intermediates, a reaction that requires Mn^{2+} or Mg^{2+} ions. Studies with tropic acid dehydrogenase were hampered by lack of availability of phenylmalonic semialdehyde, but in the absence of added divalent metal ions, both enantiomers of tropic acid were completely oxidized and we have not, by a number of approaches, found any evidence for the transient accumulation of phenylmalonic semialdehyde.**

Atropine, from *Atropa belladonna*, is one of the tropane alkaloids synthesized by solanaceous plants. In common with other alkaloids of the group, it consists of an N-heterocyclic secondary alcohol and an aromatic organic acid, tropine and *dl*-tropic acid, respectively, in this instance joined by an ester linkage. The natural alkaloid is therefore a mixture of two optical isomers. The ability of bacteria to use the compound as the sole carbon source, and in some instances as the sole nitrogen source also, has been well documented (3, 10, 11, 14, 17, 19, 22). Hydrolytic cleavage of the molecule into alcohol and acid components by an inducible esterase has been reported by several workers (3, 17, 20–22), and recently atropine esterase, purified from *Pseudomonas putida* PMBL-1, has been used as a simplified model for mammalian cholinergic receptors and serine esterases (26). The bacterial metabolism of the tropine fragment by *Corynebacterium belladonna* has been reported to involve oxidation to tropinone by an NAD^+-linked tropine dehydrogenase, followed by sequential cleavage of the carbocyclic and N-heterocyclic rings (19). In contrast, *Pseudomonas* sp. strain AT3 makes use of a different catabolic route, with initial excision of the N atom, to yield 6-hydroxycyclohepta-1,4-dione as an intermediate (1, 2, 14). The bacterial metabolism of tropic acid has received relatively little attention but has been shown, on the basis of oxidation studies with intact cells and accumulation of phenylacetic acid by mutants unable to grow on tropic acid, to occur via phenylacetic acid in *P. putida* PMBL-1 (25). NAD^+-linked dehydrogenase activity, specific for *d*- and *l*-tropic acids, was reported to be present in acetone powders prepared from atropine-grown *P. putida* by Michel et al. (17), but no evidence was presented to show whether this was due to a single enzyme capable of acting on both enantiomers or to two stereospecific enzymes. Two moles of NADH were formed for each mole of tropic acid provided, and although the authors proposed that a second dehydrogenation step leading from phenylmalonic semialdehyde to either phenylacetic acid or phenylmalonic acid was also catalyzed by tropic acid dehydrogenase, this too was not supported by any detailed enzymology. An $NADP^+$-specific phenylacetaldehyde dehydrogenase has been partially purified from phenylethylamine-grown *Arthrobacter globiformis* (24), and an NAD^+-specific enzyme is involved in the catabolism of phenylalanine by *Achromobacter eurydice* (7). In this paper, we report studies of the enzymology of the oxidation of tropic acid to phenylacetic acid by *Pseudomonas* sp. strain AT3.

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MATERIALS AND METHODS

Maintenance and growth of organism. *Pseudomonas* sp. strain AT3, isolated by elective culture with atropine from rhizosphere soil of *A. belladonna*, was maintained on nutrient agar slopes and routinely grown in liquid medium containing, per liter, 2 g of KH_2PO_4, 4 g of NaH_2PO_4, 1 g of $(NH_4)_2SO_4$, 1 g of *dl*-tropic acid, and 4 ml of trace element solution (23). Medium was adjusted to pH 7.1 with 1 M NaOH and sterilized by autoclaving. Cell crops for oxidation studies were grown in conical flasks on an orbital incubator at 150 rpm and 30°C as previously described (14). For larger crops of cells, two 500-ml batches of medium in 2-liter conical flasks were used to inoculate 10 liters of medium in a New Brunswick Microferm laboratory fermenter, which was stirred at 250 to 400 rpm and aerated with sterile air at 3 liters/min. Growth was monitored by measuring the optical density at 580 nm (OD_{580}). Further additions of tropic acid (sterile neutralized solution to give 0.5 g of free acid per liter) were made when the OD_{580} (measured in diluted samples) reached 1.5 and 3.0. Cells were harvested at an OD_{580} of 3.5 or greater while the cultures were still in the exponential phase of growth. Cell pastes were resuspended in an equal volume of 42 mM sodium and potassium phosphate buffer (pH 7.1) and either used immediately or stored at −20°C until required.

Measurement of oxygen uptake. Oxygen consumption by whole-cell suspensions was measured by conventional Warburg manometry at 30°C. Warburg flasks typically contained 10 mg (dry weight) of bacteria in 1.9 ml of 42 mM sodium/potassium phosphate buffer (pH 7.1), and reactions were started by the

addition of 3 μmol of substrates from side arms. Center wells contained 0.1 ml of 20% (wt/vol) KOH and a roll of filter paper to absorb expired CO_2.

Preparation of cell extracts. Cell suspensions were thawed at room temperature and disrupted either by passage through a French press with a pressure difference at the orifice of 140 MPa or by ultrasonic treatment with a Soniprep 150 (MSE, Crawley, Sussex, England, United Kingdom) at a probe amplitude of 16 to 18 μm with four 30-s bursts interspersed with cooling on ice. Broken cell suspensions were incubated with DNase and centrifuged at 27,000 × *g* (average) for 45 min at 4°C, and the supernatant was taken as a crude extract.

Enzyme assays. Tropic acid and phenylacetaldehyde dehydrogenases were routinely assayed spectrophotometrically by monitoring the reduction of NAD^+ at 340 nm and 30°C when either 1 μmol of *dl*-tropic acid sodium salt or 1 μmol of phenylacetaldehyde was added to a 1-ml reaction volume that contained 100 μmol of glycine-NaOH buffer (pH 9.5); 1 μmol of NAD^+, and cell extract. One unit of enzyme activity is defined as the reduction of 1 μmol of NAD^+ per min. All assays were carried out at 30°C.

Reaction product derivatization with phenylhydrazine. A reaction mixture that contained, in 3 ml, 290 μmol of glycine-NaOH buffer (pH 9.5), 10 μmol of sodium tropate, 10 μmol of NAD^+, and 1.7 U of pure tropic acid dehydrogenase was monitored at 340 nm for 30 min. The pH was then adjusted to 6.2 by the addition of 1 ml of 0.2 M Na/K phosphate buffer (pH 6.0) containing 10 μmol of phenylhydrazine, and the mixture was incubated at 30°C for 30 min. The pH was then carefully adjusted to 4 with dilute acetic acid, and phenylhydrazone derivatives were extracted twice with 1.5 ml of diethyl ether. The pooled ether layers were dried over anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 50 μl of absolute ethanol. Adams catalyst (1 mg) was added, and hydrogen, introduced by a fine glass capillary, was bubbled gently through the mixture for 6 min. Distilled water (10 μl) was added, the mixture was agitated and centrifuged to remove the catalyst, and the supernatant was analyzed by thin-layer chromatography (TLC).

Protein estimation. The protein content of crude cell extracts was routinely measured by the biuret method (8). For measurement of the protein content of column fractions and purified enzyme preparations, the more sensitive tannin-gum arabic turbidometric assay (16) was used.

Purification of tropic acid dehydrogenase. All procedures were done at 2 to 4°C.

(i) Step 1. Cell extract prepared from approximately 50 g of cells was loaded onto a DEAE-cellulose column (2.5 by 12 cm) equilibrated with 42 mM sodium/potassium phosphate buffer (pH 7.1). The column was washed with the buffer (100 ml), and a linear KCl gradient (1 liter; 0 to 0.5 M KCl in the phosphate buffer) was applied to elute the dehydrogenase. Fractions (10 ml) were collected, and selected fractions containing dehydrogenase activity, typically fractions 18 to 25, were pooled and concentrated to a volume of approximately 20 ml in an Amicon ultrafiltration cell fitted with a PM10 membrane.

(ii) Step 2. Samples of the DEAE-cellulose pool (8 ml) were loaded onto a column of Sephacryl S200 (2.5 by 80 cm), and the column was eluted with 0.1 M Tris-HCl buffer (pH 8.0) by gravity feed. Fractions (5 ml) were collected, and those containing dehydrogenase (fractions 40 to 44) were pooled. The Sephacryl column was calibrated with commercially supplied proteins covering an M_r range from 17,000 to 150,000.

(iii) Step 3. The pool fractions were concentrated to a volume of <3 ml with an Amicon ultrafiltration cell fitted with a PM10 membrane and dialyzed for 14 h against 20 mM sodium/potassium phosphate buffer (pH 7.1). The whole sample was loaded onto a column of Mimetic Orange 2 (8 by 20 mm) which was washed sequentially with 4 ml of 20 mM sodium/potassium phosphate buffer (pH 7.1) to remove unbound protein and with 6 ml of the same buffer containing 7 mM NAD^+ to displace the enzyme; 1-ml fractions were collected. Active fractions were pooled and loaded directly onto a column of Mimetic Yellow 2 (8 by 20 mm) which was washed sequentially with 6 ml of 50 mM glycine-NaOH buffer (pH 8.0) to remove unbound protein and with 6 ml of the same buffer containing 1 M KCl to displace the enzyme. Active fractions were pooled, dialyzed against 42 mM sodium/potassium phosphate buffer (pH 7.1) for 14 h, and stored at −80°C.

PAGE. Nondenaturing polyacrylamide gel electrophoresis (PAGE) of 10 to 30 μg of protein in the presence of bromothymol blue tracking dye was done on 1-mm-thick, 4 to 20% (wt/vol) gradient polyacrylamide gels in the Mini-Protean system (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) at 10 mA until the tracking dye was a few millimeters from the bottom of the gels. The gels were stained in 0.2% Coomassie brilliant blue R-250 in ethanol-water-acetic acid (9:9:2, vol/vol/vol) and destained in water-ethanol-acetic acid (13:5:2, vol/vol/vol). For activity staining, the gel was immersed in 0.1 M glycine-NaOH buffer, pH 9.5, containing 1-mg/ml each NAD^+, phenazine methosulfate, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium, and 1 mM sodium tropic until colored formazan bands appeared. The gel was then thoroughly washed in distilled water. Sodium dodecyl sulfate (SDS)-PAGE was done by the method of Laemmli (13) on 10 and 15% (wt/vol) acrylamide gels which were also stained with Coomassie brilliant blue R-250.

TLC. TLC was performed on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) with butan-1-ol–acetic acid–water (4:1:1, vol/vol/vol) as the solvent. Amino compounds were located by spraying with ninhydrin reagent.

GC-MS. Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard 5890 instrument with a 5971 mass-selective detector. For separations on an HP5 (cross-linked 5% phenylmethylsilicone) column (25 m by 0.2 mm; 0.33-μm film thickness) with helium as the carrier gas, a temperature program of 4 min at 70°C rising at 10°C/min to 275°C was used. The HP20M (polyethylene glycol) column (25 m by 0.2 mm; 0.2-μm film thickness) was used isothermally or with a temperature program of 3 min at 70°C rising at 20°C/min to 170°C. When required, compounds were derivatized by incubation with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

Laser desorption MS. Matrix-assisted laser desorption MS was done on a Vestec Lasertec bench top instrument. Enzyme solution was mixed with an equal volume of matrix solution, and 1-μl samples were analyzed. Accelerating and detector voltages were 25 and 3.4 kV, respectively, and maximum laser intensity was employed. Myoglobin was used as the external standard.

HPLC. High-performance liquid chromatography (HPLC) of tropic acid and metabolites was done on an ODS2 column (250 by 4.6 mm; 5-μm particle size) (Phase Sep, Deeside, United Kingdom) eluted at a flow rate of 1.5 ml/min with a methanol-water (1:1 vol/vol) mobile phase. Compounds were detected by measuring absorbance at 254 nm in a continuous flow cell, and information was processed by Thermochrom II software (LDC Analytical, Stone, United Kingdom).

Optical rotation. The optical rotation of separated *d*- and *l*-tropic acids was measured in aqueous solution with an automatic polarimeter, model AA-10 (Optical Activity Ltd, Ramsey, United Kingdom).

Chemicals. Platinum(IV) oxide was supplied by Aldrich (Gillingham, England, United Kingdom). Mimetic Orange 2 and Mimetic Yellow 2 were obtained from Affinity Chromatography Ltd. (Freeport, Ballasalla, Isle of Man, United Kingdom). 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium was obtained from BDH (Poole, England, United Kingdom). HPLC-grade solvents were supplied by Fisons (Loughborough, England, United Kingdom), and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide was supplied by Fluka (Gillingham, England, United Kingdom). Coomassie brilliant blue R-250 was supplied by Pharmacia (St. Albans, England, United Kingdom), and atropine, oxaloacetic acid, phenylacetaldehyde, phenylethylamine, tropine, *dl*-tropic acid, and marker proteins for SDS-PAGE were from Sigma (Poole, England, United Kingdom). *dl*-Tropic acid was purified by recrystallization from boiling diethyl ether to which petroleum ether (40 to 60°C) was slowly added until the solution became faintly turbid. The solution was cooled to 4°C, and the *dl*-tropic acid was harvested and its purity was checked by GC-MS. *d*-Tropic acid and *l*-tropic acid were separated from the *dl*-mixture by crystallization of the diastereoisomeric salt of *l*-tropic acid and D-(−)-*threo*-2-amino-1-(*p*-nitrophenyl)-1,3-propanediol and isolation of the *d*-tropic acid from the mother liquor, essentially according to the procedure of Fodor, Rakoczi, and Csepreghy (6). The *l*-tropic acid was chromatographically pure, with an $[\alpha]_D$ of −74°C (literature value, −72°). The *d*-tropic acid from several preparations was chromatographically homogeneous by GC-MS but not enantiomerically pure. The best preparation had an $[\alpha]_D$ of +50.2° (enantiomeric excess, 70%) and was used in enzymic studies.

RESULTS AND DISCUSSION

Cell growth, substrate oxidation, and studies with cell extracts. *Pseudomonas* sp. strain AT3 grew with *dl*-tropic acid as the sole carbon source and had a generation time of 4 h. The growth yields and lack of residual tropic acid in spent medium indicated that both isomers of the substrate were utilized. Tropic acid-grown cells were capable of the immediate rapid and extensive oxidation of the growth substrate, phenylacetaldehyde, and phenylacetic acid. Tropine was not oxidized, and succinate-grown cells did not oxidize any of these compounds. Crude extracts of tropic acid-grown *Pseudomonas* sp. strain AT3 catalyzed the reduction of NAD^+ in the presence of *dl*-tropic acid, with a pH optimum for the reduction of approximately 9.5. Incubation of cell extract with phenylacetaldehyde also resulted in the reduction of NAD^+, again with a pH optimum of 9.5. Both enzyme activities were inducible, being virtually absent from the extract of succinate-grown cells, but were present at intermediate levels in extracts of cells harvested during the tropic acid phase of diauxic growth on atropine (Table 1).

Incubation of 3 μmol of *dl*-tropic acid and 3 μmol of NAD^+ with 0.5 mg of crude extract protein in glycine-NaOH buffer, pH 9.5, at 30°C for 20 min, followed by diethyl ether extraction and GC-MS analysis, revealed phenylacetic acid to be the major product, but in addition to residual substrate, some phenylacetaldehyde was also present. These results showed that phenylacetaldehyde is an intermediate in tropic acid oxidation, and they extend earlier observations (25) in which *P.*

TABLE 1. Tropic acid and phenylacetaldehyde dehydrogenase activities in crude extracts of *Pseudomonas* sp. strain AT3 grown on atropine, *dl*-tropic acid, and succinate

Enzyme	Activity (U/mg of protein) in extracts of cells grown on:		
	Atropine[a]	*dl*-Tropic acid	Succinate
Tropic acid dehydrogenase	0.23	0.42	<0.01
Phenylacetaldehyde dehydrogenase	0.08	0.11	<0.01

[a] Atropine-grown cells were harvested in the tropic acid phase of diauxic growth.

putida, strain PMBL-1, was shown to convert tropic acid into phenylacetic acid, although no intermediates were identified and no enzyme-catalyzed reactions were reported.

NAD$^+$-linked dehydrogenases and tropic acid oxidation. The possibility that the two sequential dehydrogenation reactions were catalyzed by the same dehydrogenase, as was proposed for a strain of *P. putida* (17), was not supported by preliminary observations of their stabilities in a crude cell extract of *Pseudomonas* sp. strain AT3 maintained at 4°C: phenylacetaldehyde dehydrogenase was completely inactivated within 24 h, while tropic acid dehydrogenase activity was retained for several days.

Results from an initial trial enzyme separation by DEAE-cellulose chromatography confirmed the discrete identities of the two enzymes (Fig. 1). Quantitative recovery of phenylacetaldehyde dehydrogenase (peak at 0.22 M KCl) was poor, commensurate with its established instability in extracts of this organism. However, it was clearly separated from tropic acid dehydrogenase (peak at 0.33 M KCl), which displayed no detectable phenylacetaldehyde dehydrogenase activity.

Tropic acid dehydrogenase and phenylacetaldehyde formation. The involvement of two dehydrogenases in the oxidation of tropic acid to phenylacetic acid, with phenylacetaldehyde as an intermediate, necessitates the loss of one carbon atom in the conversion of tropic acid to phenylacetaldehyde. Phenylmalonic semialdehyde, a logical product of tropic acid oxidation, is the expected intermediate lying between tropic acid and phenylacetaldehyde (Fig. 2). Decarboxylation of this putative intermediate would yield phenylacetaldehyde. The situation is complicated, because phenylmalonic semialdehyde is a 3-oxoacid and thus, like oxaloacetic acid and oxalosuccinic acid, will spontaneously decarboxylate and be unstable at extremes of pH and in the presence of a variety of divalent cations (4). In addition, as an alternative to catalysis by decarboxylase or spontaneous decarboxylation of this putative intermediate, a concerted oxidative decarboxylation of the enzyme-bound phenylmalonic semialdehyde by the tropic acid dehydrogenase is also possible; parallel reactions occur in the conversion of isocitrate into 2-oxoglutarate by the NAD$^+$-linked dehydrogenase (EC 1.1.1.42) from hog heart (18) and L-malate into pyruvate by the malate dehydrogenase (EC 1.1.1.38) of *Lactobacillus arabinosus* (9, 12).

Studies of the decarboxylation phases of the reactions catalyzed by isocitrate and malate dehydrogenases were facilitated by the commercial availability of the 3-oxoacid intermediates. Phenylmalonic semialdehyde is not commercially available, and its synthesis has been neither described in the literature

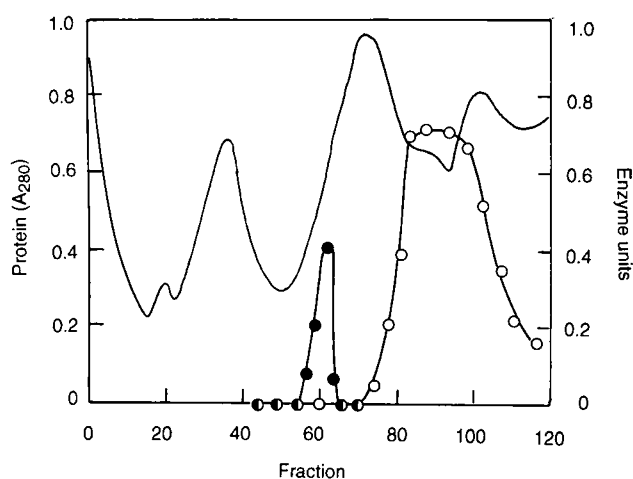


FIG. 1. Chromatography of tropic acid dehydrogenase and phenylacetaldehyde dehydrogenase on DEAE-cellulose. Crude extract protein (1.7 g) was loaded onto a DE-52 column (5 by 8 cm) and eluted with a linear KCl gradient (600 ml; 0 to 0.5 M KCl in 21 mM phosphate buffer, pH 7.1). Fractions of 5 ml were collected. ○, tropic acid dehydrogenase (units per milliliter); ●, phenylacetaldehyde dehydrogenase (units per fraction). The curve with no symbols shows protein at A_{280}.

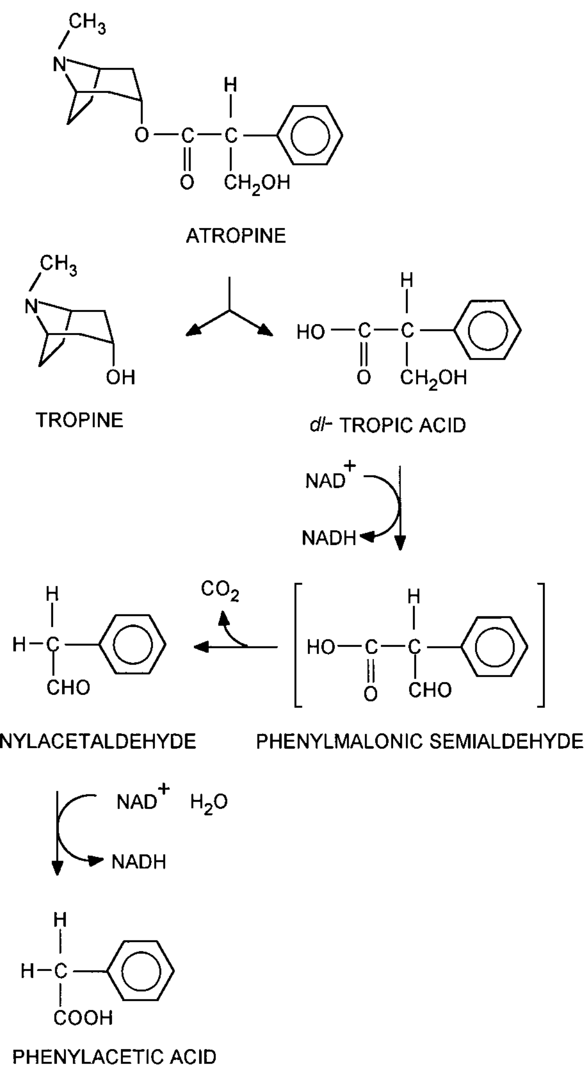


FIG. 2. The structure of atropine and reactions leading to phenylacetic acid.

TABLE 2. Purification of tropic acid dehydrogenase from *Pseudomonas* sp. strain AT3

Stage	Vol (ml)	Total protein (mg)	Total activity (U)	Overall recovery (%)	Sp act (U mg^{-1})	Overall purification (fold)
Crude extract	150	2,400	1,650	100	0.69	1
DEAE-cellulose chromatography	64	301	843	51	2.77	4.0
Sephacryl S200 chromatography[a]	7.8	54.6	657	39	12	17.4
Mimetic affinity chromatography[a]	6.3	8.4	216	13.1	25.7	37.2

[a] Sephacryl S200 and mimetic affinity chromatography of the pooled fractions from DEAE-cellulose chromatography were performed on three separate batches, and the accumulated results are presented.

nor achieved in our hands. Alternative approaches to the problem were therefore required.

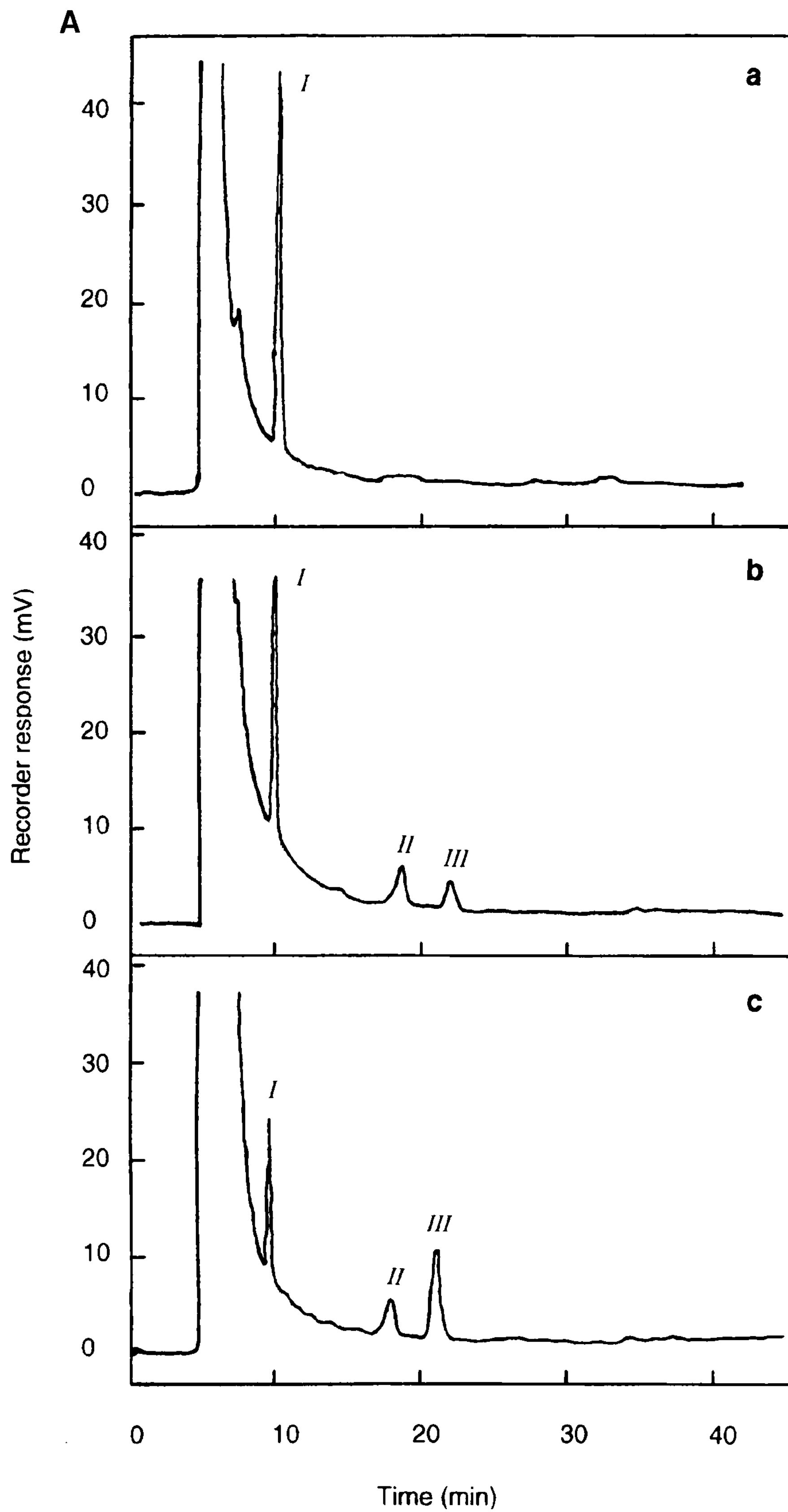
Purification of tropic acid dehydrogenase. To investigate the dehydrogenation-decarboxylation phase of tropic acid oxidation in more detail, tropic acid dehydrogenase was purified for this purpose as described in Materials and Methods (Table 2). Pure enzyme was obtained by a combination of ion-exchange, molecular-sieve, and affinity chromatography and gave a single tight protein band on polyacrylamide gels that displayed tropic acid dehydrogenase activity in situ with *dl*-tropic acid as the substrate. SDS-PAGE on 10 and 15% (wt/vol) polyacrylamide gels gave a single band with an M_r of 30,000. This correlated well with the M_r of 31,543 obtained by laser desorption MS. An M_r of 69,000 for the native enzyme, determined by Sephacryl S200 chromatography, indicated that the holoenzyme consists of two electrophoretically identical subunits. It was stable for months in 42 mM sodium/potassium phosphate buffer (pH 7.1) at −20°C without significant loss of activity.

Substrate specificity. The pure enzyme was absolutely specific for NAD^+ as the electron acceptor and was inactive with phenylacetaldehyde. Maximum activity was obtained in glycine-NaOH buffer at pH 9.5. Spectrophotometric assays in glycine-NaOH buffer at pH 9.5 were nonlinear, an initial rapid phase being followed by a slower second phase. This was not due to inhibition by phenylacetaldehyde, one possible product of the enzyme, since addition of this compound to assay mixtures did not alter the assay profile. The initial reaction rate and overall NAD^+ reduction decreased rapidly as the reaction mixture pH was lowered towards neutrality. Diethyl ether extraction of reaction mixtures in which *dl*-tropic acid was the substrate, either before or after careful acidification to pH 4 with acetic acid, yielded phenylacetaldehyde as the only product detected by TLC or GC-MS.

It was previously reported (17) that tropic acid dehydrogenase activity in crude acetone powder extracts of atropine-grown *P. putida* had NAD^+-specific dehydrogenase activity towards both isomers of tropic acid, although no evidence was presented to show that this was due to a single enzyme. Pure tropic acid dehydrogenase from *Pseudomonas* sp. strain AT3 displayed no significant differences in initial reaction rate or reaction profile with the racemic mixture or either of the isomers of tropic acid. In order to establish that activity observed with *d*-tropic acid was not due to the oxidation of the residual *l* isomer (15%) present as a contaminant, reactions with 100 nmol of tropic acid and an excess (2 μmol) of NAD^+ were allowed to run to completion. Irrespective of the tropic acid isomer provided, initial reaction rates were almost identical, and in total, 97 to 98 nmol of NAD^+ was reduced at the end of each reaction. Both isomers of tropic acid were, therefore, completely oxidized by the pure enzyme.

Search for the initial product of the tropic acid dehydrogenase reaction. Although phenylacetaldehyde was identified as the product of the tropine dehydrogenase reaction, it is possible that this is produced by the spontaneous time-dependent decarboxylation of phenylmalonic semialdehyde during incubations at 30°C, adjustment of pH, extraction of reaction products, and other manipulations. An attempt was made to trap this putative intermediate under mild conditions, using phenylhydrazine at pH 6 followed by reduction of the adduct to the corresponding stable amino acid by hydrogen in the presence of platinum oxide (Adam's catalyst). When the product of this procedure was examined by TLC, it gave a spot corresponding to authentic phenylethylamine, the derivative expected from phenylacetaldehyde, as the only detectable ninhydrin-positive material and was not present in controls omitting substrate or enzyme. However, a control in which oxaloacetic acid was processed in an identical manner gave alanine and not aspartate as the product, indicating that even these conditions result in decarboxylation. Thus, the finding of phenylethylamine as the product from tropic acid provides confirmation of the identity of phenylacetaldehyde as the ultimate reaction product but provides no evidence for or against a role for phenylmalonic semialdehyde.

Direct analysis of the products of tropic acid dehydrogenation by HPLC. Tropic acid, phenylacetaldehyde, and phenylacetic acid can be separated effectively by isocratic HPLC on an ODS2 column with methanol-water (1:1 vol/vol) as the mobile phase. A reaction mixture contained, in a volume of 5 ml, 100 μmol of glycine-NaOH buffer (pH 9.5), 9.2 μmol of NAD^+, and 7.5 μmol of *dl*-tropic acid. The reaction mixture was preincubated at 30°C, and a zero time sample (0.5 ml) was transferred to an equal volume of methanol, cooled on ice. The reaction was started by the addition of 1.1 U of enzyme, NADH formation was monitored spectrophotometrically in a 2-mm-light-path cuvette, and further 0.5-ml samples were transferred to cooled tubes, containing 0.5 ml of methanol, at timed intervals. The collected samples were filtered and analyzed directly (20 μl) by HPLC. In so far as it was possible, samples were analyzed as they were collected. A delay of 45 min existed between collection of the last sample and HPLC analysis. Repeated analysis of the sample taken at 45 min, after storage in an ice bath for a further 45 min, showed no change in the quantitative distribution of components, confirming that the procedure adopted allowed real-time analysis of the progress of the reaction. HPLC analysis (Fig. 3) showed that oxidation of tropic acid (peak *I*), concomitant with NAD^+ reduction, resulted in the initial formation of a single product (peak *II*) which was followed by the formation of a second component (peak *III*). Analysis of the progression of metabolite formation (Fig. 3) suggested a sequential reaction sequence in which tropic acid initially yielded *II* which was converted at a slower rate into *III*. Our initial assumption that *II* was phenylmalonic semialdehyde, which then decarboxylated to form phenylacetaldehyde (*III*), was shown to be erroneous when HPLC analysis of a fresh commercial sample of phenylacetaldehyde showed it to consist predominantly of *II* (75%), with 25% of a component with the same retention time as *III*. GC-MS of this fresh sample revealed only a single peak that


A
a
b
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III
Recorder response (mV)
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0
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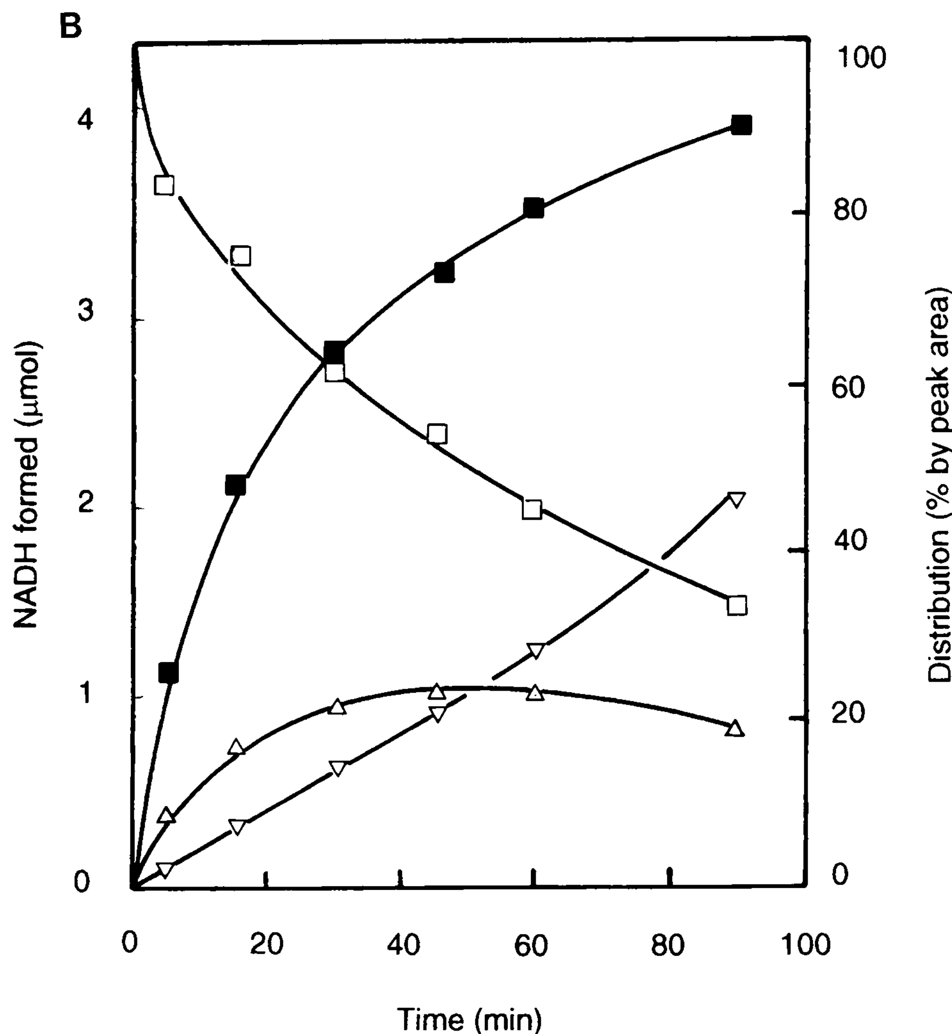

FIG. 3. Analysis of the products of tropic acid dehydrogenation. The reaction mixture contained, in 5 ml of glycine-NaOH buffer (pH 9.5), 10 μmol of NAD^+ and 7.5 μmol of *dl*-tropic acid. The reaction at 30°C was started by the addition of 0.25 U of pure enzyme. NADH formation was monitored spectrophotometrically at 340 nm in a 2-mm-light-path cuvette, 0.5-ml samples were added to 0.5 ml of ice-cold methanol at timed intervals, and 20-μl samples were analyzed by HPLC as described in Materials and Methods. (A) HPLC traces of samples from time zero (a), 30 min (b), and 60 min (c). Peaks are as follows: *I*, phenylacetaldehyde; *II*, primary dehydrogenation product which cochromatographs with authentic phenylacetaldehyde; *III*, secondary dehydrogenation product, probably the ionized phenylacetaldehyde enolate which is also formed from authentic phenylacetaldehyde in alkali solution. (B) Tropic acid oxidation, NAD reduction, and product formation. ■, NADH formed; □, peak area of tropic acid; △, peak area of product *II* (phenylacetaldehyde); ▽, peak area of product *III*. All were measured at 254 nm and expressed as the percentage of total peak area at that wavelength.

was identified, by comparison with the Wiley library of mass spectra, as phenylacetaldehyde. Although this ratio of the two components in the authentic phenylacetaldehyde remained constant for several hours in aqueous solution, incubation of authentic phenylacetaldehyde in glycine-NaOH buffer (pH 9.5) followed by HPLC analysis showed that the same time-dependent *A*-to-*B* transition as was observed during the enzyme-catalyzed oxidation of tropic acid occurred. Spectrophotometric analysis of phenylacetaldehyde in glycine-NaOH buffer (pH 9.5) showed a time-dependent transition from λ_{max} at 254 nm to λ_{max} at 287 nm and an approximately threefold increase in ε. A very similar time-dependent spectral shift was also observed when the pH of an aqueous solution was adjusted to 10 by the addition of a predetermined amount of NaOH solution. This spectral change was reversed within a few minutes when the pH of the solution was lowered to 7.5 by the addition of dilute HCl. Although it is known that phenylacetaldehyde will form Schiff bases with amino acids in aqueous solution (15), this does not provide an acceptable explanation of the observed transitions, since they also take place in alkaline aqueous solution. GC-MS analysis of commercial samples of phenylacetaldehyde, shown by HPLC to contain the second component, on both the HP5 and HP20M columns, yielded phenylacetaldehyde as the only compound detected. Phenylacetaldehyde is also known to undergo an aldol condensation, but again, the reversible nature of the transitions observed would preclude this as a significant contributory reaction. None of these transitions caused a significant change in absorbance at 340 nm.

In the absence of a precise understanding of these transformations, the crucial observation, namely that they can be observed with authentic phenylacetaldehyde and with the product of tropic acid oxidation, further reinforces the conclusion that the postulated intermediate in the dehydrogenation and decarboxylation of tropic acid, phenylmalonic semialdehyde, has no separate existence in the reaction catalyzed by tropic acid dehydrogenase from *Pseudomonas* sp. strain AT3.

Divalent metal ions and tropic acid dehydrogenase. The NAD^+-specific isocitrate and malate dehydrogenases that catalyze sequential dehydrogenation and decarboxylation of their substrates require Mn^{2+} or Mg^{2+} for the decarboxylation phase of the reaction to occur (12, 18). At near-neutral pH, the decarboxylation step serves to displace continually the dehydrogenation equilibrium, such that in the absence of Mn^{2+} required for decarboxylation, significant activity of the pig heart isocitrate dehydrogenase was not observed. Similar observations resulted from the sequestration of divalent cations by EDTA. Although tropic acid dehydrogenase activity was very pH dependent, with little activity occurring at pH 7, addition of $MnCl_2$ or $MgCl_2$ (5 to 10 mM) to spectrophotometric assays did not stimulate substrate-dependent NAD^+ reduction. Addition of EDTA (10 mM) to dehydrogenation reaction mixtures monitored spectrophotometrically in Na/K phosphate buffer (pH 7), Tris-HCl buffer (pH 8), and glycine-NaOH buffer (pH 9.5) had no detectable effect upon the initial reaction rate or the subsequent progression of the reaction.

These observations suggest that tropic acid dehydrogenase from *Pseudomonas* sp. strain AT3 is not a member of the divalent-cation-dependent group of decarboxylating dehydrogenases.

In conclusion, although we have been able to establish a role for two discrete inducible NAD^+-linked dehydrogenases in the conversion of tropic acid into phenylacetic acid by *Pseudomonas* sp. strain AT3, we have no evidence that phenylmalonic semialdehyde is formed as a free intermediate. Phenylacetaldehyde is the only detectable product formed by pure tropic acid dehydrogenase, even during rapid direct analysis of reaction mixtures by HPLC. Either tropic acid dehydrogenase from *Pseudomonas* sp. strain AT3 possesses decarboxylase activity that is independent of added Mn^{2+} or Mg^{2+} or phenylmalonic semialdehyde is very unstable over the practicable assay pH range. Either situation would result in the continuous displacement of the reaction in the direction of phenylacetaldehyde formation, and the observation that, under appropriate assay conditions, the reaction will proceed to completion is indicative that decarboxylation of the presumed intermediate, phenylmalonic semialdehyde, does occur during catalysis, although the mechanism of this decarboxylation has not been established.

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