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Degradation of Protocatechuate in *Pseudomonas testosteroni* by a Pathway Involving Oxidation of the Product of *Meta*-Fission

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In addition to catalyzing the hydrolysis of 4-carboxy-2-hydroxymuconic semialdehyde, formed by *meta*-fission of protocatechuate, *Pseudomonas testosteroni* also possesses a nicotinamide adenine dinucleotide(phosphate)-linked dehydrogenase for this compound and can degrade protocatechuate to pyruvate and oxaloacetate.

Reactions by which protocatechuate (I, Fig. 1) is degraded in *Pseudomonas testosteroni* by *meta*-fission involve hydrolysis of the ring-fission product (II), hydration to give 4-hydroxy-4-methyl-2-oxoglutarate (III), and an aldol fission yielding pyruvate (4). Recently, it was shown that *Pseudomonas putida* degrades gallic acid (IV) by hydrating its ring-fission product (V) to 4-carboxy-4-hydroxy-2-oxoadipate (VI) which then gives oxaloacetate and pyruvate by aldol fission (8). Hegeman (5) showed that an early step in the degradation of protocatechuate by *Rhodopseudomonas palustris* involved a nicotinamide adenine dinucleotide phosphate (NADP)-linked oxidation of the ring-fission product. We now summarize evidence which was obtained for the strain of *P. testosteroni* used previously (4) and which shows that a metabolic pathway, involving the oxidation of II to V (Fig. 1), exists as an alternative to hydrolytic fission of formate from II. These results may be compared with the finding that *P. putida* possesses enzymes that catalyze both hydrolytic and oxidative pathways for *meta*-cleavage of catechols (2).

Intact cells, and also extracts, of *P. testosteroni* grown with *p*-hydroxybenzoate (4) oxidized gallate and protocatechuate with equal ease. It may be noted that protocatechuate 4,5-oxygenase has been reported as failing to attack 15 substrates related to protocatechuate in structure (6); however, gallate was not tested. In a typical experiment, 0.10 μmole of protocatechuate was oxidized in 1 min by crude extract (1 mg of protein) to give 0.09 μmole of ring-fission product, II, which possesses λ_{max} 410 nm at *p*H 7 (3, 4). This absorbance decreased slowly as II was hydrolyzed (4) but was rapidly abolished on adding 0.2 μmole of either NAD or NADP. Determinations of stoichiometry were hindered by rapid enzymatic reoxidation of the reduced NAD(P) formed in the reaction. Figure 2 shows that formate, a hydrolytic product of II, was not responsible for the reduction of NADP observed; neither was reoxidation due solely to the NADH oxidase activity of extracts. However, we have shown that one of the degradative products from protecatechuate is oxaloacetate; this metabolite could serve as electron acceptor for the oxidation of reduced NAD(P) [NAD(P)H] since a powerful malate dehydrogenase was found in crude extracts. It became possible to study the oxidation of II when J. M. Wood, University of Illinois, provided a preparation of protocatechuate 4,5-oxygenase which contained the dehydrogenase for II but was free from all of the subsequent enzymes of Fig. 1 and also from malate dehydrogenase. Accordingly, II accumulated when protocatechuate was incubated with this preparation; and on addition of NAD(P), II was converted completely into a compound having λ_{max} 310 nm, which was not further metabolized. The spectrum given was that for the ring-fission product of gallic acid which has been shown to have structure V (8). On addition of crude extracts, compound V was rapidly decomposed. These observations are consistent with Fig. 1, which shows a route for protocatechuate degradation, joining that for gallate through the NAD(P)-linked conversion of II to V.

In the experiment of Fig. 3 with a crude extract, 0.1 μmole of gallate gave rise to the

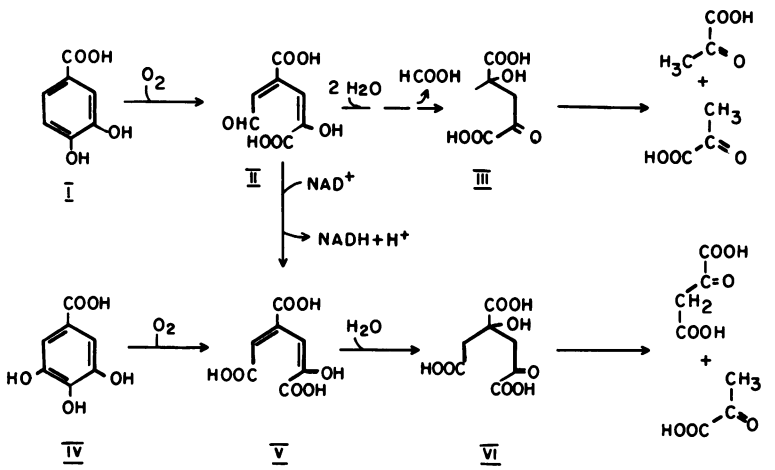

FIG. 1. *Pathways for the metabolism of protocatechuate (I) and gallate (IV) in P. testosteroni.*

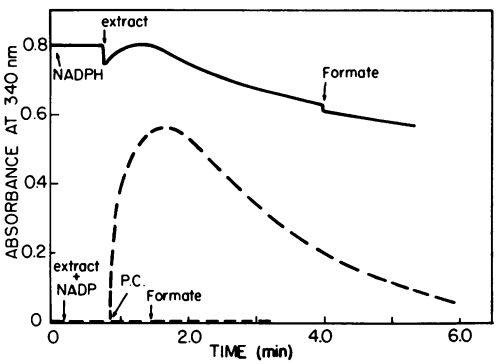

FIG. 2. *The protocatechuate-dependent reduction of NADP. Each of three cuvettes contained, in 3 ml of 0.05 M phosphate buffer (pH 7), 0.01 μmole of $FeSO_4$ and 2 mg of crude cell-extract protein. To one cuvette, successive additions of 0.4 μmole of NADPH, cell extract, and 0.2 μmole of formate were made (solid line). To the other two, the above-mentioned amounts of extract and formate were added, with 1 μmole of NADP, at times indicated. To one cuvette no further addition was made, and to the other was added 0.55 μmole of protocatechuate (PC).*

oxidation of 0.2 μmole of NADH, of which 0.115 μmole was accounted for by reduction of pyruvate on addition of lactate dehydrogenase (LDH). The remainder of the NADH oxidized is ascribed to the reduction of 0.085 μmole of oxaloacetate, catalyzed by endogenous malate dehydrogenase. No LDH could be detected in the extracts. In a similar experiment, the ring-fission product from 0.1 μmole of protocatechuate was oxidized completely by NADP, NADH was then added, and the first stage of oxidation was monitored. LDH was then added, and pyruvate was determined. The amounts of

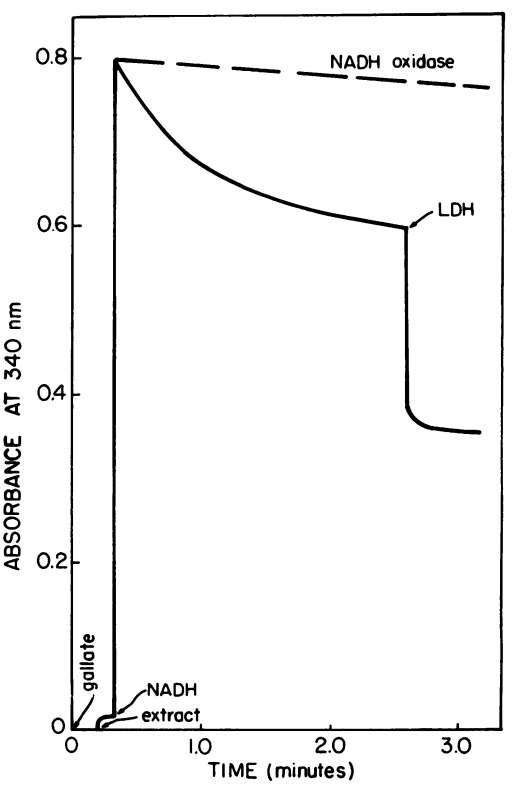

FIG. 3. *Oxidation of NADH by metabolic products from gallic acid. The cuvette (reaction volume 3 ml) contained 140 μmoles of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8, and the following were added at the times indicated: 0.1 μmole of gallate, 0.4 mg of crude extract protein, and 0.4 μmole of NADH. After 2.5 min, 125 units of lactate dehydrogenase was added.*

pyruvate and oxaloacetate, calculated from these changes in absorbance, were 80% of those expected. The end products shown in the scheme of Fig. 1 were identified as follows. Each of three reaction mixtures contained, in 12 ml of phosphate buffer, pH 7.4, 112 mg of cell extract protein and 60 μmoles of arsenite to inhibit metabolism of keto acids. To two of the mixtures were added 30 μmoles of protocatechuate and gallate, respectively; and to the third, 15 μmoles of protocatechuate with 15 μmoles of *p*-hydroxybenzoate plus 1.5 μmoles of NAD to provide for the continuous utilization of NADH, and regeneration of NAD, by the action of *p*-hydroxybenzoate hydroxylase present in the extracts. Reaction mixtures were shaken for 20 min at 30 C, protein was then precipitated, and supernatant fluids were examined for keto acids by thin-layer chromatography of their dinitrophenylhydrazones, which were also reduced to amino acids and chromatographed as described by Bayly and Dagley (1). All three reactions gave pyruvate, but oxaloacetate was formed only from gallate, or from protocatechuate continually supplied with NAD. Ribbons and Evans (7) have also shown that oxaloacetate can arise from protocatechuate.

An aldolase from *P. putida*, purified to homogeneity, accepts either III or VI as substrate (8). Both of these compounds were also readily cleaved by an aldolase partially purified from *P. testosteroni*, so that the same enzyme appears to serve both pathways.

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