Bacterial Degradation of 3,4,5-Trimethoxyphenylacetic and 3-Ketoglutaric Acids

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When grown at the expense of 3,4,5-trimethoxyphenylacetic acid, a species of *Arthrobacter* readily oxidized 3,4-dihydroxy-5-methoxyphenylacetic acid, but other structurally related aromatic acids were oxidized only slowly. Cell extracts contained a dioxygenase for 3,4-dihydroxy-5-methoxyphenylacetate, and the corresponding trihydroxy acid, which was not attacked by the enzyme, inhibited oxidation of this ring-fission substrate. Cell suspensions did not release carbon dioxide from 3,4-[methoxyl- ^{14}C]dihydroxy-5-methoxyphenylacetate but accumulated 1 mol of methanol per mol of 3,4,5-trimethoxyphenylacetate oxidized. A cell extract converted the ring-fission substrate into stoichiometric amounts of pyruvate and acetoacetate, formed from 3-ketoglutarate by the action of an induced decarboxylase. 3-Ketoglutaric acid served as sole source of carbon for many soil isolates.

In the preceding paper (4), we demonstrated that methanol is formed as the end product of metabolism of 3,4,5-trimethoxycinnamic acid (TMCA). Methanol arises from one of the methoxyl groups which is retained in the ring-fission substrate, 3-*O*-methylgallate. Thus, the pathway for degrading TMCA converges on that described earlier for 3,4,5-trimethoxybenzoic acid (TMBA; 3). From a survey of several bacteria isolated on various 3,4,5-substituted aromatic acids, it appears that convergence of this sort is common (4). Suspensions of cells grown on compounds with three carbons in the side chain ($\text{C}_6\text{-C}_3$ acids) all attack the corresponding benzoic acid ($\text{C}_6\text{-C}_1$ acids), and cell extracts oxidize 3-*O*-methylgallate without addition of reduced pyridine nucleotides. It appears, therefore, that non-oxidative metabolism of aromatic methoxyl groups to methanol (3, 4) may be common in nature.

This paper describes the degradation of a homologous compound, 3,4,5-trimethoxyphenylacetic acid (TMPA; Fig. 1, I). It was found that the side chain of this compound, unlike those of methoxylated cinnamic and propionic acids, was not shortened before oxidative demethoxylation, and ring cleavage occurred. Accordingly, TMBA was not a catabolite; however, TMPA was degraded by a similar sequence of reactions involving intermediates that were the methylene homologs of those found in the TMBA pathway (3).

MATERIALS AND METHODS

The organism, a strain of *Arthrobacter* sp. designated *Arthrobacter* sp. strain TMP, was isolated from soil by elective culture with TMPA as sole carbon source. The following procedures used in this investigation have been described previously (3, 4): growth of cells, preparation of extracts, determination of protein, enzymatic determination of pyruvate, chromatography and detection of phenolic compounds, preparation of 2,4-dinitrophenylhydrazones and 3,5-dinitrobenzoates, measurements of oxygen consumption either by Warburg respirometry or by the oxygen electrode, and quantification of methanol. Solutions of 3-ketoglutarate for growth media were sterilized by membrane filtration (Millipore Corp., Bedford, Mass.). Acetoacetate was measured enzymatically by using β -hydroxybutyrate dehydrogenase with NADH. 3-Ketoglutarate decarboxylase, which produces acetoacetate, was also assayed by using this dehydrogenase with 0.5 mM 3-ketoglutarate in 0.1 M sodium phosphate buffer, pH 7.2. Homoprotocatechuic acid was determined in the oxygen electrode, using extracts prepared from *Pseudomonas putida* grown on *p*-hydroxyphenylacetic acid (12). 2,4-Dinitrophenylhydrazones were identified by thin-layer chromatography, using as solvents benzene-ethyl acetate-formic acid (5:5:1) and benzene-ethyl acetate-formic acid (7:3:1). In these solvents, R_f values for a standard derivative of 3-ketoglutarate were 0.25 and 0.34, respectively. Radiochromatograms were scanned on a Packard model 7201 radiochromatogram scanner. Scintillation counting was performed as described previously (4).

Synthesis of phenolic compounds. 3,4,5-Trihydroxyphenylacetic acid was prepared from TMPA by the demethylation method of Blakley and Simpson

(1). Recrystallization from ethyl acetate gave 3,4,5-trihydroxyphenylacetic acid with melting point of 156°C (6; 158°C), pure by thin-layer chromatography and by gas chromatography of the trimethylsilyl derivative. A molecular ion of the expected mass (m/e 472) was obtained in the mass spectrometer.

3,4-Dihydroxy-5-methoxyphenylacetic acid (Fig. 1, II) was prepared from the corresponding trihydroxy compound by the method of Scheline (11); this compound (2.47 g, 13.4 μmol) was dissolved in 200 ml of 5% sodium tetraborate hexahydrate in a three-necked, round-bottomed flask and sparged with N_2 . While the flow of N_2 continued, 5.5 g of sodium hydroxide in 20 ml of water and 12.7 ml of dimethyl sulfate were added dropwise to the stirred solution. The rate of base addition was adjusted to maintain the pH near 11. After the mixture stood overnight under N_2 , examination by thin-layer chromatography revealed two compounds, judged to be the 3-*O*-methyl derivative and its methyl ester, based on their mobilities and reaction with 2,6-dibromoquinone-4-chloroimide. The mixture was filtered, the filtrate was acidified and extracted with ethyl acetate, and the ethyl acetate was removed under reduced pressure. The methyl ester was saponified by dissolving the resulting oil in 20% aqueous sodium hydroxide which had been sparged exhaustively with N_2 ; the solution was then heated in a steam bath for 1 h under N_2 , acidified, and continuously extracted with ether. After drying over Na_2SO_4 , the ether was evaporated under reduced pressure, and the residual solid was recrystallized from ethyl acetate to give 3,4-dihydroxy-5-methoxyphenylacetic acid (1.43 g, 54%), melting point, 139 to 141°C . The infrared spectrum showed bands at $3,480$ and $3,380\text{ cm}^{-1}$ (aryl O—H), $3,200$ to $2,800\text{ cm}^{-1}$ (acid O—H), $1,450\text{ cm}^{-1}$ (OCH_3 symmetrical deformation), and four strong peaks between $1,230$ and $1,110\text{ cm}^{-1}$ (C—O and C—O—C). For the trimethylsilyl derivative, the mass spectrometer showed ions with m/e of 414 (0.93, molecular ion), 297 (0.47, benzyl fragment), and 209 (0.83, monotrimethylsilylbenzyl fragment).

To prepare 3,4-[methoxyl- ^{14}C]dihydroxy-5-methoxyphenylacetic acid, the corresponding trihydroxy compound (100 mg) was methylated in the same manner as described above, using [^{14}C]dimethyl sulfate (Amersham Corp., Arlington Heights, Ill.) diluted to a specific activity of 7 μCi per mmol. The products of the reaction were extracted from the acidified reaction mixture and separated by descending paper chromatography on Whatman no. 3 chromatography paper, using benzene-propionic acid-water (2:1:1 by volume; upper layer). A radiochromatogram scan showed

two peaks of radioactive material, the major peak comigrating with the authentic monomethoxy compound; the second, which migrated further, was probably due to its methyl ester. The band corresponding to the free acid was cut from the chromatogram and eluted with ethyl acetate. After the solvent was evaporated in a stream of N_2 , the residue was dissolved in 1.0 ml of distilled water. The concentration of this solution of 3,4-[methoxyl- ^{14}C]dihydroxy-5-methoxyphenylacetic acid was estimated enzymatically in the oxygen electrode by means of an extract of *Arthrobacter* sp. TMP; the stoichiometry of oxidation was assumed to be that recorded below for the compound. The product appeared to be pure when examined by thin-layer chromatography. Its specific activity was 6.9 μCi per mmol.

Synthesis of the monomethyl ester of 3-ketoglutarate. The monomethyl ester of 3-ketoglutarate was synthesized from 3-ketoglutarate anhydride; this was prepared by the methods of Kaushal (8) and Findlay (5). From 4.0 g of 3-ketoglutarate, 2.3 g (63%) of crude anhydride was obtained with a melting point of 136 to 137°C (7; 138°C). To prepare the monomethyl ester, 128 mg (1 mmol) of the anhydride was dissolved in 1.0 ml of methanol which had been dried over 0.4-nm molecular sieves. A single product was observed on thin-layer chromatography. Infrared spectrometry of the methanol solution showed two carbonyl absorptions, $1,740\text{ cm}^{-1}$ (ester) and $1,720\text{ cm}^{-1}$ (ketone).

Other materials. The commercial sources of enzymes, coenzymes, hydroxyl- and methoxyl-substituted aromatic acids, and isotopically labeled reagents were those given previously (3).

RESULTS

Selection of organisms. Most of the organisms selected for growth on methoxylated aromatic acids with one or three carbon acidic side chains were pseudomonads (4). By contrast, when TMPA was used as sole carbon source, no pseudomonads were found, and most of the isolates were species of *Arthrobacter*. Although varying in characteristics, they all grew more slowly than the pseudomonads studied earlier (3, 4); cell extracts also were generally less active.

The strain selected for study (*Arthrobacter* sp. TMP) failed to grow on the 3,4,5-trimethoxy derivatives of benzoic or cinnamic acids. When grown on TMPA, cells oxidized a limited range

FIG. 1. Reaction sequence proposed for the degradation of 3,4,5-trimethoxyphenylacetate (I) to methanol, pyruvate, and acetoacetate. The precursor of acetoacetate is 3-ketoglutarate and the ring-fission substrate is 3,4-dihydroxy-5-methoxyphenylacetate (II).

of compounds in the Warburg respirometer (Table 1). A brief lag occurred before intact cells began to oxidize substrates, and it was not possible, therefore, to use the oxygen electrode to assay their ability to oxidize formaldehyde. Since this compound is also toxic at the concentrations used in the Warburg vessels (4), oxidation of formaldehyde was shown by radioassay with low concentrations of [^{14}C]formaldehyde either alone or in the presence of TMPA (Table 2). The inability of these cells to oxidize methanol was also confirmed by using [^{14}C]methanol.

Identification of the ring-fission substrate. Extracts of TMPA-grown *Arthrobacter* sp. TMP failed to oxidize 3,4,5-trihydroxyphenylacetic acid, whereas 3,4-dihydroxy-5-methoxyphenylacetic acid (Fig. 1, II) was oxidized rapidly, with consumption of 0.96 mol of O_2 per mol of substrate. The dioxygenase catalyzing this reaction was absent in succinate-grown cells but was induced to a specific activity of 0.92 μmol per min per mg of protein in cells grown on TMPA. The trihydroxy compound, besides not serving as substrate, strongly inhibited oxidation of 3,4-dihydroxy-5-methoxyphenylacetic acid. In the presence of 0.2 mM ring-fission substrate, the concentration required to reduce the activity of the dioxygenase by one-half (9; I_{50}) was 51 μM 3,4,5-trihydroxyphenylacetate. Homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid) was not attacked by the dioxygenase. Oxidative demethoxylation activity was not detectable in cell extracts. However, the strict substrate specificity of the dioxygenase suggested that one methoxyl group of TMPA, remaining after oxidative demethylation, was metabolized nonoxidatively and that release of methanol could be expected as in previous studies (3, 4).

Products of metabolism of 3,4-dihydroxy-5-methoxyphenylacetic acid (compound II). When 3-*O*-methylgallate is degraded, oxaloacetate and pyruvate are formed; oxaloacetate is then decarboxylated to generate a second molecule of pyruvate (3, 13). Since 3,4-dihydroxy-5-methoxyphenylacetate is the methylene homolog of 3-*O*-methylgallate, similar reactions would be expected to generate the homolog of oxaloacetate, namely, 3-ketoglutarate, which would then give acetoacetate by decarboxylation (Fig. 1). To increase yields of predicted end products, it was necessary to dialyze 1.0 ml of cell extract overnight against 1 liter of 0.05 M $\text{K}^+\text{-Na}^+$ phosphate buffer, pH 8.0, containing 10% glycerol and 5 mM ascorbic acid. Reaction mixtures contained 0.2 mg of dialyzed extract protein, 60 nmol of ring-fission substrate, and 0.25 μmol of magnesium chloride in 1 ml of phosphate-glycerol-ascorbate buffer, pH 7.5. After 2.5 h, the solutions were assayed for ac-

TABLE 1. Rates of oxidation by washed cells

Compound oxidized	Oxygen uptake ^a	
	TMPA ^b	Succinic acid ^b
TMPA	3.7	<0.1
3,4-Dihydroxy-5-methoxyphenylacetic acid	1.0	0.4 ^c
3,4,5-Trihydroxyphenylacetic acid	0.3 ^c	0.6 ^c
3,4-Dimethoxyphenylacetic acid	0.3	0.2
Homovanillic acid	0.3	<0.1
3,4,5-Trimethoxybenzoic acid		<0.1
Methanol	<0.1	ND ^d

^a Expressed as microliters of O_2 per minute, measured after subtracting endogenous respiration (0.9 μl of O_2 per min). Warburg flasks contained, in 2.9 ml of phosphate buffer (pH 7.0), 50 mg (wet weight) of cells, 140 μg of chloramphenicol, and 3 μmol of substrate. Center wells of flasks contained 0.1 ml of 5 N sodium hydroxide.

^b Compound upon which cells were grown.

^c Discoloration consistent with autooxidation occurred.

^d ND, Not determined.

TABLE 2. Distribution of radioactivity after oxidation of ^{14}C -labeled substrates by cell suspensions

Compound	% of final cpm	
	Center well ^a	Main compartment
3,4-[methoxy- ^{14}C]dihydroxy-5-methoxyphenylacetic acid ^b	4	96
[^{14}C]methanol	6	94
Plus TMPA ^c	4	96
Without cells	6	94
[^{14}C]formaldehyde ^d	51	49
Plus TMPA ^c	52	48
Without cells	0	100

^a Trap for $^{14}\text{CO}_2$.

^b 2.5 μmol of substrate was oxidized to completion, and incubation was continued for another 0.5 h before analysis.

^c Unlabeled TMPA; 3 μmol .

^d A 0.3- μmol (0.3- μCi) amount of [^{14}C]formaldehyde. Possible traces of [^{14}C]formate were removed by passing the solution through Dowex-1 (formate form) before use; 97% of the radioactivity was recovered after treatment.

cumulated pyruvate or acetoacetate; the amounts found were, respectively, 54.5 and 52.8 nmol. Neither compound was formed in controls when either extract or substrate was omitted.

The role of 3-ketoglutarate as precursor of

acetoacetate was substantiated by two experiments. First, extracts of *Arthrobacter* sp. TMP contained an inducible enzyme that catalyzed the rapid decarboxylation of 3-ketoglutarate (specific activity, $0.195 \mu\text{mol per min per mg of protein}$). Oxaloacetate was decarboxylated at less than one-tenth the rate for 3-ketoglutarate. Further, the monomethyl ester of 3-ketoglutarate was not metabolized to acetoacetate by extracts of TMPA-grown cells, since they did not contain an esterase for this compound. Extracts of succinate-grown cells decarboxylated oxaloacetate slowly but did not attack 3-ketoglutarate.

Further support for the metabolic status of 3-ketoglutarate was obtained by using a different strain of *Arthrobacter* sp. that grew relatively poorly on TMPA. This strain cleaved only compound II, and cell extracts also generated pyruvate and acetoacetate, but yields were less than stoichiometric. The strain possessed an induced decarboxylase for 3-ketoglutarate, but the specific activity ($0.027 \mu\text{mol per min per mg of protein}$) was much lower. This permitted small amounts of 3-ketoglutarate to accumulate when compound II was metabolized by cell extracts; 3-ketoglutarate was then identified by chromatography of its 2,4-dinitrophenylhydrazone.

Formation of methanol. There was no significant release of radioactivity as $^{14}\text{CO}_2$ when 3,4-[methoxyl- ^{14}C]dihydroxy-5-methoxyphenylacetic acid (labeled ring-fission substrate) was oxidized by cell suspensions (Table 2). The cells did not oxidize [^{14}C]methanol, and it was assumed that, as in previous investigations (3, 4), two adjacent methoxyl groups of the growth substrate were oxidized and the one remaining group gave rise to methanol. This was confirmed by using the isotope dilution method (4) to determine the amount of methanol formed when a cell suspension of *Arthrobacter* sp. TMP oxidized $6 \mu\text{mol}$ of TMPA to completion in the Warburg respirometer. When uptake of O_2 had ceased, $6 \mu\text{mol}$ of trideuteromethanol (CD_3OH) was added; analysis with the mass spectrometer (4) showed formation of $6.2 \mu\text{mol}$ of unlabeled methanol (CH_3OH). In a control experiment with $6 \mu\text{mol}$ of pyruvate, $0.75 \mu\text{mol}$ of methanol was found. Thus, $5.45 \mu\text{mol}$ of methanol was formed by the oxidation of $6 \mu\text{mol}$ of TMCA, corresponding to $0.91 \mu\text{mol}$ of methanol per mol of TMCA, in good agreement with the degradative pathway shown in Fig. 1.

DISCUSSION

The only aromatic acid tested that was oxidized by cell suspension of *Arthrobacter* sp. TMP at a rate comparable to that of the growth substrate, TMPA, was 3,4-dihydroxy-5-methoxyphenylacetic acid (Fig. 1, II). The latter com-

pound was substrate for a dioxygenase which did not attack, but was inhibited by, 3,4,5-trihydroxyphenylacetic acid. This ring-fission substrate is the methylene analog of 3-*O*-methylgallate which serves a similar function in the degradation of 3,4,5-trimethoxybenzoic acid (3, 4), and we found that *Arthrobacter* sp. TMP also accumulated about 1 mol of methanol per mol of TMPA oxidized. It was not possible to show directly that two adjacent methoxyl groups were oxidized to hydroxyls since cell extracts showed no demethylase activity toward TMPA. At low concentrations, formaldehyde, an intermediate in oxidative demethylation (10), was oxidized to carbon dioxide by intact cells (Table 2); the radioactivity remaining when oxidation ceased was probably due to the presence of paraformaldehyde in the sample of [^{14}C]formaldehyde used.

The view that reactions of the type shown in Fig. 1 provide a significant source of methanol in the biosphere (3, 4) is supported by the observation that growth of *Arthrobacter* sp. TMP on TMPA strongly induced a decarboxylase for 3-ketoglutarate, the methylene analog of a metabolite of the TMBA pathway, oxaloacetate (3). The present study also supports the proposal that methanol is released during, or immediately after, ring fission: the methyl ester of 3-ketoglutarate was not hydrolyzed by cell extracts of *Arthrobacter* sp. TMP, whereas cell extracts of another species of *Arthrobacter* formed detectable amounts of the free acid from the ring-fission substrate.

A survey conducted in these laboratories 8 years ago by Van Buskirk (B.S. Honors thesis, University of Minnesota, Minneapolis-St. Paul, 1973) showed that many species of *Pseudomonas* and *Acinetobacter* grew readily with 3-ketoglutarate as the sole source of carbon. The ability of 3-ketoglutarate utilizers to grow with 4-hydroxyphenylacetic acid was also examined: 17 soil isolates used the homogentisate pathway (7) and 5 used the homoprotocatechuate pathway (12). 3-Ketoglutarate participates in neither sequence, although its decarboxylation product, acetoacetate, is a catabolite of homogentisate. No evidence was obtained that 3-ketoglutarate is a degradation product of homoprotocatechuate, as was formerly reported for a single strain of *Pseudomonas* sp. that is no longer available (2); it appears likely, therefore, that the main significance of 3-ketoglutarate in aromatic degradation is that of a catabolite of TMPA. Van Buskirk did not examine strains for ability to grow with TMPA; however, none of the utilizers of 3-ketoglutarate were species of *Arthrobacter*, and we isolated no pseudomonads that grew with TMPA.

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