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The Degradation of *trans*-Ferulic Acid by *Pseudomonas acidovorans**

Anne Toms and J. M. Wood

ABSTRACT: Washed cell suspensions of *Pseudomonas acidovorans*, grown with *trans*-ferulic acid as sole carbon source, oxidized vanillic acid, vanillin, protocatechuic acid, caffeic acid, and *cis*-ferulic acid at similar rates to *trans*-ferulic acid. Vanillic acid and vanillin were extracted from culture filtrates of *trans*-ferulic acid grown cells. Cell extracts were shown to convert [2-^{14}C]*trans*-ferulic acid into [2-^{14}C]acetate and vanillic acid only in the presence of nicotinamide–adenine dinucleotide.

Oxidized nicotinamide–adenine dinucleotide was required for the conversion of vanillin into vanillic acid *via* an oxidoreductase. Vanillic acid was oxidized to protocatechuic acid and formate by a monooxygenase requiring oxidized nicotinamide–adenine dinucleotide, reduced glutathione (GSH), ferrous ions, and formaldehyde. This mixture provided the reduced nicotinamide–adenine dinucleotide generating system required for mixed-function oxidation. Enzymes which convert formaldehyde into formate, requiring oxidized nicotinamide–adenine dinucleotide and reduced glutathione, and formaldehyde into methanol, requiring reduced nicotinamide–adenine dinucleotide, were separable by fractionation with ammonium sulfate. A reaction sequence for the complete degradation of *trans*-ferulic acid in which 3-methoxy-4-hydroxyphenyl-β-hydroxypropionic acid is an intermediate is proposed.

The microbial degradation of aromatic acids which contain the phenylpropane- (C_6–C_3) type structure may occur by (1) dihydroxylation of the benzene nucleus followed by ring fission leaving the side chain intact (Coulson and Evans, 1959; Dagley *et al.*, 1963, 1965; Blakely and Simpson, 1964; Seidman *et al.*, 1969); or (2) shortening of the side chain by a two-carbon fragment before ring fission (Webley *et al.*, 1962; Henderson, 1955; Cartwright and Smith, 1967).

trans-Ferulic acid is regarded as one of the simplest model compounds found in lignin (Siegel, 1954; Ishikawa and Takaichi, 1955). For example, α-conidendrin is believed to be synthesized by the condensation of dehydrogenated *trans*-ferulic acid with a quinone methide radical produced by dehydrogenation of a second molecule of coniferyl alcohol (Freudenberg and Geiger, 1963). When *Pseudomonas fluorescens* was grown with *trans*-ferulic acid as sole source of carbon, cell suspensions oxidized vanillin, vanillic acid, and protocatechuic acid in addition to the growth substrate (Cartwright and Smith,

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1967). Protocatechuic acid was isolated and characterized (by melting point) as a product of the action of a partially purified demethoxylating enzyme for vanillic acid (Cartwright and Buswell, 1967).

Details of the specificity of *trans*-ferulic acid degradation are scarce, and the chemical characterization of vanillin and vanillic acid as definite intermediates has thus far not been established. Cell-free metabolism of *trans*-ferulic acid has proved difficult owing to the instability of the first enzyme in this degradative sequence, coupled with the slow kinetics of the demethoxylating enzyme (Cartwright and Smith, 1967). In this communication we establish vanillin and vanillic acid as intermediates both chemically and enzymatically, examine the stereospecificity of the first enzyme, and present evidence for the mechanism of the release of [2-^{14}C]acetate from [2-^{14}C]-*trans*-ferulic acid.

Materials and Methods

A nonfluorescent pseudomonad was isolated from soil by elective culture with *p*-OH-*trans*-cinnamic acid as the carbon source. This organism was identified with the *Pseudomonas acidovorans* group (Stanier *et al.*, 1966). Cells grown with *p*-OH-*trans*-cinnamic acid, *trans*-ferulic acid, or *p*-OH-benzoic acid induced protocatechuate 4:5-oxygenase (Dagley *et al.*, 1968). Stock cultures were maintained on nutrient agar slants and subcultured every 2 weeks. Cells were grown at 30° with *trans*-ferulic acid by sequential inoculation from 50 ml to 1 to 16 l. The medium consisted of 0.7 g/l. of *trans*-ferulic acid, 2.0 g/l. of KH_2PO_4, 1.0 g/l. of $(NH_4)_2SO_4$, 50 mg/l. of $MgSO_4$, and 10 mg/l. of $FeSO_4$ adjusted to pH 7.2 with 5 N NaOH. *Ps. acidovorans* was harvested in the late exponential growth phase (16 hr) in a Sharples air-driven centrifuge. The cell paste was washed with 0.05 M Tris buffer (pH 7.2) prior to disruption. For cells grown with vanillin (0.7 g/l.) growth conditions were identical with those for *trans*-ferulic acid.

The determination of [^{14}C]CO_2 from [2-^{14}C]*trans*-ferulic acid by washed cell suspensions was accomplished by the gas chromatographic technique of Wood *et al.* (1965).

Materials. cis-Ferulic acid was synthesized by ultraviolet irradiation of *trans*-ferulic acid by an adaptation of the method of Noyce and Avarbock (1962). The latter authors synthesized *cis-p*-methoxycinnamic acid from *trans-p*-methoxycinnamic acid. *trans*-Ferulic acid (3.0 g) was dissolved in 400 ml of 0.2 M Na_2CO_3 and irradiated with a 450-W ultraviolet lamp, under N_2, for 24 hr. After acidification to pH 2.0 with 10 N H_2SO_4, *cis*- and residual *trans*-ferulic acids were extracted into diethyl ether. Careful evaporation of the ether layer caused crystallization of *trans*-ferulic acid. When the volume was reduced to 2.0 ml, *cis*-ferulic acid remained as a yellow oil. This oil (40 mg) was dissolved in deuterated acetone and examined for purity by nuclear magnetic resonance. Integration of the methoxyl peaks of the nuclear magnetic resonance spectrum for *cis*- and *trans*-ferulic acids present in this oil showed that a mixture consisting of 79% *cis* contaminated with 21% *trans* was the result of this synthesis. δ Values for *cis* were OCH_3, 3.87; H on C_3, 5.83; H on C_2, 6.90. Whereas δ values for *trans* were OCH_3, 3.95; H on C_3, 6.38; H on C_2, 7.67.

[2-^{14}C]*trans*-Ferulic acid (0.164 mCi/mmole) was synthesized from vanillin and [2-^{14}C]malonic acid (0.198 mCi/mmole) by the method of Pearl and Beyer (1951), yield 79%.

Preparation of Cell Extracts. Cell paste was suspended in 0.05 M Tris buffer (pH 7.2) (1 g wet weight/2.0 ml of buffer) and subjected to the maximum frequency of a Branson sonic probe for 2 min at 0°. Cell walls and other debris were removed by centrifugation at 26,000*g* for 45 min. Extracts prepared in this way contained active protocatechuate 4:5-oxygenase, methanol dehydrogenase, and formaldehyde oxidoreductase activity. Early enzymes for the conversion of *trans*-ferulic acid through protocatechuate were denatured by the above procedure. The activities of these early enzymes could only be demonstrated by cell extracts prepared using a Hughes press without abrasive (Hughes, 1951). Methanol dehydrogenase was conveniently separated from formaldehyde oxidoreductase by fractionation with saturated $(NH_4)_2SO_4$. Methanol dehydrogenase precipitated between 50 and 70% saturation.

Analytical Methods. Oxygen uptake was used as a measure of aromatic oxidation. Experiments were conducted using conventional manometric techniques. Formaldehyde oxidoreductase and methanol dehydrogenase activities were assayed at 340 mμ on a Cary Model 14 recording spectrophotometer. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer using a scintillation fluid for aqueous samples (Bray, 1960). Infrared spectra were obtained with a Perkin–Elmer 137 spectrophotometer. Samples were mulled in Nujol and placed between NaCl disks. Melting points (uncorrected) were determined with a Fisher–Johns melting point apparatus. Nuclear magnetic resonance spectra were recorded in deuterated acetone on a Varian A 60. Thin-layer chromatography was conducted on Eastman chromagram sheets type K/30R using benzene–dioxane–acetic acid (45:12.5:2.5, v/v). Compounds concerned in *trans*-ferulic acid degradation were visualized under ultraviolet light and by spraying with a 2% solution of 2,6-dichloroquinone-4-chloroimide in ethanol.

Isolation of Intermediates. Vanillin and vanillic acid were isolated from culture filtrates of *trans*-ferulic acid grown cells. Culture filtrate (16 l.) was acidified with 20 ml of concentrated H_2SO_4 followed by hand extraction into 12 l. of ethyl acetate. Water was removed from this organic solvent by filtration through anhydrous sodium sulfate followed by evaporation to dryness. The residue was dissolved in 30 ml of chloroform and chloroform-insoluble material was dissolved in diethyl ether. The chloroform layer was shown to contain both vanillin and vanillic acid as adjudged by thin-layer chromatography (vanillin R_F = 0.58 and vanillic acid, R_F = 0.46). Vanillin was separated from vanillic acid by elution with 100% chloroform from a silica gel column (45 × 2 cm). Fractions of 20 ml were collected, and vanillin was eluted in fractions 21–30. Insufficient material was isolated for recrystallization; therefore use was made of the reaction of the aldehyde with 2,4-dinitrophenylhydrazine. 2,4-Dinitrophenylhydrazine (0.5 g) was dissolved in 2.0 ml of methanol and diluted to 10.0 ml with 2 N HCl. This reagent was added to the residue from fractions 21–30 followed by incubation at 30° for 1 hr. An orange-colored precipitate formed which was removed by centrifugation, dissolved in chloroform, and passed down a Bentonite–Celite column (80:20, w/w) (20 × 2 cm). Unreacted 2,4-dinitrophenylhydrazine adhered strongly to the column, but the neutral 2,4-dinitrophenylhydrazone derivative eluted and was recrystallized from chloroform. This derivative was dried over P_2O_5 for infrared spectrophotometry (mp 264° dec with no depression on admixture with the authentic 2,4-dinitrophenylhydrazone derivative of vanillin). Although the chloroform-

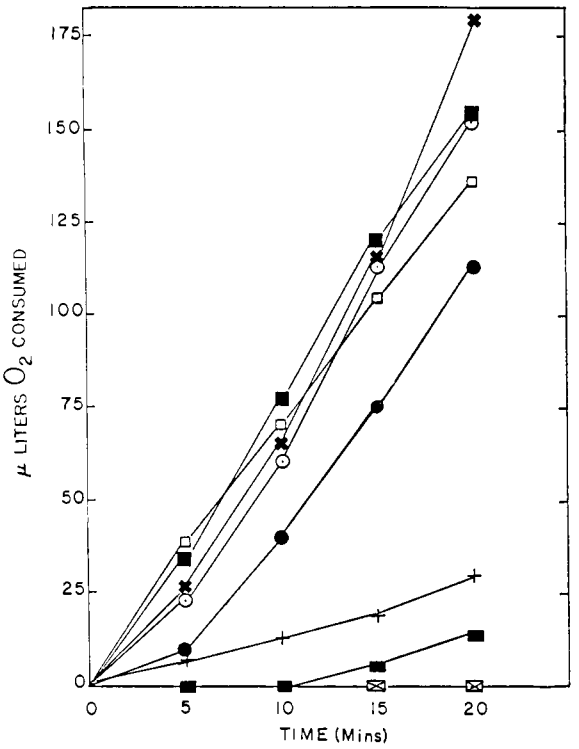


FIGURE 1: Oxidation of aromatic compounds by washed cell suspensions of *trans*-ferulate-grown *Ps. acidovorans*. Warburg flasks contained in a final volume of 3.0 ml: Tris buffer (pH 7.2), 90 μmoles; cell suspension 1.0 ml; and substrate, 5.0 μmoles. Results are corrected for endogenous respiration in the absence of substrate; *trans*-ferulic acid (×—×); vanillic acid (■—■); vanillin (⊙—⊙); caffeic acid (□—□); protocatechuic acid (●—●); 3,4-di-OH-β-phenylpropionic acid (+—+); homoprotocatechuic acid (▣—▣); catechol (×—×); and 4-methylcatechol (□□—□□).

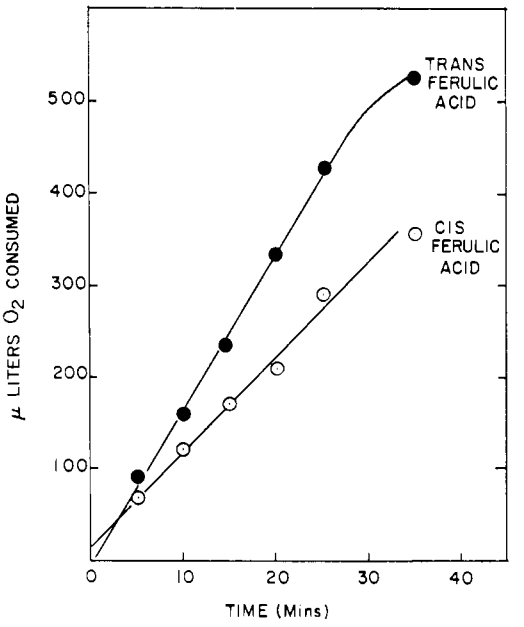


FIGURE 2: Oxidation of *cis*- and *trans*-ferulic acids by washed cell suspensions of *trans*-ferulate-grown *Ps. acidovorans*. Warburg flasks contained in a final volume of 3.0 ml: Tris buffer (pH 7.2), 90 μmoles; cell suspension, 1.0 ml; and substrate, 5.0 μmoles. Results are corrected for endogenous respiration.

soluble fraction of the culture filtrate extract contained traces of vanillic acid, the ether-soluble fraction contained the majority of this compound. Vanillic acid was eluted from a silica gel column (prepared in diethyl ether) with a mixture of 95% diethyl ether and 5% methanol. On evaporation of the ether methanol mixture a yield of 547 mg of vanillic acid was obtained (mp 210°, not depressed on admixture with authentic vanillic acid).

[2-^{14}C]Acetic acid was readily separated from reaction mixtures, which contained unreacted [2-^{14}C]*trans*-ferulic acid, by gel filtration in 0.01 N HCl on a Bio-Gel P-10 column (35 × 2 cm). Aliquots of column fractions were taken to determine radioactivity and unreacted [2-^{14}C]*trans*-ferulic acid was followed spectrophotometrically at 320 mμ. Calibration of this Bio-Gel P-10 column with [2-^{14}C]acetate and [2-^{14}C]*trans*-ferulic acid showed that acetate eluted well ahead of the aromatic compound.

[2-^{14}C]Malonic acid was obtained from Volk Radiochemical Co., Burbank, Calif. Silica gel (0.05–0.20 mm) was obtained from E. Merck AG, Darmstadt, Germany. Bio-Gel P-10 was obtained from Bio-Rad, Richmond, Calif. NAD^+, NADH, and GSH[1] were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of highest purity commercially available.

[1] Abbreviation used is: GSH, reduced glutathione.

Results

Experiments with Whole Cells. Washed cell suspensions of *Ps. acidovorans* grown with *trans*-ferulic acid as the source of carbon, oxidized vanillic acid, vanillin, protocatechuic acid, and caffeic acid at approximately the same rate as the growth substrate. Homoprotocatechuic acid and 3,4-$(OH)_2$-β-phenylpropionic acid were oxidized slowly and only after a sufficient lag to allow enzyme derepression. Catechol and 4-methylcatechol were not oxidized (Figure 1).

cis-Ferulic acid was oxidized by washed cell suspensions at 60% of the rate of the *trans* isomer. This reaction rate for *cis*-ferulic acid could not be attributed to contaminating *trans* isomer (21%), because a greater stoichiometry for O_2 consumption was observed than would be expected for 1 μmole of contaminating *trans* isomer (Figure 2).

After a lag of 20 min, washed cell suspensions catalyzed the rapid evolution of [^{14}C]CO_2 from [2-^{14}C]*trans*-ferulic acid. Addition of a tenfold excess of acetate to an identical reaction vessel caused dramatic dilution of [^{14}C]CO_2 liberated. These data suggest that C_2 of the *trans*-ferulic acid side chain enters C_2 metabolism in *Ps. acidovorans* (Figure 3).

When *Ps. acidovorans* was grown with vanillin as the source of carbon, washed cell suspensions oxidized vanillic acid and protocatechuic acid at equal rates to the growth substrate, but did not oxidize *trans*-ferulic acid. Clearly, the enzymes involved in the degradation of *trans*-ferulic acid to protocatechuic acid in *Ps. acidovorans* are not derepressed as a group as they are for shortening of the side chain of mandelic acid by *Pseudomonas putida* (Hegeman, 1966) (Figure 4).

Isolation of Intermediates from Culture Filtrates. Vanillic acid and vanillin (isolated as its 2,4-dinitrophenylhydrazone derivative) were isolated from culture filtrates of *trans*-ferulic acid grown cells. Complete characterization of these com-

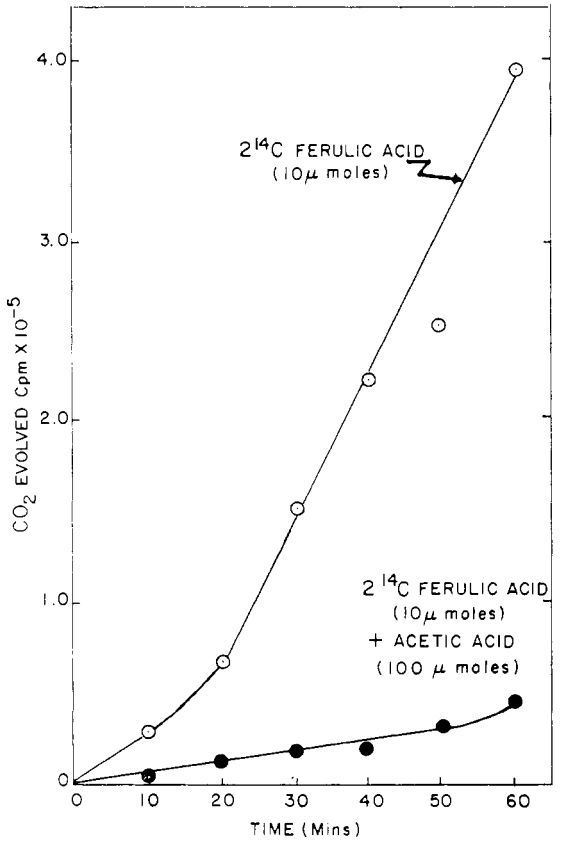


FIGURE 3: Evolution of [^{14}C]CO_2 from [2-^{14}C]*trans*-ferulic acid by washed cell suspensions of *trans*-ferulate-grown *Ps. acidovorans*. Warburg flasks were stoppered with serum caps, and 0.4-ml samples of gas were taken every 10 min. Flasks contained: Tris buffer (pH 8.0), 90 μmoles; cell suspension, 1.0 ml; and substrate where indicated.

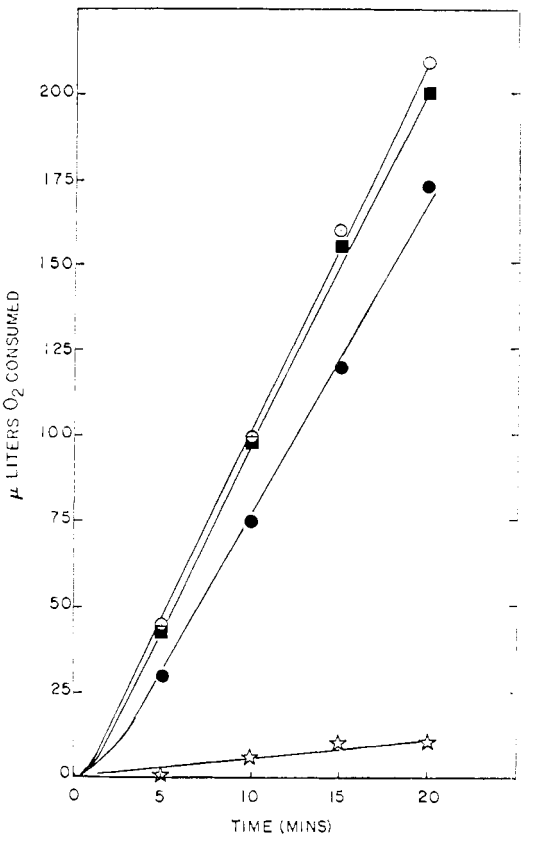


FIGURE 4: Oxidation of aromatic compounds by washed cell suspensions of vanillin-grown *Ps. acidovorans*. Warburg flasks contained in a final volume of 3.0 ml: Tris buffer (pH 7.2), 90 μmoles; cell suspension, 1.0 ml; and substrate, 5.0 μmoles. Results are corrected for endogenous respiration; vanillic acid (⊙—⊙); vanillin (■—■); protocatechuic acid (●—●); and *trans*-ferulic acid (—).

pounds was achieved by infrared spectrophotometry. Both compounds gave spectra identical with those of their respective authentic compounds in the range of 4000 to 650 cm^{-1}.

Experiments with Cell Extract. Crude cell extracts would not oxidize vanillic acid unless formaldehyde, NAD^+, Fe^{2+}, and GSH were added to the reaction mixture. These extracts contained formaldehyde oxidoreductase which would catalyze continuous production of NADH, which is required in the initial demethoxylating reaction. Each mole of vanillic acid consumed 2.0 moles of O_2; 1 mole for demethoxylation, and 1 mole for cleavage *via* protocatechuate 4:5-oxygenase.

Protocatechuate was rapidly oxidized with the consumption of 1 mole of O_2/mole of substrate (Figure 5) to give α-hydroxy-γ-carboxymuconic semialdehyde as the first aliphatic intermediate (λ_{max} 410 mμ in alkali) (Dagley *et al.*, 1968).

Both formaldehyde oxidoreductase and methanol dehydrogenase activities were present in crude cell extracts, but were difficult to demonstrate owing to dismutation; each mole of NADH generated by oxidoreductase provided cofactor for methanol dehydrogenase. These enzymes were conveniently separated by fractionation with ammonium sulfate (Figure 6). Formaldehyde oxidoreductase activity was demonstrated by running reactions in anaerobic cuvets in the presence of catalytic amounts of GSH. Formate dehydrogenase activity was not present in the above cell extracts, but was found to be associated with the membrane fraction as with other pseudomonads (Dagley and Gibson, 1965; Dagley *et al.*, 1968).

Crude cell extracts would only oxidize *trans*-ferulic acid or vanillin in the presence of catalytic amounts of NAD^+. Oxygen (1 mole) was required for the oxidation of 2 moles of either *trans*-ferulic acid or vanillin (Figure 7). These data show that the release of a C_2 fragment from *trans*-ferulic acid to give vanillin as an aromatic intermediate does not require any oxidation step.

The identity of the C_2 fragment released during the metabolism of *trans*-ferulic acid by cell extracts was determined in the following experiments. [2-^{14}C]*trans*-Ferulic acid (50.0 μmoles) was incubated in a total volume of 15.0 ml with crude cell extract (150.0 mg) and DPN^+ (1.0 μmole) at 30° for 70 min. After this time, protein was removed by precipitation with 5.0 ml of 5 N H_2SO_4. The precipitate was removed by centrifugation and the $^{14}C_2$ fragment plus unreacted [2-^{14}C]-*trans*-ferulic acid was extracted into 100 ml of diethyl ether for 18 hr using a Kutscher–Steudal liquid–liquid extraction apparatus. Acids were extracted from the ether layer into 10.0 ml of 0.05 N KOH, and the pH was adjusted to 7.0 with 6 N HCl prior to lyophilization. The residue was dissolved in 1.0 ml of water and applied to a Bio-Gel P-10 column which had been calibrated with [^{14}C]acetate and [^{14}C]ferulate (3.0-ml frac-

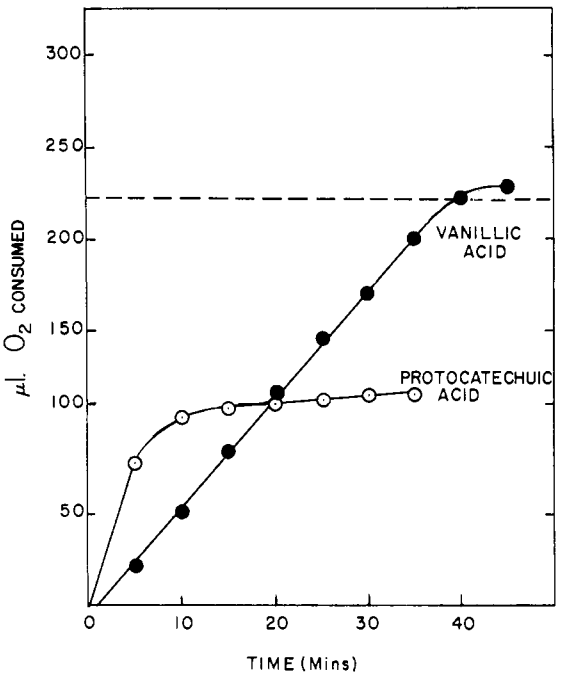


FIGURE 5: Oxidation of vanillic acid and protocatechuic acid by cell-free extracts of *trans*-ferulate-grown *Ps. acidovorans*. Warburg flasks contained in final volume of 3.0 ml: Tris buffer (pH 7.2), 90 μmoles; protein, 26.3 mg; and substrate, 5.0 μmoles (where indicated). No cofactors were required for protocatechuate oxidation. Vanillate oxidation required an NADH-generating system consisting of: formaldehyde, 5.0 μmoles; GSH, 10 μmoles; NAD^+, 1.0 μmole; and Fe^{2+}, 1.0 μmole. Each result was corrected for endogenous flasks which contained all additions except substrate.

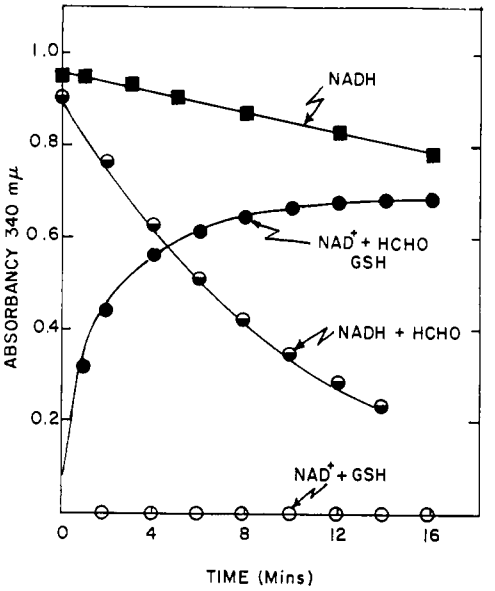


FIGURE 6: Spectrophotometric demonstration of formaldehyde oxidoreductase and methanol dehydrogenase activities by $(NH_4)_2SO_4$-fractionated extracts of *trans*-ferulate-grown *Ps. acidovorans*. For methanol dehydrogenase cuvets contained in a total volume of 3.0 ml: NADH, 0.3 μmole; formaldehyde, 5.0 μmoles; Tris buffer (pH 7.2), 127 μmoles; and protein, 1.8 mg. For formaldehyde oxidoreductase anaerobic cuvets contained in a total volume of 3.0 ml: NAD^+, 0.3 μmole; formaldehyde, 5.0 μmoles; GSH, 0.05 μmole; Tris buffer (pH 7.2), 120 μmoles; and protein, 1.1 mg. Reactions were started by the addition of NADH (methanol dehydrogenase) or NAD^+ (formaldehyde oxidoreductase).

tion). [^{14}C]Acetate was readily separated from [2-^{14}C]*trans*-ferulic acid under these conditions (Figure 8). Fractions 23–29 were pooled, adjusted to pH 7.0 with 6 N KOH, and lyophilized. Acetate was characterized by running two-dimensional cellulose chromatography sheets with ethanol–ammonia–water (20:1:4, v/v) and 2-propanol–ammonia–water (200:10:20). Acetate (as the ammonium salt) was detected by spraying with bromocresol purple, and by radioautography with Kodak No-Screen X-Ray film.

Discussion

The majority of research on the structure of lignin indicates that it is a polymer derived from the phenylpropanoid compound coniferyl alcohol, and the degradation of these polymers by molds and bacteria in the soil leads to the release of this alcohol and oxidation products such as *trans*-ferulic acid and vanillin (Freudenberg and Neish, 1968). In the biosynthesis of lignin there is good evidence from isotope studies that *trans*-ferulic acid is synthesized from the aromatic amino acids phenylalanine and tyrosine (El-Basyouni *et al.*, 1964). [2-^{14}C]*trans*-ferulic acid was shown to be subsequently reduced to coniferyl alcohol in vascular plants (Higuchi and Brown, 1963).

Studies on the bacterial degradation of *trans*-ferulic acid have been limited to manometric studies with washed cell suspensions and paper chromatographic analysis of products accumulating in culture filtrates. On the above basis, vanillin

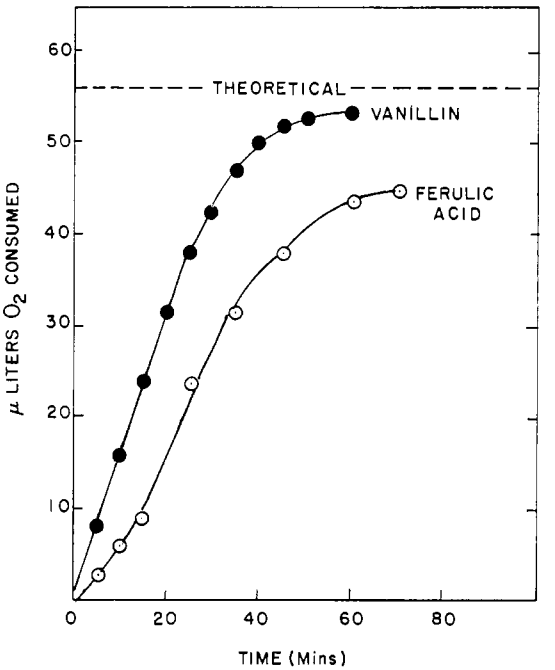
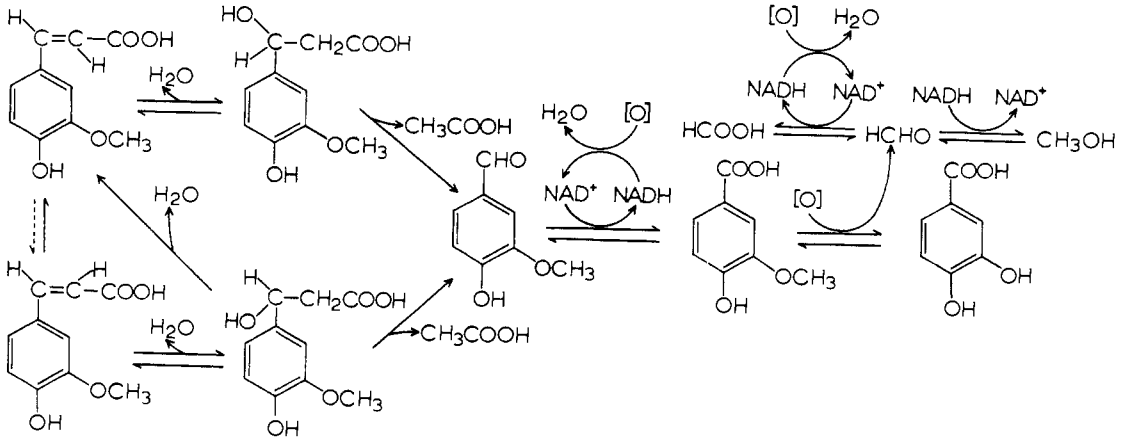


FIGURE 7: Oxidation of *trans*-ferulic acid and vanillin by cell-free extracts of *trans*-ferulate-grown cells of *Ps. acidovorans*. Warburg flasks contained in a final volume of 3.0 ml: Tris buffer, 90 μmoles; protein, 46.3 mg; NAD^+, 1.0 μmole; and substrate, 5.0 μmoles (where indicated). Reactions were corrected for endogenous oxygen consumption.

SCHEME I



and vanillic acid have been implicated as intermediates in *trans*-ferulic acid degradation in both yeasts (Henderson, 1955) and bacteria (Cartwright and Smith, 1967). Protocatechuic acid was isolated and identified (by mp) as the reaction product formed by the action of a partially purified demethoxylating enzyme for vanillic acid from *trans*-ferulate-grown *Ps. fluorescens* (Cartwright and Buswell, 1967).

Washed cell suspensions of *Ps. acidovorans* grown with *trans*-ferulate-oxidized *trans*-ferulic acid, vanillin, vanillic acid, protocatechuic acid, and caffeic acid without lag. Caffeic acid was not oxidized by cell extracts indicating that protocatechuate 4:5-oxygenase is specific in *Ps. acidovorans*. In *Ps. fluorescens* grown with *p*-OH-*trans*-cinnamic acid, caffeic acid 3:4-oxygenase was shown to cleave caffeic acid and protocatechuic acid at similar rates (Seidman *et al.*, 1969). However, when *Ps. fluorescens* was grown with *p*-OH-benzoate, specificity for protocatechuic acid *via* a 3:4-cleavage mechanism was observed; no activity toward caffeic acid could be demonstrated under these conditions. The oxidation of caffeic acid by washed cell suspensions of *Ps. acidovorans* may occur either as a result of the lack of specificity of the enzymes which remove acetate from the propanoid side chain, or by conversion of this catechol into *trans*-ferulic acid by [$^{14}CH_3$]-*S*-adenosylmethionine as in plants (Finkle and Nelson, 1963). However, methylation of caffeic acid in washed cell suspensions seems unlikely since a large pool of *S*-adenosylmethionine would have to be present in these cells to give such rapid and complete oxidation of this catechol. Confirmation that vanillin and vanillic acid (by infrared spectra and mp) are produced in cultures of *trans*-ferulate-grown *Ps. acidovorans* indicates that shortening of the propanoid side chain occurs.

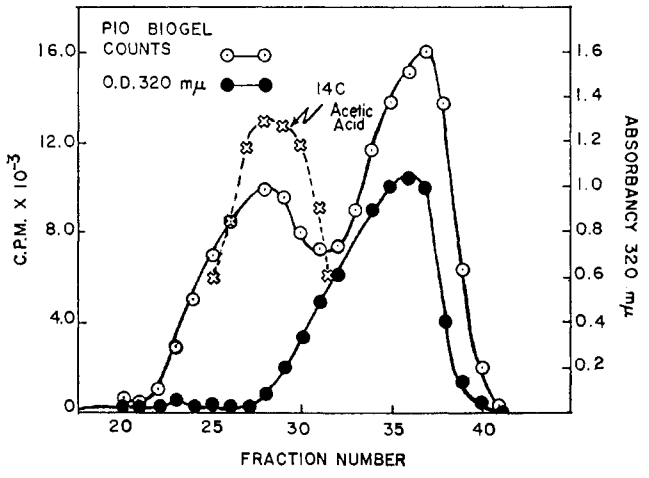


FIGURE 8: Separation of [^{14}C]acetic acid from the reaction products of [2-^{14}C]*trans*-ferulate oxidation by cell-free extracts of *Ps. acidovorans*.

Cell extracts consumed the same amount of oxygen when converting *trans*-ferulic acid or vanillin into vanillic acid. Therefore no oxidative step is involved in the conversion of *trans*-ferulic acid into vanillin plus acetate. A reaction scheme for the conversion of *trans*-ferulic acid into protocatechuic acid by *Ps. acidovorans* is proposed (Scheme I).

In this reaction sequence, hydration of the *trans* double bond to give 4-OH-3-OCH$_3$-β-OH-phenylpropionic acid as a transient intermediate, followed by aldolase cleavage to vanillin and acetate seems feasible. Numerous attempts were made to synthesize 4-OH-3-OCH$_3$-β-OH-phenylpropionic acid. 4-*O*-Acetyl-3-OCH$_3$-β-OH-phenylpropionic acid ethyl ester was successfully synthesized by a Reformatsky reaction with *O*-acetylvanillin and bromoacetic ethyl ester. However, all attempts to hydrolyze this ester to the free acid were unsuccessful because base attacks the phenolic hydrogen on the parahydroxy group forming a quinone and causing rapid dehydration back to *trans*-ferulic acid. Attempts to synthesize this compound by oxidation to the keto acid, hydrolyzes and reduction failed due to decarboxylation of the β-keto acid. Enzymatic synthesis is probably the only way to make this β-OH acid.

Rapid oxidation of both *cis*- and *trans*-ferulic acids indicates that cells either contain an isomerase, or that hydration of *cis* occurs to give an intermediate which is rapidly converted into *trans* (Noyce and Avarbock, 1962). A third possible explanation cannot be ruled out since aldolase may show a lack of specificity for the two isomers of the β-OH acid which would be formed on hydration of *cis*- or *trans*-ferulic acids. Dagley *et al.* (1968) have shown that *Ps. testosteroni* induces an aldolase which shows a lack of specificity for α-oxo-γ-hydroxy-γ-car-

boxyvaleric acid which is an intermediate in the degradation of protocatechuic acid *via* protocatechuate 4:5-oxygenase. Finally specificity may be maintained if a racemase similar to mandelate racemase was present.

The cofactor requirements for vanillic acid oxidation by cell-extracts of *trans*-ferulate-grown *Ps. acidovorans* are similar to those reported for the oxidation of this compound by *Ps. fluorescens*. In *Ps. acidovorans* NAD^+ was found to be a more effective cofactor than $NADP^+$, which was the cofactor of choice in *Ps. fluorescens* (Cartwright and Smith, 1967). Also, in *Ps. fluorescens* 2.5 moles of O_2 was consumed per mole of vanillic acid whereas in *Ps. acidovorans* 2.0 moles of O_2 was consumed per mole of this aromatic acid. This difference in stoichiometry can be explained on the basis of the lack of formate dehydrogenase activity in extracts of *Ps. acidovorans*. A second minor difference demonstrated in the metabolism of these two organisms is the apparent lack of methanol dehydrogenase in *Ps. fluorescens*. This discrepancy is difficult to reconcile in terms of the overall stoichiometry of demethoxylation of vanillic acid. In our hands 1 mole of O_2 is required by the mixed-function oxygenase for each mole of vanillic acid converted into protocatechuic acid, 1 atom being incorporated into formaldehyde and 1 atom being reduced to water with NADH. Cartwright and Smith (1967) suggest that 1 atom of oxygen is required for this demethoxylation reaction and that the second atom is consumed *via* formaldehyde oxidoreductase in the oxidation of formaldehyde to formic acid.

From the results of this study, we can conclude that *Ps. acidovorans* is capable of oxidizing *trans*-ferulic acid to small molecules common to basic metabolism. For every molecule of *trans*-ferulic acid oxidized, 1.5 moles of O_2 is consumed to give 1 mole each of protocatechuate, formate, and acetate. The further metabolism of protocatechuate by a 4:5-oxygenase mechanism would be expected to give rise to 2.0 moles of pyruvate, 1 mole of formate, with a further consumption of 1 mole of oxygen (Dagley *et al.*, 1968).

Acknowledgment

We thank Francis Engle for technical assistance.

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