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Degradation of Substituted Mandelic Acids by *meta* Fission Reactions

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A strain of *Acinetobacter lwoffii* degraded 4-hydroxymandelic and 4-hydroxy-3-methoxymandelic acids to their corresponding benzoates, which were then hydroxylated by specific monooxygenases to yield, respectively, protocatechuic and 3-*O*-methylgallic acids; these were substrates for *meta* fission dioxygenases. The product formed from 3-*O*-methylgallate underwent slow spontaneous cyclization at pH 7 to release methanol.

Mandelic acid (phenylglycollic acid) is a naturally occurring compound which served as the growth substrate in the first investigations that used rates of respiration for delineating pathways of bacterial aromatic catabolism (14). *Pseudomonas putida* converts this substrate into benzaldehyde by successive dehydrogenation and decarboxylation reactions, and benzaldehyde is then oxidized to benzoic acid by one of two dehydrogenases requiring either NAD or NADP. Together with a racemase that enables some strains to utilize D-mandelate (10), these four enzymes form a group which is the product of one operon (5–7) and which can also accept 4-hydroxymandelate (HM) as a substrate, yielding 4-hydroxybenzoate (15). These studies with pseudomonads were extended to the bacterium NCIB 8250 (a member of the *Acinetobacter-Moraxella* group [4]), and 4-hydroxy-3-methoxymandelic acid was included in the list of substrates investigated. Kennedy and Fewson (8, 9) proposed catabolic pathway A of Fig. 1 for this compound, which is also of clinical interest, often being referred to as vanilmandelic acid (VM): its D isomer is a metabolite of epinephrine and norepinephrine, and under certain pathological conditions it is excreted in abnormal amounts in urine. It was this consideration that influenced our choice of soil from a St. Paul cattle yard for elective culturing of an organism that grew with DL-VM as the sole carbon source. This isolate was identified in the laboratories of the American Type Culture Collection, Rockville, Md., as a strain of *Acinetobacter lwoffii*. The organism also grew on DL-HM, but benzoic acid and the isomers of mandelic acid were not growth substrates; methanol was not metabolized. Amounts of VM and HM present in cultures during growth were measured by means of high-pressure liquid chromatography, and both isomers of each substrate were found to be fully utilized.

For this study, *A. lwoffii* was grown at 30°C in shake flasks containing 0.2% NH_4Cl, 0.005% Casamino Acids, 0.005% yeast extract, 0.01% $MgSO_4$, and 0.05% carbon substrate in 0.5 M sodium-potassium phosphate buffer (pH 7). The nuclear magnetic resonance spectrometer and the instruments and procedures used for high-pressure liquid chromatography and thin-layer chromatography were as previously described, as was the preparation of [*methoxyl*-^{13}C]3-*O*-methylgallate (2, 11, 12).

Enzyme assays of cell preparations (5, 9) indicated that most enzymes of the "mandelate group" of rather nonspecific enzymes (10) were present in this bacterium. A "particulate" dehydrogenase and a soluble dehydrogenase for L-(+)-mandelate and D-(−)-mandelate, respectively, attacked only one-half of DL-VM or DL-HM. The ring fission dioxygenases of cell extracts autoinactivated rapidly under assay conditions, but active NAD-dependent dehydrogenases for 4-hydroxybenzaldehyde or vanillin (Fig. 1, compound III) produced spectrophotometrically determined stoichiometric yields of 4-hydroxybenzoic acid or vanillic acid (Fig. 1, compound IV). Compound II (Fig. 1) was not available, but soluble cell extracts contained a benzoylformate decarboxylase that was stimulated severalfold by additions of thiamine PP_i.

The foregoing experiments show that *A. lwoffii* closely resembles previously investigated species in possessing a mandelate group of enzymes, converting VM and HM into their substituted benzoates. However, at this point pathways diverge as a consequence of differences in the substrate specificities of oxygenases. *A. lwoffii* did not elaborate any oxygenases for benzoate: this is the probable reason for the lack of growth on mandelate. *A. lwoffii* also did not possess the *O*-demethylase (enzyme 4) for vanillate that would make pathway A in Fig. 1 available (9). Instead, VM-grown cells used pathway B, initiated by an NADPH-dependent hydroxylase (enzyme 6), yielding 3-*O*-methylgallic acid (compound V), while HM-grown cells used another NADPH-dependent hydroxylase that oxidized 4-hydroxybenzoate to protocatechuate. The enzymes were substrate specific: thus, the first monooxygenase did not attack 4-hydroxybenzoate, and the second did not attack vanillate. None of the enzymes in Fig. 1 were detected in succinate-grown cells. 3-*O*-Methylgallic acid (compound V) is the substrate of an unstable dioxygenase (enzyme 7) which is also present in other bacterial species after growth with various methoxyl group-substituted benzoates that are formed when lignin is degraded (2, 3). It has been found that in all reaction sequences involving 3-*O*-methylgallate (compound V), a *meta* fission pathway invariably follows ring opening (11, 12), but the dioxygenase itself has not been characterized because it is so unstable and is usually accompanied by a very active hydrolase that removes the ring fission product, compound VI (3). However, when *A. lwoffii* was grown with HM, cell extracts at pH 8 were found to produce a yellow color (maximum wavelength, 408 nm in alkali) with protocatechuate; the color was bleached upon the addition of ammonium ions. This enzyme was also unstable, but the observed properties

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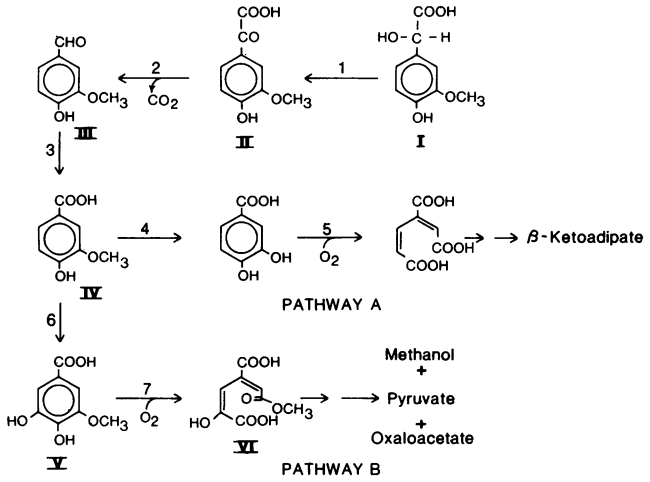

FIG. 1. Degradation of VM (compound I) to yield vanillic acid (compound IV). Organisms previously investigated convert vanillate to protocatechuate by an *O*-demethylase (enzyme 4), and degradation then proceeds by *ortho* fission (pathway A). *A. lwoffii* hydroxylates (by enzyme 6) vanillate to yield 3-*O*-methylgallate (compound V), and subsequent reactions proceed by *meta* fission (pathway B).

clearly indicate *meta* fission. There have been many studies of the catabolism of mandelic acid over the past 40 years, and in every case except the present, *ortho* fission rather than *meta* fission of the benzene nucleus has been either demonstrated or assumed to take place.

In view of the difficulties attending work with cell extracts, we decided to use suspensions of intact cells of VM-grown *A. lwoffii* in subsequent studies of reactions involving 3-*O*-methylgallate. Formation of this compound from VM was shown by inhibiting ring fission with 2,2′-bipyridyl (1). VM-grown cells (0.33 g, wet weight) were shaken with 31 mg of inhibitor in 20 ml of phosphate buffer (pH 7) at 30°C for 45 min. Cells were then removed by centrifugation, 2,2′-bipyridyl was removed by ethyl acetate extraction, and reaction products were extracted into ethyl acetate after acidification of the aqueous solution. Analysis of this extract by thin-layer chromatography and gas chromatography-mass spectrometry showed that the substrate had been converted into 3-*O*-methylgallic acid plus a smaller quantity of vanillic acid.

The metabolism of [*methoxyl*-^{13}C]3-*O*-methylgallate by dense suspensions of VM-grown *A. lwoffii* was monitored by nuclear magnetic resonance spectroscopy essentially by the procedure of Walker et al. (16). Oxygen was bubbled through 1.5 g (wet weight) of cells suspended in phosphate buffer (pH 7) in a final volume of 3 ml containing 11 mg of ^{13}C-labeled substrate and 25% D_2O to provide a lock signal. Chemical shifts were expressed relative to that of methanol, for which a value of 49.9 ppm was assumed (13). The initial peak at 57.1 ppm from the [^{13}C]methoxyl group of the substrate disappeared after 40 min and was replaced by two peaks of approximately the same height at 53.0 and 49.9 ppm (methanol). This change was not observed when no O_2 was bubbled. The position of the peak at 53.0 ppm was that of a methyl ester such as compound VI; thus, peaks for the monomethyl esters of succinate and fumarate were observed, respectively, at 53.2 and 53.6 ppm. The measured coupling constant for the quartet arising from the 53.0-ppm peak was 148.3 Hz, also in agreement with those for the two monomethyl esters, namely, 148.0 and 148.8 Hz. Although compound VI (Fig. 1) has been proposed as a catabolite in experiments with *P. putida* TM (2), it appeared to be rapidly hydrolyzed and therefore did not accumulate. We confirmed these observations by using dense suspensions of 3,4,5-trimethoxybenzoate-grown *P. putida* TM instead of *A. lwoffii*. The substrate peak at 57.1 ppm diminished and then disappeared, to be replaced by the single peak of methanol; no intermediate at 53.0 ppm was detected.

Compound VI was shown to undergo spontaneous cyclization to 2-pyrone-4,6-dicarboxylic acid (Fig. 2, compound VII) with the release of methanol. A cell suspension of *A. lwoffii* used in the foregoing experiment was centrifuged, and the clear supernatant was passed through an Amicon PM-10 filter (excluding molecular weights below 10,000) to remove any extracellular enzyme that might catalyze the reaction. The peak from compound VI in the solution slowly disappeared (half-life, approximately 70 min) with the formation of [^{13}C]methanol. No other peak was observed, but the UV spectrum of the solution exhibited the characteristics of 2-pyrone-4,6-dicarboxylate, and the presence of this compound was confirmed by thin-layer chromatography (12). Extracts of VM-grown cells also contained a hydrolase for the pyrone (12); this enzyme was absent when succinate was the growth substrate. While spontaneous cyclization of compound VI can evidently take place, it was not possible to determine its significance under normal conditions of growth. At pH 7 the nonenzymatic process was slow, but upon the addition of cell extract, the peak at 53.0 ppm diminished much more rapidly and the peak from methanol simultaneously increased. These observations suggest that the alternative hydrolytic route involving an esterase (Fig. 2), as found in *P. putida* TM (2, 3), is also used by growing cultures of *A. lwoffii*. However, while precise rates were not recorded, it was observed that the nonenzymatic reaction was greatly accelerated when the pH was raised to 8 or more. This effect is explained by the mechanism suggested in Fig. 2, in which cyclization is initiated by the attack of an enolate oxygen on the ester carbonyl group. It therefore remains a possibility that this nonenzymatic process may contribute to the elimination of methanol and halides when various organisms grow at the expense of substituted protocatechuates (11).

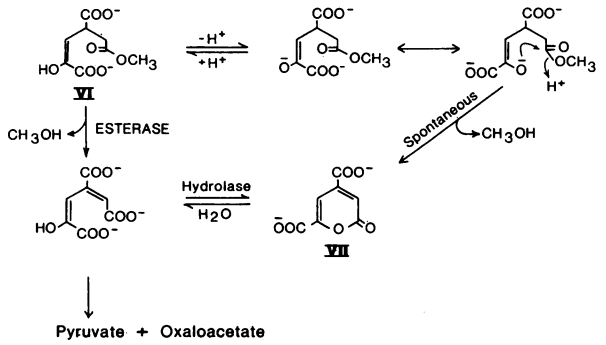

FIG. 2. Degradation of the ring fission product (compound VI) of 3-*O*-methylgallate (Fig. 1, compound V). The enzymatic *meta* fission route is initiated by an esterase that releases methanol. An alternative possibility involves spontaneous cyclization of the enolate of compound VI with the release of methanol. Cells contain a hydrolase for 2-pyrone-4,6-dicarboxylic acid (compound VII).

LITERATURE CITED

1. **Dagley, S., and P. J. Chapman.** 1971. Evaluation of methods used to determine metabolic pathways, p. 217–268. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 6A. Academic Press, Inc., New York.
2. **Donnelly, M. I., and S. Dagley.** 1980. Production of methanol from aromatic acids by *Pseudomonas putida*. J. Bacteriol. **142:**916–924.
3. **Donnelly, M. I., and S. Dagley.** 1981. Bacterial degradation of 3,4,5-trimethoxycinnamic acid with production of methanol. J. Bacteriol. **147:**471–476.
4. **Fewson, C. A.** 1967. The identity of the gram-negative bacterium NCIB 8250 (vibrio O1). J. Gen. Microbiol. **48:**107–110.
5. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway of *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. J. Bacteriol. **91:**1140–1154.
6. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. II. Isolation and properties of blocked mutants. J. Bacteriol. **91:**1155–1160.
7. **Hegeman, G. D.** 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. III. Isolation and properties of constitutive mutants. J. Bacteriol. **91:**1161–1167.
8. **Kennedy, S. I. T., and C. A. Fewson.** 1968. Metabolism of mandelate and related compounds by bacterium NCIB 8250. J. Gen. Microbiol. **53:**259–273.
9. **Kennedy, S. I. T., and C. A. Fewson.** 1968. Enzymes of the mandelate pathway in bacterium NCIB 8250. Biochem. J. **107:**497–505.
10. **Kenyon, G. L., and G. D. Hegeman.** 1979. Mandelate racemase. Adv. Enzymol. Relat. Areas Mol. Biol. **50:**325–360.
11. **Kersten, P. J., P. J. Chapman, and S. Dagley.** 1985. Enzymatic release of halogens or methanol from some substituted protocatechuates. J. Bacteriol. **162:**693–697.
12. **Kersten, P. J., S. Dagley, J. W. Whittaker, D. M. Anciero, and J. D. Lipscomb.** 1982. 2-Pyrone-4,6-dicarboxylic acid, a catabolite of gallic acids in *Pseudomonas* species. J. Bacteriol. **152:**1154–1162.
13. **Levy, G. C., R. L. Lichter, and G. L. Nelson.** 1980. Carbon-13 nuclear magnetic resonance spectroscopy, 2nd ed. John Wiley & Sons, Inc., New York.
14. **Stanier, R. Y.** 1947. Simultaneous adaptation: a new technique for the study of metabolic pathways. J. Bacteriol. **54:**339–348.
15. **Stevenson, I. L., and J. Mandelstam.** 1965. Induction and multisensitive end-product repression in two converging pathways degrading aromatic substances in *Pseudomonas fluorescens*. Biochem. J. **96:**354–362.
16. **Walker, T. E., C. H. Han, V. H. Kollman, R. E. London, and N. A. Matwiyoff.** 1982. ^{13}C nuclear magnetic resonance studies of the biosynthesis by *Microbacterium ammoniaphilum* of L-glutamate selectively enriched with carbon-13. J. Biol. Chem. **257:**1189–1195.