Metabolism of Gallic Acid and Syringic Acid by *Pseudomonas putida*®

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**SUMMARY**

Cell-free extracts of *Pseudomonas putida*, grown with syringic acid as carbon source, catalyzed the oxidation of 1 mole of gallate (3,4,5-trihydroxybenzoate) by 1 mole of oxygen to give 2 moles of pyruvate and 1 mole of carbon dioxide. Oxaloacetate, formed as an intermediate in reactions, could be reduced quantitatively when NADH and sufficient malate dehydrogenase were added, despite the presence in extracts of oxaloacetate decarboxylase. With these conditions of assay it was shown that oxaloacetate was the precursor of half of the pyruvate formed from gallate. Similarly, when 1 mole of gallate was first oxidized by protocatechuolate 4,5-oxygenase purified from *Pseudomonas testosteroni* and was then degraded by cell-free extracts, 1 mole of oxaloacetate and 1 mole of pyruvate were again formed. Although gallate oxygenase proved too labile to isolate, it was concluded from these results that the enzyme gave the same ring fission product as that obtained by the action of protocatechuolate 4,5-oxygenase on gallate, namely 4-carboxy-2-hydroxy-cis,cis-muconic acid. This compound appeared to undergo enzymic hydration to give 4-carboxy-4-hydroxy-2-oxoacid which was then cleaved to oxaloacetate and pyruvate. Cell-free extracts oxidized 3-O-methylgallate, but protocatechuolate (3,4-dihydroxybenzoate), 5-carboxy-3,4-dihydroxy benzoate and 3,4-dihydroxy-5-methylbenzoate were not attacked. Syringic acid was oxidized only in the presence of substrate amounts of NADH, when 2 moles of oxygen were taken up and 1 mole of pyruvate appeared; additional pyruvate was formed by a much slower reaction. There was no requirement for added NADH when concentrated cell extracts were used to oxidize syringate. These and other observations indicate that the C-5 methoxyl group of syringate was oxidized by an O-demethylase. The benzene nucleus of the resulting 3-O-methylgallate was then cleaved between C-3 and C-4 to give, as final products, pyruvate and a compound presumed to be a monomethyl ester of oxaloacetic acid. The substrate specificity of syringate O-demethylase was studied in experiments with intact cells.

Gallic acid occurs in the free state in tea and many other plants (1) and is also encountered in the form of esters, notably in gallo-tannins (2). A biosynthetic pathway that involves the dehydrogenation of 5-dehydroshikimate has been proposed for gallic acid (3), but little information is available concerning its enzymatic degradation. Beveridge and Hugo (4) isolated α-ketoglutarate as its 2,4-dinitrophenylhydrazone from cultures of *Pseudomonas campestris* metabolizing gallic acid, and they suggested that this compound might be formed directly after fission of the benzene nucleus. However, no experiments were performed with cell-free extracts, and the possibility was not excluded that the α-ketoglutarate obtained might arise from the operation of the tricarboxylic acid cycle. Micro-organisms capable of utilizing gallic acid as their sole source of carbon for growth are not readily isolated (4); moreover, solutions of this substrate rapidly darken in color when shaken in air at neutral or alkaline pH. Accordingly, we isolated for use in this investigation a strain of *Pseudomonas putida* which oxidized gallic acid readily to completion after growth at the expense of syringic acid, namely the 3,5-dimethyl ether of gallic acid. Syringic acid is stable in aqueous solution and is also a natural product which has been extracted from certain lignins when exposed to the action of wood-rotting fungi (5).

**MATERIALS AND METHODS**

**Organism** The strain utilized in these experiments was isolated from soil in St. Paul, Minnesota by elective culture in media containing syringic acid. It formed gram-negative, motile rods and produced soluble yellow-green pigments during growth with syringate as source of carbon. When the organism was streaked out on the media of King et al. (6) a fluorescent pigment was observed on Medium B after 24 hours, whereas there was no significant pigmentation on Medium A. The organism grew at the expense of syringate, namely the 3,5-dimethyl ether of gallic acid. Syringic acid is stable in aqueous solution and is also a natural product which has been extracted from certain lignins when exposed to the action of wood-rotting fungi (5).

1 The tests used (7) were gelatin liquefaction (−), and growth: creatinine (+), hippurate (+), inositol (−), trehalose (−).
added, as sterile solutions, after cooling. Sodium syringate solutions were sterilized by Millipore filtration. Cultures were grown at 30°C.

**Preparation of Cell-free Extracts**—Cells were grown in batches of 40 liters in a fermentor provided with stirring and vigorous forced aeration. The growth medium was inoculated with two cultures, each of 50 ml, which had grown overnight in 250-ml Erlenmeyer flasks placed on a gyratory shaker. After growth in the fermentor for 14 hours, a second addition was made of 20 g of sodium syringate and the culture was harvested 4 hours later; yield, 70 g wet weight of cells. Cells were washed and then crushed without abrasive in the bacterial press of Hughes (8) and the precipitate removed by centrifuging. The clear supernatant, referred to as a heat-treated extract, still contained active enzymes that metabolized the ring fission product of gallic acid to oxalacetate and pyruvate. These enzymes, and also gallate oxidase, were effectively absent from crude extracts of cells grown with sucinate as carbon source.

**Chemicals**—Gallic acid, of analytical reagent grade, was from Mallinckrodt Chemical Works, St. Louis, and syringic acid was from Aldrich Chemical Company. 3-O-Methylgallic acid was prepared by acid-catalyzed condensation of pyruvic acid (12, 13); the compound had a melting point of 112-114° and showed the ultraviolet absorption characteristics reported by Reusch and Starkley (14). The 1,4-lactone of 4-carboxy-4-hydroxy-2-oxoadipic acid was prepared according to Martius and Reusch (15). After several recrystallizations from ethyl acetate-petroleum ether, the compound had a melting point of 171-173°, darkening at 168°, and its ultraviolet spectrum in ethanol closely resembled that of pyruvate alde, with λ max 237 shifted to λ max 274 on adding NaOH. Solutions of 4-hydroxy-4-methyl-2-oxoglutarate and 4-carboxy-4-hydroxy-2-oxoadipate were prepared from their lactones by mild alkaline hydrolyses with NaOH (13). 5-Carboxy-3,4-dihydroxybenzoic acid and 3,4-dihydroxy-5-methylbenzoate were the kind gifts of Dr. D. J. Hopper. Lactate dehydrogenase (EC 1.1.1.27; 5 mg of protein and 3,450 units per ml) and malate dehydrogenase (EC 1.1.1.37; 9 mg of protein and 11,100 units per ml) were from Calbiochem.

**Uptake of Oxygen**—Measurements of oxygen consumption were made either by Warburg respirometry with air as the gas phase (16) or by means of the oxygen electrode as described by Dagley et al. (9).

**Chromatography**—The procedures used to identify the 2,4-dinitrophenylhydrazone of pyruvate by means of thin layer and paper chromatography were those described by Dagley and Gibson (16).

**RESULTS**

**Oxidation of Substrates by Intact Cells**—Suspensions of intact cells, grown at the expense of syringic acid (II, Scheme 1), oxidized gallic acid (I), and 3-O-methylgallic acid (II) at almost equal rates, although the final uptake of oxygen for II was less than that for I (Fig. 1). Syringic acid was oxidized more slowly, but when uptake ceased the amount of oxygen consumed was approximately the same as for gallic acid. With vanillic acid (IV) and 3,5-dimethoxybenzoic acid (V) the oxygen consumed was about 75% of that required for converting one methoxyl group of each substrate into a hydroxyl group, carbon dioxide, and water. Isovanillic acid (VI), 3,4,5-trimethoxybenzoic acid and protocatechuic acid (3,4-dihydroxybenzoic acid) were not oxidized.

**Oxidation of Substrates by Cell-free Extracts**—Oxygen was rapidly consumed when reaction mixtures of 3 ml, containing 54 mg of extract protein and 5 μmoles of either gallate, 3-O-methylgallate, or syringate, were shaken in a Warburg respirometer. However, this technique proved to be of limited value for investigating stoichiometry, since extracts at this concentration showed substantial oxygen uptake in the absence of added substrates. On dilution, the gallate oxygenase activities of extracts diminished and a proportion of the gallate remained unchanged when amounts of substrate convenient for respirometry were shaken with diluted extracts. Similar observations have been made for protocatechuic acid, 5-oxoanise (9) and 3,4-dihydroxyphenylacetate, 3-oxoanise (17) which also lose activity during...
catalysis when diluted. Measurements of oxygen used in ring fission and O-demethylation were therefore made by means of the oxygen electrode, which requires much lower concentrations of substrates and permits the use of dilute extracts. Under these conditions 0.4 μmole of oxygen was consumed by 0.4 μmole of gallic and 3-O-methylgallic acids, and there was no attack on 3,4-dihydroxy-5-methylbenzoic and 5-carboxy-3,4-dihydroxybenzoic acids in which the hydroxyl group at C-5 (or C-3) of gallic acid is replaced by methyl and carboxyl, respectively (Fig. 2e).

Syringic acid was not oxidized unless NADH was present; 0.4 μmole of oxygen was then taken up by 0.2 μmole of substrate (Fig. 2f). By contrast, no increase in oxygen consumption was observed for 3-O-methylgallic acid when NADH was present (Fig. 2f). There was no requirement for NADH when syringate was oxidized in the Warburg respirometer. It is probable that, under these conditions, NADH required for O-demethylation (18) is furnished continuously by reactions catalyzed by other enzymes present in the concentrated crude extracts used, such as formaldehyde dehydrogenase (EC 1.2.1.1). Formaldehyde is a product of O-demethylation (18), so that once this reaction is initiated, its NADH requirement may then be satisfied by coupling with the oxidation of formaldehyde.

Formation of Pyruvate from Substrates—The amount of pyruvate formed enzymically from gallic acid was determined spectroscopically in cuvettes that contained: 0.15 μmole of gallic acid, 0.41 mg of NADH, 2.47 ml of Tris-HCl buffer of pH 7.0, and water to a final volume of 3 ml. Reactions were started by adding 0.1 ml of cell extract (29.6 mg of protein per ml) and were observed for 2 min, at which time the rate of change of absorbance at 340 nm had decreased to that for a control reaction lacking gallic acid. From 0.15 μmole of gallic acid in a typical experiment, 0.29 μmole of pyruvate was produced. When gallicate was replaced by 3-O-methylgallicate, 0.15 μmole of pyruvate was formed from 0.15 μmole of substrate. For syringic acid the procedure was modified, since NADH is required to initiate oxidation (Fig. 2b). Six reaction mixtures were therefore set up, each of which contained in a final volume of 3 ml, 0.25 μmole of syringic acid, 0.15 μmole of Tris-HCl buffer of pH 7.5, 2.8 mg of cell extract protein, and 0.5 μmole of NADH. The mixtures were then divided into three pairs. The first pair were incubated for 15 min at 30°, the second for 50 min, and the third for 45 min. All reactions were stopped by heating in a water bath for 2 min at 100°, denatured protein was removed by centrifuging, and 2-ml aliquots were then withdrawn for spectrophotometric determination of pyruvate using lactate dehydrogenase. In each determination, a cuvette containing the aliquot from one reaction mixture of a pair received no NADH, and this solution served as a blank when oxidation of NADH was measured in the second aliquot. From the mixtures incubated for 15 min, 30 min, and 45 min, the amounts of pyruvate formed from 1 μmole of syringic acid were, respectively, 1.16, 1.24, and 1.36 μmoles. In Fig. 2b, the oxidation of syringic acid was complete within 3 min. Assuming that 1 μmole of pyruvate was formed from 1 μmole of substrate in this period, as was the case for 3-O-methylgallicate, it appears that further reactions occurred which gave rise to an increase in the pyruvate concentration of about 1% per min.

Formation of Oxaloacetate from Gallicate—Carbon dioxide was evolved when gallicate was oxidized by crude cell-free extracts. When the reaction was conducted in a Warburg respirometer that contained 12 mg of extract protein, gas exchange ceased when 1 μmole of carbon dioxide had been evolved and 1 μmole of oxygen consumed. In Scheme 2 it is proposed that 1 mole of carbon dioxide is liberated from the oxaloacetate formed from 1 mole of gallicate, a reaction readily catalyzed by cell extracts. This decarboxylase activity was an obstacle in demonstrating directly the conversion of gallicate into oxaloacetate, since fractionation procedures that removed the decarboxylase invariably resulted in simultaneous loss of gallicate dehydrogenase. However, when a high concentration of malate dehydrogenase was added to reaction mixtures, oxaloacetate was reduced faster than it was decarboxylated, and it became possible to observe a stoichiometric oxidation of added NADH. Thus, 2 μmoles of NADH/1 μmole of gallicate were oxidized when cell extract was added to a reaction mixture containing lactate dehydrogenase. When this enzyme was replaced by malate dehydrogenase, only 1 μmole of NADH was oxidized; but a further 1 μmole of NADH reacted when lactate dehydrogenase was then added (Fig. 3). A similar experiment showed that 4-carboxy-4-hydroxy-2-oxoacidate (VIII, Scheme 2) was also cleaved by cell extracts to give equimolar amounts of oxaloacetate and pyruvate. Although, as previously observed, 1 μmole of NADH was oxidized rapidly when lactate dehydrogenase was incubated with 3-O-methylgallicate and cell extract, a different result was obtained for malate dehydrogenase. When this enzyme was present with 3-O-methylgallicate in the incubation mixture, the oxidation of NADH proceeded initially at only one-fifth of the rate shown for gallicate in Fig. 3 and virtually ceased when about 0.5 μmole of NADH had been oxidized for 1 μmole of 3-O-methylgallicate present.

Enzymic Ring Fission of Gallicate—The loss of gallicate oxygenase activity during fractionation was also an obstacle to the use of cell extracts for preparing the ring fission product of gallic acid. Inactivation may have been due to the labile nature of the protein. Alternatively, since activity was abolished completely when 1 mM of α,α′-dipyridyl was added to reaction mixtures, it appeared that this enzyme, like certain other dioxygenases (19) is Fe³⁺-dependent. However, when attempts were

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**Fig. 2. Oxygen uptake catalyzed by diluted extracts of syringacate-grown cells.** An oxygen electrode vessel contained 0.5 ml of phosphate buffer of pH 7.0, 27 mg of extract protein and the following: 1, gallic acid, 0.6 μmole; 2, gallic acid, 0.4 μmole; 5, 3,4-dihydroxy-5-methylbenzoate, 0.4 μmole; 6, 5-carboxy-3,4-dihydroxybenzoate, 0.4 μmole. The remaining measurements were made with 5.1 mg of extract protein and the following: 3, 3-O-methylgallicate, 0.4 μmole; 4, 3-O-methylgallicate, 0.4 μmole plus NADH, 0.5 μmole; 7, syringic acid, 0.2 μmole; 8, syringic acid, 0.2 μmole plus NADH, 0.5 μmole.
made to activate the enzyme by treating with Fe^{++} ions, it was found necessary to remove the excess since ferrous sulfate reacted rapidly with the substrate, gallic acid, to give a dark green complex. It was also difficult to achieve this removal effectively without simultaneous inactivation, since Fe^{++} ions appeared to be very loosely bound to the enzyme. An alternative experimental approach became available when it was found that gallic acid was attacked by protocatechuate 4,5-oxygenase (EC 1.13.1.8). First, this enzyme, which has been purified from Pseudomonas testosteroni (9), was used to accumulate a ring fission compound from gallic acid. Second, this same compound was shown to be metabolized rapidly and quantitatively by extracts of Pseudomonas putida to give, like gallic acid, equimolar amounts of oxaloacetate and pyruvate. It was therefore concluded that protocatechuate 4,5-oxygenase and gallic acid oxygenase cleaved the benzene nucleus of gallic acid to give the same ring fission product. These experiments were conducted as follows, using a preparation of protocatechuate 4,5-oxygenase (24 mg of protein per mg) which was kindly provided by Dr. J. M. Wood, University of Illinois. An extract of syringate-grown Pseudomonas pulida was also used which, after heat treatment, was devoid of gallic acid oxygenase but retained activity towards other metabolites in the proposed reaction sequence (Scheme 2). A cuvette contained, in a final volume of 3 ml, 123 μmoles of Tris-HCl buffer of pH 8.0, 0.41 mg of NADH, 1.2 mg of protocatechuate 4,5-oxygenase, and 5 μl of lactate dehydrogenase. No oxidation of NADH was observed when 0.2 μmole of gallic acid was added. After incubating for 1.5 min, during which period the absorbance remained unchanged, an addition of 0.05 ml of heat-treated extract (0.42 mg of protein) was made and this was followed immediately by a decrease in absorbance at 340 nm. When lactate dehydrogenase was omitted, and replaced by 5 μl of malate dehydrogenase, oxidation of NADH was again dependent upon the addition of heat-treated extract. With lactate dehydrogenase the decrease in absorbance corresponded to reduction of 0.38 μmole of pyruvate. In the second experiment with malate dehydrogenase, 0.19 μmole of oxaloacetate reacted; and when lactate dehydrogenase was then added, a further decrease in absorbance indicated the presence of 0.19 μmole of pyruvate. Therefore, from 1 μmole of gallic acid, oxidized to the ring fission product of protocatechuate 4,5-oxygenase, approximately 2 μmoles of pyruvate were formed by the heat-treated extract of Pseudomonas putida; and of this yield, 1 μmole of pyruvate arose from oxaloacetate which could be trapped by reduction to malate.

These results support the proposed structure (VII, Scheme 2) for the product of ring fission of gallic acid. Further evidence was obtained by observing the changes in ultraviolet absorption when 50 μl of protocatechuate 4,5-oxygenase were added to 0.5 μmole of gallic acid in 3 ml of 0.1 M phosphate buffer, pH 7.0. An immediate decrease in absorbance at 260 nm, the wave length of maximum absorbance for gallic acid at pH 7.0, was accompanied by an increase at 310 nm; these changes were complete after 2 min. On addition of sufficient NaOH to bring the pH to 12.6, the peak at 310 nm was shifted to 355 nm with increased absorbance (Fig. 4); the shift was freely reversible on acidification. For 2-hydroxymuconic acid the following values have been recorded (20); at pH 7.0, λ_{max} 295 nm (log ε 4.06); at pH 12.4, λ_{max} 350 nm (log ε 4.43). We obtained for VII: at pH 7.0, λ_{max} 310 nm (log ε 3.62); at pH 12.6, λ_{max} 355 nm (log ε 4.00). These results are consistent with the proposal that VII is 4-carboxy-2-hydroxymuconic acid, the spectral shift in alkaline being due to base-promoted ionization of the enol tautomer; this reacted with ferric chloride to give a purple solution. Protocatechuate, 4,5-oxygenase also oxidized 3-O-methylgallic acid to a compound having λ_{max} 310 nm at pH 7.0 and λ_{max} 355 nm at pH 12.6.

**DISCUSSION**

The metabolic pathway proposed in Scheme 2 accords with the observation that crude cell extracts catalyzed the conversion of
1 µmole of gallate into 2 µmoles of pyruvate with the consumption of 1 µmole of oxygen. Such extracts contained a powerful de-

Oxygen reacted readily with 3-O-methylgallate, although the rate was somewhat less than that for gallate (Fig. 2b). It appears, for the following reasons, that the benzene nucleus was cleaved between C-4 and C-3, which bears the hydroxyl group. (a) One of the products formed from 3-O-methylgallate (pyruvate) was rapidly reduced by NADH when lactate dehydrogenase was added; but the other product differed from oxaloacetate and lactate dehydrogenase to reduce oxaloacetate faster than it was decarboxylated. Evidence of the involvement of 4-carboxy-2-hydroxy-6,6-muconic acid (VII in Scheme 2) was less direct, and stemmed from the finding that gallate was oxidized by protocatechuic 4,5-oxidase, purified from another organism (Pseudomonas testosteroni). From the known properties of this enzyme (18, 21) it is highly probable that the benzene nucleus of gallate is cleaved between C-3 and C-4 (or C-4 and C-5) to give VII, although fission between C-2 and C-3, to give an aldehyde acid, might be a formal possibility. However, this second alternative is discounted by the spectroscopic properties of the ring fission product, which were those of a substituted 2-hydroxymuconic acid, such as VII. When gallate was incubated, first with protocatechuic 4,5-oxidase and then with an extract of syringate-grown Pseudomonas putida that lacked gallete oxygenase, it was found that approximately 1 µmole each of oxaloacetate and pyruvate were formed from 1 µmole of gallate. Although, therefore, gallete and protocatechuic oxidases are separate enzymes, differing at least insofar as gallete oxygenase does not attack protocatechuic acid, it appears that they oxidize gallete to the same ring fission product, VII.

4-Carboxy-4-hydroxy-2-ketoacidic acid (VIII) was rapidly cleaved to oxaloacetate and pyruvate by an aldolase present in extracts of syringate-grown Pseudomonas putida. This enzyme has now been purified (22). Insofar as it involves enzyme hydration and retroaldol cleavage, the reaction sequence of Scheme 2 resembles those for the meta-fission pathways studied previously (23), but it differs as follows. The compounds formed by oxidative ring fission of catechol or 3-methylcatechol or 4-methylcatechol all give rise to an oxoenoic acid, either by hydrolytic fission (24) or by oxidation followed by decarbosylation (25). The oxoenoic acid so formed is then hydrated. In Scheme 2, however, direct hydration of the ring fission product is shown.

The oxidation of syringic acid by cell extracts was not initiated until NADH was added (Fig. 2b). This requirement for NADH indicates that ring fission is preceded by O-demethylation according to the following equation:

\[
\text{Syringate} + \text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{3-O-methylgallate} + \text{HCHO} + \text{NAD}^+
\]

Ribbons (18) showed that an analogous equation described O-demethylation of vanillate, veratrate and m-methoxybenzoate by extracts of Pseudomonas aeruginosa. We found that extracts catalyzed an NAD⁺-linked oxidation of formaldehyde; and the ability of concentrated cell extracts to oxidize syringate without additions of NAD⁺ may be accounted for by the coupling of demethylation with the reaction:

\[
\text{Formaldehyde} + \text{H}_2\text{O} + \text{NAD}^+ \rightarrow \text{formate} + \text{NADH} + \text{H}^+
\]

Of the 2 µmoles of oxygen taken up by 1 µmole of syringate in the oxygen electrode experiments (Fig. 2b), 1 µmole of oxygen would be required in O-demethylation and 1 µmole for ring fission of the 3-O-methylgallate formed; that is, only one methoxyl group was oxidized. It was also found (Fig. 1) that only one of the two meta-placed methoxyl groups of 3,5-dimethoxybenzoate was oxidized by intact cells. Degradation of 3-O-methylgallate would account for the rapid formation of 1 µmole of pyruvate observed when syringate was incubated with cell extracts, and the slow appearance of more pyruvate on prolonged incubation (about 0.01 µmole per min) may be due to the slow release of oxaloacetate from its monomethyl ester, followed by decarbosylation. We have no information as to the significance of this suggested hydrolysis in the metabolism of syringate by the organism, although it may be noted that when rapid oxidation ceased at 150 min (Fig. 1) the total amount of oxygen consumed by intact cells was significantly less for 3-O-methylgallate than for gallete, despite the fact that 1 mole of the former substrate provides more carbon than the latter. This observation suggests that methylation has rendered a position of the gallic acid molecule less susceptible to rapid degradation. We found that an NAD⁺-linked methanol dehydrogenase was present in cell extracts, which might be used to metabolize methanol eventually released from a methyl ester.

\[
\text{GALLIC ACID} \xrightarrow{+ \text{O}_2} \text{VII} \quad \text{VIII} \quad \text{OXALOACETATE} \xrightarrow{+ \text{H}_2\text{O} \rightarrow \text{CO}_2} \text{PYRUVATE}
\]

SCHEME 2
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