Catabolism of 2,4,5-Trimethoxybenzoic Acid and 3-Methoxycrotonic Acid

YAO-LING T. LEE, VELTA L. SPARNINS, AND STANLEY DAGLEY*

Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108

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4-Methoxygentisic acid was an intermediate formed when Arthrobacter degraded 2,4,5-trimethoxybenzoic acid. Isolates of Pseudomonas and Arthrobacter from soil grew at the expense of 3-methoxycrotonic acid. Evidence is presented that enzymatic hydration, with elimination of methanol, accounted for replacement of the methoxyl group of 3-methoxycrotonic acid and also of one methoxyl group of 2,4,5-trimethoxybenzoic acid.

Many aromatic natural products contain methoxyl groups which, in the course of degradation, are oxidized to hydroxyl groups by bacterial O-demethylases as follows (1, 5):

\[ \text{O}_2 + 2 \text{[H]} + \text{aromatic CH}_3 \rightarrow \text{H}_2\text{O} + [\text{aromatic CH}_3\text{OH}] \rightarrow \text{aromatic OH} + \text{HCHO} \]

A different sequence for demethylation has emerged from studies of the bacterial catabolism of 3,5-dimethoxy-4-hydroxybenzoate (syringate). Of the two methoxyl groups, only one was converted to hydroxyl; and it appeared that after degradation, the other was retained in the monomethyl ester of oxalacetic acid, from which it was later released as methanol by hydrolysis (7, 8). For organisms able to utilize methanol this sequence yields, overall, more energy than when degradation is initiated by attack upon both of the methyl groups by monoxygenases, since each consumes reducing equivalents. A third demethylating process, involving hydration, is suggested by the work of Chapman (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q90, p. 276), who demonstrated conversion of 3-hydroxy-4-methoxytoluene, and also isovanillic acid, into 4-methoxyn gentisic acid, which was then degraded to pyruvic acid plus methoxymaleic acid. For the last-named compound, a feasible reaction for further metabolism would involve enzymatic hydration to give oxalacetic acid and methanol, but this has not yet been established.

Our present studies indicate that 4-methoxyn gentisic acid (Fig. 1, compound II) and methoxymaleic acid (IV) are also metabolites of a species of Arthrobacter, isolated from soil by enrichment with 2,4,5-trimethoxybenzoic acid (I) and grown for these experiments in mineral salts media supplying this compound (0.05%) as the sole source of carbon. Figure 1a adopts a feature from the catabolism of syringate (7, 8), insofar as preliminary oxidative attack is restricted to specific methoxyl groups of the growth substrate. Thus, we propose that only two of three such groups in compound I are attacked so that, upon oxidation, they provide the hydroxyls necessary for ring fission of 4-methoxyn gentisate (II). Supporting observations were as follows. (i) In the presence of 5 mM \( \text{a,a'-bipyridyl} \), cell suspensions oxidized I with accumulation of II. The procedures for respirometry of treated cells, continuous ether extraction of the product, and thin-layer chromatography were essentially as described by Hopper and Chapman (3). The mass spectrum of the trimethylsilyl derivative (6) of the isolated product was identical to that of the derivative of authentic 4-methoxyn gentisic acid. (ii) In respirometry experiments, cell extracts oxidized II with an uptake of 1 mol of \( \text{O}_2 \) per mol, giving III which showed a spectrum having a single peak at 317 nm (pH 13), abolished on acidification: similar behavior is shown by solutions of maleylpyruvic acid (4), of which III is a methoxyl-substituted analog. (iii) Extracts also oxidized gentisic acid, but at only 56% of the rate for II; 4-methylgentisate was oxidized (rate, 40%), but 4-hydroxygentisate was scarcely attacked. It is therefore unlikely that all three of the methoxyl groups of I were demethylated before ring fission, since 4-hydroxygentisate would then be given. (iv) Pyruvate was determined using lactate dehydrogenase (6) immediately after disappearance of the light absorption due to III, formed transiently when cell extracts oxidized II; approximately 1 mol of pyruvate was given per mol of substrate.

After the hydrolytic release of pyruvate, it is proposed in Fig. 1a that methoxymaleic acid (IV) is hydrated. It was possible to show the formation of both methanol and oxalacetae.
Metabolic pathways proposed for degradation of (a) 2,4,5-trimethoxybenzoic acid and (b) 3-methoxycrotonic acid.

(V) when 4-methoxygentisate (II) was incubated with cell extracts for various intervals of time. Methanol was determined colorimetrically using chromotropic acid (2), and V was determined by means of malate dehydrogenase (8), but good stoichiometry was not observed. Extracts were not free from oxaloacetate decarboxylase activity, whereas the hydratase for IV appeared to be somewhat labile. We therefore sought additional evidence in another system for methanol elimination coupled with dehydration. The methyl ether of acetoacetic acid (3-methoxycrotonic acid; Fig. 1, VI) was obtained by saponifying ethyl 3-methoxycrotonate, synthesized from ethylacetoacetate and trimethoxyorthoformate (H. Brinkhoff, British patent 1,137,466, 1968); the free acid (VI) was then precipitated from solution on acidification with concentrated HCl. To the best of our knowledge, compound VI is not a natural metabolite; however, it supported growth of 10 strains (Pseudomonas and Arthrobacter) from our laboratory collection which had been isolated from soil originally by enrichment with other compounds. In all cases, growth was relatively slow at 30°C in 100 ml of shaken mineral salts medium (6) with 0.05% compound VI: the stationary phase was reached in 4 days, whereas under similar conditions Arthrobacter grew overnight at the expense of compound I. Catabolism of VI is formulated as shown in Fig. 1b, based on the following observations. The slow initial reaction appears to be catalyzed by an enzyme that normally hydrates some other substrate. Thus, although several different species grew slowly with VI, its rate of oxidation remained low (Fig. 2a), whereas, by contrast, acetoacetate was attacked rapidly. Acetate-grown cells oxidized both acetoacetate and VI slowly (Fig. 2b). This pattern of adaptation suggests that acetoacetate, a compound often encountered in bacterial metabolism, was formed in cells exposed to the novel, synthetic compound, VI. Enzymes for catabolizing acetoacetate were then induced. That the low rate of
oxidation of VI was due solely to low permeability of cells is unlikely, since attack on this substrate by cell extracts was barely detectable.

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LITERATURE CITED


