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The Metabolism of Nitrilotriacetate by a Pseudomonad

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1. An organism that grows on nitrilotriacetate as sole source of carbon and energy was isolated in pure culture and was identified as a pseudomonad. 2. Cell-free extracts of the nitrilotriacetate-grown pseudomonad contain an enzyme that catalyses the NADH- and O_2-dependent oxidation of nitrilotriacetate to iminodiacetate and glyoxalate. This enzyme is absent from extracts of glucose-grown cells. 3. Compared with growth on glucose, growth on nitrilotriacetate results in increased activities of enzymes of glycine and serine metabolism, namely serine hydroxymethyltransferase, glycine decarboxylase, serine–oxaloacetate aminotransferase and hydroxypyruvate reductase. 4. Cell-free extracts of the nitrilotriacetate-grown organism contain the enzyme glyoxalate carboligase and, when supplemented with NADH, Mg^{2+} and thiamin pyrophosphate, can catalyse the anaerobic conversion of glyoxalate into glycerate. 5. These results are incorporated in a scheme which shows the oxidative metabolism of nitrilotriacetate by the successive removal of C_2 units to form 2mol of glyoxalate and 1mol of glycine per mol of nitrilotriacetate degraded. The glyoxalate and glycine are then both metabolized to glycerate by separate pathways, via tartronic semialdehyde and serine respectively. The role of this scheme in the growth of the organism on nitrilotriacetate is discussed.

The polyphosphates of detergents have been implicated as a contributing factor in the eutrophication of lakes and ponds (see, e.g., Hammond, 1971) and alternative non-phosphate materials have been sought by the detergent industries. One such phosphate substitute is nitrilotriacetic acid [$N(CH_2CO_2H)_3$] and this compound has been included in some detergent formulations. However, it has been suggested by Epstein (1970) that the large-scale use of nitrilotriacetate might not be desirable because of the possible conversion of it, or its degradation products, into potentially carcinogenic nitrosamines by reaction with biologically produced nitrite. It is therefore important to obtain knowledge of the fate of nitrilotriacetate in the environment, and in particular to study its degradation by micro-organisms, so that these possible hazards can be properly assessed.

Nitrilotriacetate has been shown to be rapidly biodegradable, after suitable adaptation periods, in activated-sludge systems (Swisher *et al.*, 1967; Pfeil & Lee, 1968) and in river water (Thompson & Duthie, 1968; Warren & Malec, 1972) with no accumulation of organic intermediates. Also, micro-organisms have been isolated that can utilize nitrilotriacetate as a growth substrate under both aerobic (Forsberg & Lindqvist, 1967; Focht & Joseph, 1971; Tiedje *et al.*, 1973) and anaerobic (Enfors & Molin, 1971) conditions. Biochemical studies have shown that nitrilotriacetate is degraded to CO_2 and biomass with the release of the N atom as NH_4^+ (Focht & Joseph, 1971; Tiedje *et al.*, 1973). Only trace amounts of nitrite were detected. In addition, simultaneous adaptation studies have suggested that the metabolism of nitrilotriacetate proceeds by the successive removal of the C_2 fragments of the molecule to form iminodiacetic acid [$HN(CH_2CO_2H)_2$] and glycine and not by a mechanism involving decarboxylation and the formation of *N*-methyliminodiacetate or *N*-methylglycines (Tiedje *et al.*, 1973).

The present paper describes further studies on the biochemical reactions involved in nitrilotriacetate metabolism by an organism which was isolated by virtue of its ability to utilize this compound as sole source of carbon and nitrogen for growth. This organism was tentatively identified as a pseudomonad and given the designation *Pseudomonas* sp. T23. A preliminary account of some of the results has been presented (Cripps & Noble, 1972).

Experimental

Chemical reagents

Nitrilotriacetic acid, iminodiacetic acid, glyoxalate, pyridoxal phosphate and thiamin pyrophosphate were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.: oxaloacetate, DL-serine *O*-phosphate, DL-glycerate, acetyl-CoA, nicotinamide nucleotides

(oxidized and reduced) and H_4PteGlu* were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; lithium hydroxypyruvate was from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and alcohol dehydrogenase was from C. F. Boehringer und Soehne G.m.b.h., Mannheim, Germany. Phosphohydroxypyruvate dimethylketal tricyclohexylammonium salt (Sigma) was converted into the free acid by the method of Ballou & Hesse (1956) by treatment with Dowex 50 (H^+ form) followed by hydrolysis at 40°C for 4 days. The solution was neutralized with NaOH and assayed for total and inorganic phosphorus by the method of Rockstein & Herron (1951). Total phosphorus was measured after hydrolysis with 9M-H_2SO_4 for 3min at 100°C. This procedure showed that less than 3% of the phosphate group had been released during the hydrolysis.

All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Specifically radioactively labelled nitrilotriacetate was synthesized by reaction of the appropriate ^{14}C-labelled glycine with chloroacetic acid in NaOH solution as described by Bentov *et al.* (1967) and purified by crystallization from water. The radiochemical purity of the product was shown by t.l.c. on cellulose plates in phenol–water–formic acid (75:25:1, by vol.; R_F 0.70) and diethyl ether–formic acid–water (7:2:1, by vol.; R_F 0.20) when only a single radioactive peak was detected.

The last traces of O_2 were removed from O_2-free N_2 (British Oxygen Co. Ltd., Greenwich, London S.E.10, U.K.) by passage over copper turnings at 350°C. This purified gas was used to obtain anaerobic conditions in reaction flasks.

Growth media

Pseudomonas sp. T23 was isolated on modified Hutner's mineral medium, pH 7.0 (Hegeman, 1966), in which the normal concentration of nitrilotriacetate (0.02%) was increased to 0.1% (w/v, expressed as the free acid) and this medium was used as a routine for the growth of the organism on this substrate. Growth on other substrates was carried out in the mineral medium described by Cripps (1973), all carbon sources being added to a final concentration of 0.1% (w/v). Solutions were sterilized by autoclaving at 121°C for 15min, except those containing glyoxalate, which were sterilized by filtration.

Bulk growth of the organism for physiological studies

The organism was grown in 2 litre conical flasks containing 1 litre of growth medium incubated at 30°C on an orbital shaker. After 40h, when the culture was nearing the end of the exponential phase, the organisms were harvested by centrifuging (5600*g*, 15min, 5°C), washed twice in 67mM-sodium–potassium phosphate buffer, pH 7.0, and resuspended in the same buffer. Suspensions were used for whole-cell studies on the day of preparation.

* Abbreviation: H_4PteGlu, tetrahydropteroylglutamic acid.

Manometry

O_2 uptake and CO_2 production were measured by conventional manometric techniques (Umbreit *et al.*, 1972) at 30°C.

Preparation of cell-free extracts

Cell-free extracts were prepared from freshly harvested cells by sonication with a MSE 100W ultrasonic disintegrator at an amplitude of 8μm peak-to-peak. Samples of washed suspensions (5ml, 0.4–0.5g dry wt.) were held in an ice-cooled vessel and were sonicated for 2×30s, the periods of sonication being separated by intervals of 1min to allow for cooling. The broken-cell preparations were clarified by centrifuging at 20000*g* for 10min at 5°C and the pellets were resuspended in buffer and again subjected to sonication as above. Both batches of extract contained similar amounts of protein (15–20mg/ml) and possessed similar specific activities of all enzymes tested.

Protein determinations

The protein concentration in extracts was determined by the method of Lowry *et al.* (1951) with bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as a standard.

Buffer solutions

Sodium–potassium phosphate and Tris–HCl buffers were prepared as described by Dawson *et al.* (1969).

Radioactivity measurements

Liquid scintillation counting was carried out in an Intertechnique SL30 liquid-scintillation spectrometer (Intertechnique Ltd., Portslade, Sussex, U.K.) by using toluene scintillator [5g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre of toluene] or dioxan scintillator [8g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole and 85g of naphthalene/litre of dioxan]. Radioactive areas on chromatograms were located by a Packard model 7201 Radiochromatogram Scanner (Packard Instruments Ltd., Caversham, Berks, U.K.). After paper electrophoresis (see below) the amount of radioactivity in the radioactive areas on the paper strips was determined by combustion

to $^{14}CO_2$ by using a Packard Tri-Carb Sample Oxidiser (model 305) as recommended by the manufacturers.

Chromatography and electrophoresis

T.l.c. was carried out on 0.1mm-thick precoated cellulose and silica-gel plates (E. Merck, Darmstadt, Germany), the latter plates being activated at 105°C for 30min before use. Paper electrophoresis was carried out with a Pherograph 64 (Northern Media Supply Company, Hull, U.K.) at −5°C on Whatman 3MM paper.

Compounds were detected by the following spray reagents; organic acids (including nitrilotriacetate) by 0.04% (w/v) Bromothymol Blue in ethanol, amino acids and iminodiacetate by 0.25% (w/v) ninhydrin in acetone and glycerate by the periodate–Schiff's reagent of Baddiley *et al.* (1956). Phenylhydrazones were detected with a u.v. lamp (Hanovia Ltd., Slough, Bucks., U.K.).

Enzyme assays

Spectrophotometric enzyme assays were carried out in either a Unicam SP.1800 spectrophotometer or a Gilford model 240 spectrophotometer in 10mm-light-path cuvettes. Assays were performed at 30°C except for serine hydroxymethyltransferase (EC 2.1.2.1), which was determined at 37°C by the method of Scrimgeour & Huennekens (1962). Malate synthase (EC 4.1.3.2) was determined by the method of Dixon & Kornberg (1962), glyoxalate carboligase (EC 4.1.1.–) by the method of Kornberg & Gotto (1961), hydroxypyruvate reductase (EC 1.1.1.29) by the method of Large & Quayle (1963), phosphoserine phosphohydrolase (EC 3.1.3.3) by the method of Heptinstall & Quayle (1970) except that the liberated P_i was determined by the method of Rockstein & Herron (1951), β-hydroxyaspartate synthase (EC 4.1.2.–) by the method of Gibbs & Morris (1964), serine dehydratase (EC 4.2.1.13) by the method of Robinson & Labow (1971) and malate–NAD oxidoreductase (malate dehydrogenase, EC 1.1.1.37) by the method of Reeves *et al.* (1971). Citrate synthase (EC 4.1.3.7) was determined by the method of Reeves *et al.* (1971) after dialysis of the cell-free extract against 500vol. of 67mM-sodium–potassium phosphate buffer, pH7.0, for 16h at 4°C.

Phosphoglycerate dehydrogenase (phosphohydroxypyruvate reductase) (EC 1.1.1.–). This enzyme was assayed by measuring the oxidation of NADH at 340nm consequent on the reduction of phosphohydroxypyruvate. Reaction mixtures contained (in 3ml): 300μmol of sodium–potassium phosphate buffer, pH6.0; 4μmol of $MgCl_2$; 0.56μmol of phosphohydroxypyruvate; 0.5μmol of NADH; extract (1–2mg of protein). NADH oxidase activity was determined before substrate addition.

Glycollate–NADP oxidoreductase (glyoxalate reductase) (EC 1.1.1.–). This enzyme was assayed by measuring the oxidation of NADPH at 340nm consequent on the reduction of glyoxalate. Reaction mixtures contained (in 3ml): 100μmol of sodium–potassium phosphate buffer, pH7.0; 0.5μmol of NADPH; 2.5μmol of glyoxalate; extract (0.5–2mg of protein). NADPH oxidase activity was determined before glyoxalate addition.

Aminotransferase activities. L-Serine–glyoxalate aminotransferase (EC 2.6.1.–) was determined by the method of Harder & Quayle (1971).

L-Serine–oxaloacetate aminotransferase (EC 2.6.1.–) was assayed spectrophotometrically by coupling the reaction to endogenous malate dehydrogenase. Reaction mixtures contained (in 3ml): 67μmol of sodium–potassium phosphate buffer, pH7.0; 0.03μmol of pyridoxal phosphate; 0.5μmol of NADH; 2.5μmol of aspartate; 2.5μmol of hydroxypyruvate; extract (2–3mg of protein). The reaction was initiated by the addition of aspartate and followed at 340nm. The rate of oxidation of NADH observed was corrected for the rate found in reaction mixtures lacking aspartate. Malate dehydrogenase activity was never less than 30 times the aminotransferase activity.

L-Glutamate–glyoxalate aminotransferase (EC 2.6.1.–) was determined in reaction mixtures containing (in 0.5ml): 17μmol of sodium–potassium phosphate buffer, pH7.0; 1μmol of [1-^{14}C]glyoxalate (99μCi/mmol); 0.015μmol of pyridoxal phosphate; 3.75μmol of glutamate; extract (4–5mg of protein). The reaction was started by the addition of extract after anaerobic conditions had been established by gassing with purified N_2 for 15min. After 30min at 30°C, 0.25ml of 0.4% (w/v) dinitrophenylhydrazine in 2M-HCl was added and the precipitated protein removed by centrifuging. Samples (10μl) of the supernatant were subjected to t.l.c. on cellulose plates in diethyl ether–formic acid–water (7:2:1, by vol.; Myers & Huang, 1969) and the radioactive areas located (R_F of glycine, 0.08; R_F of glyoxalate dinitrophenylhydrazone, 0.93). The area corresponding to glycine was scraped off the plate directly into a counting vial containing dioxan scintillator and the radioactivity was determined. Control incubation mixtures, lacking glutamate, were treated similarly. The efficiency of the process (t.l.c. and radioactivity counting) was 70.2% and was determined with known amounts of [1-^{14}C]glycine, which were taken through the same procedure. Attempts to demonstrate other aminotransferase activities were made by similar methods in which the [1-^{14}C]-glyoxalate was replaced by [1-^{14}C]glycine (56μCi/μmol) and the glutamate by 2-oxoglutarate, oxaloacetate or pyruvate.

Nitrilotriacetate mono-oxygenase. (i) Spectrophotometric method. Reaction mixtures were made containing 5.7ml of 67mM-sodium–potassium phosphate buffer, pH7.5, 0.2ml of 0.1M-$MgCl_2$ and 0.2ml of extract (4–5mg of protein); 2.85ml of this mixture was placed in each of two cuvettes. Nitrilotriacetate (2.5μmol in 0.1ml of 67mM-sodium–potassium phosphate buffer, pH7.0) was added to one cuvette and 0.1ml of the phosphate buffer to the other. These solutions were used to zero the (Gilford) spectrophotometer at 340nm before the reaction was initiated by the addition of NADH (0.05ml, 0.5μmol) to each cuvette. The oxidation of NADH was measured in both reaction mixtures by the use of the automatic cell-changer facility. The difference in rates of NADH oxidation provided a measure of nitrilotriacetate mono-oxygenase activity.

(ii) Radiochemical method. The rate of formation of glyoxalate by the nitrilotriacetate mono-oxygenase reaction was measured in a coupled reaction with endogenous glyoxalate carboligase by determining the rate of production of $^{14}CO_2$ from nitrilotri-[1-^{14}C]acetate. Reaction mixtures contained (per ml): 46μmol of sodium–potassium phosphate buffer, pH7.5; 0.5μmol of thiamin pyrophosphate; 5μmol of $MgCl_2$; 1μmol of NADH; 1.25μmol of nitrilotri-[1-^{14}C]acetate (11.03μCi/mmol); extract (2–3mg of protein, 34–50munits of glyoxalate carboligase). Samples (0.9ml) were taken after 20min incubation at 30°C and placed in stoppered Warburg flasks. $^{14}CO_2$ was liberated by the immediate addition of 0.2ml of 3.75M-H_2SO_4 from the side arm and collected in 0.1ml of a mixture of 2-phenylethylamine–methanol–toluene (2:1:1, by vol.), contained in the centre well, over a 3h period. The centre-well contents were transferred quantitatively to a counting vial by two washes with 0.25ml of methanol and radioactivity was determined after the addition of 10ml of toluene scintillator. The overall efficiency of the procedure was determined as 74.0% with standard solutions of $Na_2{}^{14}CO_3$.

Glycine decarboxylase (glycine-cleavage enzyme) The activity of this enzyme system was determined by the release of $^{14}CO_2$ from [1-^{14}C]glycine (cf. McGilvray & Morris, 1969). Reaction mixtures contained (in 1ml): 60μmol of sodium–potassium phosphate buffer, pH7.5; 5μmol of [1-^{14}C]glycine (2.5μCi/mmol); 0.22μmol of H_4PteGlu (as a freshly prepared solution in 0.1ml of 67mM-phosphate buffer); 0.04μmol of pyridoxal phosphate; 1μmol of NAD^+; 10μmol of mercaptoethanol; extract (6–7mg of protein). After 30min incubation at 30°C, the $^{14}CO_2$ that had been formed was determined as described above.

Enzyme units. One unit of enzyme activity is defined as the quantity of enzyme that catalyses the formation of 1μmol of product or the disappearance of 1μmol of substrate/min in the assays described above. The molar extinction coefficient of NADH and NADPH at 340nm was taken to be 6.22×10^3 litre $\cdot mol^{-1}\cdot cm^{-1}$ (Dawson *et al.*, 1969).

Formation of glyoxalate from nitrilotriacetate

Incubation mixtures were set up containing (in 5ml): 280μmol of sodium–potassium phosphate buffer, pH7.0; 10μmol of nitrilotriacetate; 5μmol of NADH; 30μmol of phenylhydrazine; extract (7–8mg of protein). After incubation at 30°C for 2h, 1ml of 2M-HCl was added and the precipitated protein removed by centrifuging. The supernatant was extracted with 2×5ml of ethyl acetate and the separated ethyl acetate layer was extracted with 2×5ml of a saturated solution of Na_2CO_3. The Na_2CO_3 layer was acidified with conc. HCl and was then re-extracted with 2×5ml of ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4, evaporated to dryness with a stream of N_2 and the residue dissolved in a small volume of ethanol. Glyoxalate phenylhydrazone was shown to be present in this solution by t.l.c. with authentic material on silica-gel plates in butan-1-ol–ethanol–water (5:1:4, by vol.; Cavallini *et al.*, 1949), 2-methylbutan-1-ol–ethanol–water (5:1:4, by vol.; Altmann *et al.*, 1951) and 2-methylbutan-1-ol–0.25M-NH_3 (20:1, v/v; Dancis *et al.*, 1963).

Formation of iminodiacetate from nitrilotriacetate

The formation of iminodiacetate from ^{14}C-labelled nitrilotriacetate was followed in incubations lasting several hours by using the reaction mixture described under 'Radiochemical assay method' for nitrilotriacetate mono-oxygenase except that the NADH was replaced by a generating system consisting of (per ml of reaction mixture): 1μmol of NAD^+; 5μmol of ethanol; 30μg of alcohol dehydrogenase. The mixture was shaken at 30°C and the reaction terminated after a suitable incubation period by the addition of $HClO_4$ to a concentration of 3% (w/v). The precipitated protein was removed by centrifuging. Iminodiacetate was shown to be present in the supernatant by comparison, with authentic material, of its mobility on electrophoresis in acetic acid–formic acid–water (78:25:897, by vol.; Leggett-Bailey, 1967) and pyridine–acetic acid–water (2:1:97, by vol.; Leggett-Bailey, 1967) and by t.l.c. on cellulose plates in 80% (w/v) phenol containing 1% (v/v) formic acid.

Conversion of glyoxalate into glycerate

The procedures of Kornberg & Gotto (1961) were used except that the glycerate was identified by t.l.c. on the cellulose plates in ethanol–conc. NH_3 (sp.gr. 0.880)–water (20:1:4, by vol.; Long *et al.*,

1951) and in 80% (w/v) phenol. Anaerobic conditions were maintained throughout by gassing with purified N_2.

Results

Growth and whole-cell studies

Pseudomonas sp. T23 grew well in minimum media containing nitrilotriacetate as the sole source of carbon and nitrogen with a generation time in the range 4.7–6h at 30°C. The growth rate was not altered by the presence of 7.5mM-$(NH_4)_2SO_4$ in the culture medium. Growth with iminodiacetate, glycollate, glycine or glyoxalate as sole carbon sources was very poor and non-reproducible, but equimolar mixtures of glycollate and glycine or glyoxalate and glycine supported good growth with generation times of approx. 12h.

Washed cell suspensions of the organism grown on nitrilotriacetate oxidized this compound with an O_2-uptake rate of 100μl/h per mg dry wt. of cells. The O_2 consumption and CO_2 production were 2.5 and 2.9mol/mol of substrate respectively. O_2-uptake rates of less than 5% of that for nitrilotriacetate were observed with iminodiacetate, glycine, glycerate and glyoxalate. After growth of the organism on acetate, cells oxidized nitrilotriacetate with an O_2 uptake rate of 10μl/h per mg dry wt. of cells.

Nitrilotriacetate metabolism by cell-free extracts

An NADH-dependent degradation of nitrilotriacetate was observed in extracts of *Pseudomonas* sp. T23 grown on this substrate and could be assayed spectrophotometrically (see the Experimental section). The activity determined by this method was 7munits/mg of protein. Extracts prepared from glucose-grown cells contained an activity of 1munit/mg of protein. However, since a relatively high NADH oxidase activity (10munits/mg of protein) was also present in all extracts, the spectrophotometric assay of nitrilotriacetate degradation was not suitable for a detailed study of the reaction. Hence, the more sensitive radiochemical method (see the Experimental section) was developed. Both assay methods gave similar reaction rates with all extracts.

The cofactor requirements of the reaction were determined by the radiochemical method (Table 1). A specific requirement for NADH was demonstrated, NADPH being an ineffective substitute and, in addition, the reaction was totally dependent on the presence of O_2. These observations suggest that nitrilotriacetate is metabolized by a monooxygenase reaction. Thiamin pyrophosphate and Mg^{2+} were necessary for the production of $^{14}CO_2$. These are cofactors of the glyoxalate carboligase reaction (Kornberg & Gotto, 1961). The $^{14}CO_2$ evolved was derived almost totally from the carboxyl group of nitrilotriacetate for when [2-^{14}C]-labelled substrate was used in the assay, very little $^{14}CO_2$ was produced (Table 1). All activity was retained in the supernatant after centrifuging at 140000*g* for 2h.

Table 1. *Formation of $^{14}CO_2$ from nitrilotri[1^{14}C]-acetate by cell-free extracts of Pseudomonas sp. T23*

Nitrilotriacetate breakdown was assayed by the radiochemical method described in the Experimental section with the modifications to the reaction mixture noted below.

Expt. no.	Composition of reaction mixture	$^{14}CO_2$ produced/20min (c.p.m.)
1	Complete	686
	Minus Mg^{2+}	117
	Minus thiamin pyrophosphate	84
	Minus Mg^{2+} and thiamin pyrophosphate	57
	Complete but with nitrilotri[2-^{14}C]acetate	11
2	Complete	609
	Minus NADH	14
	Minus NADH plus NADPH	27
3	Complete	607
	Complete (anaerobic)	19

Products of nitrilotriacetate metabolism by cell-free extracts. The disappearance of nitrilotriacetate from reaction mixtures was accompanied by the appearance of iminodiacetate (Fig. 1). Small amounts of a second radioactive product were also detected. The identity of this compound was not established, although it was shown not to be glyoxalate, glycine, glycollate, glycerate or oxalate. This compound was further metabolized by the system.

The inclusion of phenylhydrazine in incubation mixtures containing nitrilotriacetate, NADH and cell-free extract (see the Experimental section) resulted in the formation of glyoxalate phenylhydrazone. This compound was not formed in the absence of NADH.

The demonstration of the existence of a monooxygenase reaction and the formation of iminodiacetate and glyoxalate during nitrilotriacetate metabolism are consistent with the reaction shown in eqn. (1).

$$N(CH_2CO_2H)_3 + NADH + H^+ + O_2 \rightarrow HN(CH_2CO_2H)_2 + CHO\cdot CO_2H + NAD^+ + H_2O \quad (1)$$

Further evidence for this reaction was obtained by the demonstration that, in studies with radioactively labelled substrate, approximately two-thirds

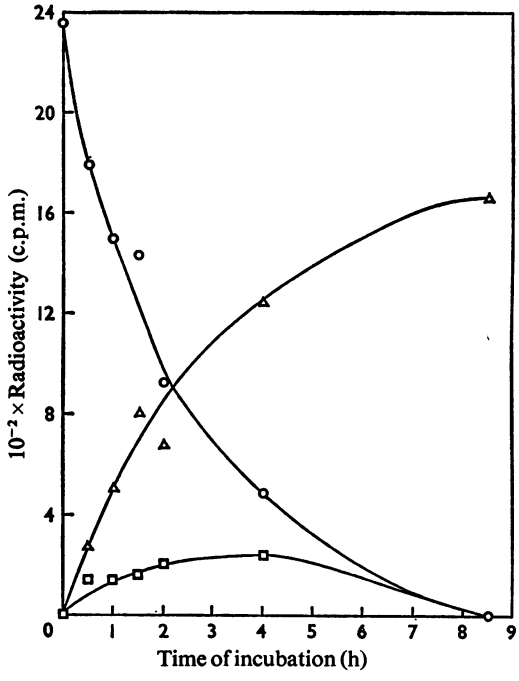


Fig. 1. *Conversion of nitrilotriacetate into iminodiacetate by a cell-free extract of Pseudomonas sp. T23*

Reaction conditions were as described in the Experimental section with 6.25μmol of nitrilotri-[2-^{14}C]acetate (19.0μCi/mmol) and 18mg of protein in a total volume of 5ml. Samples (0.5ml) were taken at the intervals shown and the reaction was terminated by the addition of 60μl of 25% (w/v) $HClO_4$. The radioactive compounds in 0.1ml of the deproteinized mixtures were separated by paper electrophoresis in formic acid–acetic acid–water (25:78:897, by vol) and determined by combusting the radioactive areas on the paper to $^{14}CO_2$. ○, Nitrilotriacetate; △, iminodiacetate; □, unknown radioactive product.

of the radioactivity of the nitrilotriacetate consumed in the reaction appeared in the product iminodiacetate (Table 2) as is required by eqn. (1). The formation of iminodiacetate by this system was totally dependent on the presence of O_2 and NADH (or an NADH-generating system) and omission of Mg^{2+} resulted in a 70% decrease in iminodiacetate formation during a 7h incubation period. No conversion of nitrilotriacetate into iminodiacetate was observed in reaction mixtures containing extract which had been boiled for 5min. The thiamin pyrophosphate which was included in the incubation mixtures as a routine was shown to have no effect on iminodiacetate formation.

Table 2. *Conversion of nitrilotriacetate into iminodiacetate by a cell-free extract of Pseudomonas sp. T23*

Reactions and determinations were carried out as described in the legend to Fig. 1 except that various concentrations of nitrilotri[2-^{14}C]acetate were used, each concentration being set up in duplicate. Samples were taken at zero time and after 16h incubation. Electrophoresis was carried out with triplicate samples (0.1ml) of the deproteinized reaction mixtures. Results given are the mean of the values obtained for the radioactive contents (as $^{14}CO_2$) of the six corresponding radioactive peaks.

Radioactivity (d.p.m./ml of reaction mixture)			
Nitrilotriacetate at zero time	Nitrilotriacetate at 16h	Iminodiacetate at 16h	Iminodiacetate formed / Nitrilotriacetate consumed
3.06	0.23	1.94	0.69
7.00	0.65	4.10	0.65
10.3	1.50	5.95	0.68

Enzymes of glycine and glyoxalate metabolism in cell-free extracts

The specific activities of known enzymes involved in glycine and glyoxalate metabolism were determined in cell-free extracts prepared from cells grown on nitrilotriacetate or glucose (Table 3). Extracts prepared from acetate-grown cells exhibited similar activities to those obtained from glucose-grown cells. Significantly increased activities of serine hydroxymethyltransferase, hydroxypyruvate reductase, serine–oxaloacetate aminotransferase, glyoxalate carboligase and glyoxalate reductase were noted after growth on nitrilotriacetate, whereas the activities of malate synthase and citrate synthase were not changed. Attempts to demonstrate β-hydroxyaspartate synthase and serine dehydratase activities were unsuccessful.

A 60-fold increase in the activity of glycine decarboxylase was observed after growth on nitrilotriacetate (Table 3). However, the specific activity of this enzyme was always lower than the other induced activities measured, so further studies were performed. It was shown that the activity, determined by the release of $^{14}CO_2$ from [1-^{14}C]glycine (see the Experimental section), was totally dependent on H_4PteGlu and NAD^+ ($NADP^+$ could not replace NAD^+) and was decreased by 24% when pyridoxal phosphate was omitted from the reaction mixture. These requirements are in accord with a glycine-cleavage enzyme catalysing the formation of CO_2, NH_3 and 5,10-methylene-H_4PteGlu, similar to those

Table 3. *Enzyme activities in cell-free extracts of Pseudomonas sp. T23 grown on nitrilotriacetate and glucose*

All values are specific activities expressed as munits/mg of protein. N.T., Not tested.

Growth substrate ... Enzyme	Activity: Nitrilotriacetate	Activity: Glucose
Glyoxalate carboligase	17	1
Glyoxalate reductase	8	1
β-Hydroxyaspartate synthase	0	N.T.
Glycine decarboxylase	2.40	0.04
Serine hydroxymethyltransferase	42	16
Serine dehydratase	0	N.T.
Hydroxypyruvate reductase	37	4
Serine-oxaloacetate aminotransferase	36	0
Phosphohydroxypyruvate reductase	13	76
Phosphoserine phosphohydrolase	9	11
Malate synthase	133	31
Citrate synthase	160	220

described by Sagers & Gunsalus (1961), Kochi & Kikuchi (1969), McGilvray & Morris (1969) and Hall & Crosbie (1971).

Enzymes involved in the biosynthesis of serine from glycolytic intermediates by the 'phosphorylated' pathway (Pizer, 1963) were also examined (Table 3). It was shown that the activity of phosphohydroxypyruvate reductase was repressed by growth on nitrilotriacetate whereas the activity of phosphoserine phosphohydrolase was unaffected.

Conversion of glyoxalate into glycerate

When supplemented with thiamin pyrophosphate, Mg^{2+} and NADH, extracts from nitrilotriacetate-grown cells catalysed the conversion of glyoxalate into glycerate under anaerobic conditions. In this process 0.5mol of CO_2 was produced/mol of glyoxalate utilized by the glyoxalate carboligase reaction. When NADH was omitted a product accumulated whose dinitrophenylhydrazone possessed the spectral properties of the tartronic semialdehyde derivative (Kornberg & Gotto, 1961). This product was metabolized rapidly in the presence of NADH. Hence the presence of tartronic semialdehyde reductase (EC 1.1.1.60) was inferred. A measure of the activity of this enzyme was obtained by allowing tartronic semialdehyde to accumulate in reaction mixtures contained in anaerobic cuvettes, adding NADH, and then following the oxidation of this cofactor at 340nm. The rate observed was 182munits/mg of protein.

Attempts to show interconversion of glyoxalate and glycine

It proved extremely difficult to demonstrate the interconversion of glyoxalate and glycine by aminotransferase-type reactions in cell-free extracts Serine–glyoxalate aminotransferase activity was undetectable in extracts of nitrilotriacetate-grown cells and studies with radioactively labelled glycine and glyoxalate with several oxo and amino acids, including oxaloacetate, pyruvate, 2-oxoglutarate and glutamate, showed very low interconversion rates. Highest activities were recorded in incubation mixtures containing [1-^{14}C]glyoxalate and glutamate, when radioactively labelled glycine was formed at a rate of 101nmol/h per mg of protein.

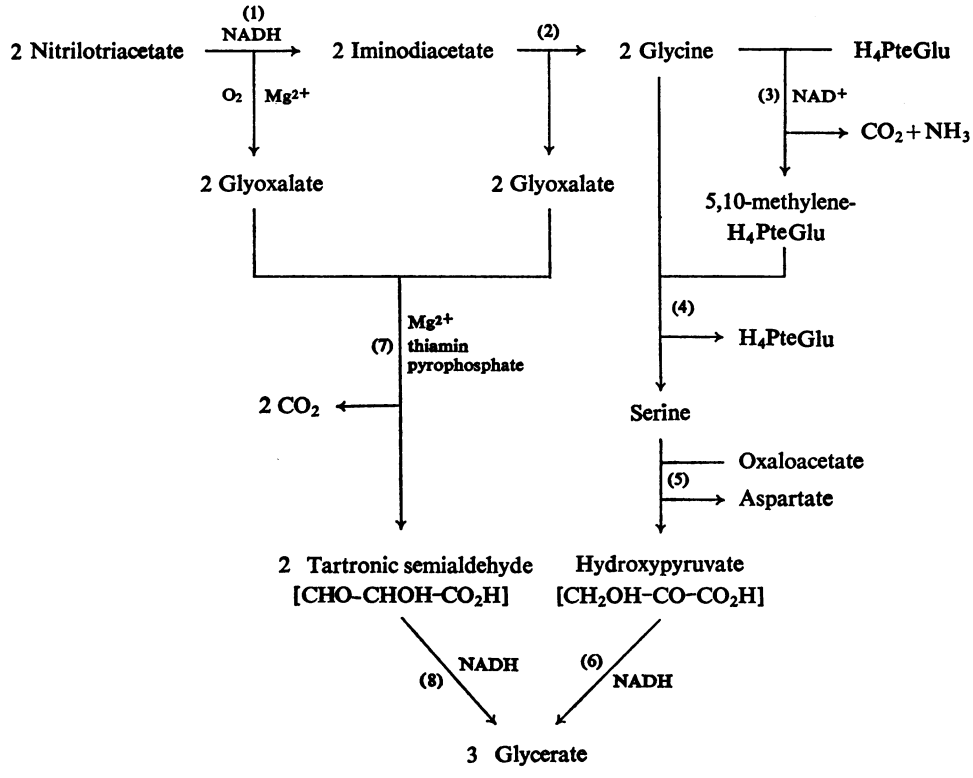
Iminodiacetate metabolism by cell-free extracts

Repeated attempts to show the metabolism of iminodiacetate by extracts from nitrilotriacetate-grown *Pseudomonas* sp. T23 were unsuccessful. The two major types of biochemical reaction by which secondary amines may be metabolized are mediated by mono-oxygenases or by dehydrogenases (Large, 1971*a*). No evidence was obtained for a mono-oxygenative attack on iminodiacetate with either NADH or NADPH as electron donors, and negative results were also obtained during attempts to demonstrate dehydrogenase activity by using phenazine methosulphate, 2,6-dichlorophenol-indophenol, ferricyanide or cytochrome *c* as electron acceptors.

Discussion

The data presented can be interpreted in terms of the reaction sequence shown in Scheme 1, in which 2mol of nitrilotriacetate are converted into 3mol of glycerate and 3mol of CO_2. This sequence was deduced largely from the induced enzyme activities found in extracts of *Pseudomonas* sp. T23 grown on nitrilotriacetate. Whole-cell studies afforded no evidence to support this pathway, as nitrilotriacetate-grown cells were able to oxidize only the growth substrate at appreciable rates and showed little O_2 uptake when presented with other intermediates shown in Scheme 1.

The first enzyme of the sequence, a Mg^{2+}-requiring, NADH-dependent mono-oxygenase, converts nitrilotriacetate into iminodiacetate and glyoxalate (reaction 1, Scheme 1). Other tertiary-amine mono-oxygenases of microbial origin have been reported (Large *et al.*, 1972; Colby & Zatman, 1973), which



Scheme 1. *Proposed pathway of nitrilotriacetate metabolism by Pseudomonas sp. T23*

The numerals in parentheses refer to reaction pathways mentioned in the text. The numerals preceding various chemicals refer to the amount (mol) of compound metabolized.

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are active with trimethylamine, but, in these cases, the electron donor was NADPH. The conversion of trimethylamine into dimethylamine by these enzymes was shown to be a two-stage process. The first product was trimethylamine *N*-oxide, which was subsequently non-oxidatively demethylated to form the secondary amine and formaldehyde (Large, 1971*b*; Large *et al.*, 1972; Colby & Zatman, 1973). The possibility that nitrilotriacetate *N*-oxide is an intermediate in the scheme presented has not been excluded.

The conversion of iminodiacetate into glycine and glyoxalate as shown in Scheme 1 (reaction 2) has not been demonstrated. Previous reports of the conversion of secondary amines into primary amines and aldehydes have all involved a single-stage monooxygenase reaction (see e.g., Eady *et al.*, 1971), although the possibility of a dehydrogenase-type reaction, similar to that described by Colby & Zatman (1973) for two obligate methylotrophs grown on trimethylamine, cannot be ruled out. Neither type of activity was demonstrated in *Pseudomonas* sp. T23. A possible alternative to reaction (2), which is not excluded by the data, is a decarboxylation of iminodiacetate forming sarcosine followed by oxidation of the sarcosine to glycine and formaldehyde. However, the simultaneous adaptation studies of Tiedje *et al.* (1973) suggested that sarcosine was not an intermediate of nitrilotriacetate oxidation by their pseudomonad. Further work is necessary to resolve this point.

Much circumstantial evidence indicated that glycine was formed as an intermediate during growth on nitrilotriacetate. The observation of elevated activities of glycine decarboxylase (reaction 3), serine hydroxymethyltransferase (reaction 4), serine-oxaloacetate aminotransferase (reaction 5) and hydroxypyruvate reductase (reaction 6) in nitrilotriacetate-grown cells is strongly indicative of the involvement of a pathway converting 2 mol of glycine into 1 mol each of glycerate and CO_2 during nitrilotriacetate catabolism. In addition, the activity

of one of the key enzymes of the 'phosphorylated' pathway of serine biosynthesis, phosphohydroxypyruvate reductase, was repressed by growth on nitrilotriacetate, suggesting that some other route for the provision of serine and glycine was operating. Induction and repression are not normally encountered as control mechanisms in the 'phosphorylated' pathway. In *Escherichia coli* and *Salmonella typhimurium* metabolic control is mediated by the end-product inhibition of phosphohydroxypyruvate reductase by serine (Umbarger & Umbarger, 1962; Pizer, 1963; Pizer & Potochny, 1964). A similar lack of induction and repression has been shown in *Pseudomonas* AM1 (Heptinstall & Quayle, 1970). Pizer & Potochny (1964), however, did show a decrease in the amount of phosphohydroxypyruvate reductase in *E. coli* when the growth medium was supplemented with a mixture of L-threonine, L-methionine, L-leucine and DL-isoleucine, although the reason for this effect is not understood. As in the present study, the activity of phosphoserine phosphohydrolase remained unchanged under all growth conditions.

The enzymic composition of extracts indicated that the glyoxalate formed from nitrilotriacetate was metabolized via tartronic semialdehyde to glycerate (reactions 7 and 8). This overall conversion was directly demonstrated and spectral evidence for the involvement of tartronic semialdehyde was obtained. An alternative pathway of glyoxalate and glycine metabolism found in *Micrococcus denitrificans*, the β-hydroxyaspartate pathway (Gibbs & Morris, 1964), was shown not to operate during nitrilotriacetate metabolism by *Pseudomonas* sp. T23, since a key enzyme of this pathway, β-hydroxyaspartate synthase, was absent. An enzymic activity capable of glyoxalate metabolism, glyoxalate reductase, was found in extracts of nitrilotriacetate-grown cells. However, this may be of no physiological significance, as this activity has been reported to be associated with hydroxypyruvate reductase (Large & Quayle, 1963).

Scheme 1 shows the metabolism of nitrilotriacetate to glycerate, the latter being presumably further metabolized via phosphorylated intermediates to pyruvate and acetyl-CoA and hence the tricarboxylic acid cycle. However, a further possibility exists for the terminal metabolism of the glyoxalate derived from nitrilotriacetate, namely oxidation by the dicarboxylic acid cycle initiated by malate synthase (Kornberg & Sadler, 1961). In this case, the reactions shown in Scheme 1 would form part of an anaplerotic sequence leading to the formation of acetyl-CoA, which would function to replenish the intermediates of the dicarboxylic acid cycle used in biosynthesis. In *E. coli* strain W, the operation of the dicarboxylic acid cycle during growth on glycollate (a precursor of glyoxalate) was accompanied by raised activities of malate synthase compared with citrate synthase (Kornberg & Sadler, 1961). During growth on acetate, the tricarboxylic acid cycle operates and the activity of citrate synthetase was shown to be greater than that of malate synthase, the latter enzyme fulfilling an anaplerotic role in this case. Little variation in activities of these two enzymes was found with *Pseudomonas* sp. T23 grown on nitrilotriacetate, glucose or acetate, and they appear to be constitutive for this strain. Hence further studies will be necessary to determine whether Scheme 1 represents the major oxidative pathway of nitrilotriacetate or whether it forms part of an anaplerotic sequence.

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