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Metabolism of Gallate and Phloroglucinol in *Eubacterium oxidoreducens* via 3-Hydroxy-5-Oxohexanoate

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The pathway for the anaerobic catabolism of gallic acid by *Eubacterium oxidoreducans* was studied by using both in vivo and cell-free systems. Cells grown with gallate and crotonate, but with no formate or H_2, excreted pyrogallol and phloroglucinol into the medium. Gallate was decarboxylated by crude cell extracts, with pyrogallol as the only detectable product. Whole cells converted pyrogallol to phloroglucinol. A phloroglucinol reductase catalyzed the conversion of phloroglucinol to dihydrophloroglucinol when NADPH was used as the source of electrons. Both formate dehydrogenase (EC 1.2.1.43) and hydrogenase (EC 1.18.99.1) were present in cell extracts of gallate-formate-grown cells. These two enzymes were both NADP linked. Since either H_2 or formate is required for cell growth with gallate or phloroglucinol, these results suggest that the oxidation of the reduced substrate may be indirectly linked to the reduction of phloroglucinol. A dihydrophloroglucinol hydrolase was present, which hydrolyzed dihydrophloroglucinol to 3-hydroxy-5-oxohexanoate. This six-carbon ring cleavage product then presumably can be broken down by a series of reactions similar to β-oxidation. These reactions cleaved the six-carbon acid to 3-hydroxybutyryl-coenzyme A yielding acetate and butyrate as end products. A number of key enzymes involved in β-oxidation and substrate-level phosphorylation were demonstrated in cell extracts.

Recently, *Eubacterium oxidoreducens* ("*Eubacterium oxidoreducans*") G41 was isolated from the rumen after enumeration and enrichment with pyrogallol. It catabolizes trihydroxybenzenes anaerobically to acetate and butyrate as the major products (5). This new isolate differs from previously known trihydroxybenzene degraders in its requirement for formate or hydrogen as a cosubstrate. Other trihydroxybenzene degraders include a rumen *Coprococcus* sp. which degrades phloroglucinol to acetate and CO_2 (16). *Pelobacter acidigallici*, isolated from sewage and freshwater sediments, degrades gallate, pyrogallol, phloroglucinol, and 1,2,4-trihydroxybenzene anaerobically to three molecules of acetate (11). The initial steps in the pathway of trihydroxybenzene catabolism by *P. acidigallici* have been elucidated (10).

Whittle et al. (21) have examined the pathway of the anaerobic catabolism of phloroglucinol by a *Rhodopseudomonas* sp. Their incomplete pathway description indicates the reduction of phloroglucinol to dihydrophloroglucinol with 2-oxo-4-hydroxyadipate as a subsequent intermediate.

The objective of the present investigation was to determine the pathway of the anaerobic metabolism of gallate by *E. oxidoreducens*. A preliminary report of this investigation has been previously presented (6).

MATERIALS AND METHODS

Cultivation and preparation of cells and cell extracts. *E. oxidoreducens* G41 was grown in basal medium containing 30 mM sodium formate and 20 mM sodium gallate or in medium containing 60 to 100 mM sodium crotonate. The composition was as described previously (5), including the addition of 0.2% vitamin-free Casitone (Difco Laboratories, Detroit, Mich.). Cells were harvested by centrifugation at 16,300 × *g* and were washed at least once with either 100 mM potassium phosphate, pH 7.0, or 100 mM Tris hydrochloride, pH 7.5. Cell extracts were prepared by the passage of the cells, which were suspended in a small amount of buffer, once through a French pressure cell. When anaerobic conditions were required, buffers were sparged with nitrogen, and all manipulations were all done in an anaerobic glove box. 2-Mercaptoethanol (10 mM) was included in anaerobic buffers.

Identification of pathway intermediates. Isolated intermediates were identified by gas chromatography-mass spectrometry (GC-MS) and, when indicated, by proton magnetic resonance (PMR) spectroscopy. For GC analysis, trimethylsilyl derivatives of phenolic compounds were prepared. Dried samples were dissolved in 0.1 ml of pyridine, and 0.1 to 0.2 ml of bis-trimethylsilyltrifluoroacetamide (plus 1% trimethyltrichlorosilane) was added. The samples were heated for 20 min at 60°C. Compounds were chromatographed on an OV-17 column with the temperature increasing from 110 to 270°C at a rate of 10°C/min. GC-MS was performed with a GC-MS spectrometer (5985B; Hewlett-Packard Co., Palo Alto, Calif.) with the CI-EI source set at 70 eV and at 200°C. For PMR experiments, a Varian XL-200 nuclear magnetic resonance spectrometer was used. Underivatized samples were dissolved in D_2O, and derivatized samples were dissolved in $CDCl_3$, with trimethylsilylpropionate and tetramethylsilane, respectively, as references.

Enzyme determinations. All assays were done with a Response spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) at 37°C with 1-ml cuvettes containing 0.5 ml of reaction mixture. When assays were performed anaerobically, the cuvettes were filled in an anaerobic glove box under 95% N_2–5% H_2 and were sealed with a piece of gum-rubber tubing. After the cuvettes were prewarmed in the cuvette holder, a final addition of substrate or enzyme

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was made by syringe to start the reaction. All reaction rates were linear during the assay period.

The specific enzyme type and reaction mixtures used to assay the enzymes were as follows. Gallate decarboxylase (EC 4.1.1.59) was assayed anaerobically with 100 μM gallic acid, 1 mM dithiothreitol, and 100 mM potassium phosphate, pH 7.0. Diluted cell extract (100 μl) was added to start the reaction, and the A_{259} was read for 2 min. The blank contained no gallic acid. Phenolic carboxylic acids have an absorbance maximum between 250 and 300 nm which is not present in their decarboxylated counterparts. By using this principle, the following compounds were tested as substrates for gallate decarboxylase at the indicated wavelength: protocatechuate, 288 nm; α-resorcylate, 295 nm; β-resorcylate, 292 nm; *m*-hydroxybenzoate, 288 nm; vanillate, 289 nm; and syringate, 262 nm.

The phloroglucinol reductase assay was done under air and was found to be more reproducible when 1 mM dithiothreitol was added. Other components in the assay solution were 2 mM phloroglucinol, 200 μM NADPH or NADH, 50 mM potassium phosphate, and 10 μl of cell extract. The decrease in A_{340} was read for 30 s to 1 min. A blank contained no phloroglucinol.

Dihydrophloroglucinol hydrolase was assayed with a reaction mixture containing 1 mM dihydrophloroglucinol, 3 mM dithioerythritol, and 50 mM Tris hydrochloride, pH 7.5. The assay was performed anaerobically, with 100 μl of cell extract added to start the reaction. The rate of decrease in A_{278} was determined. Formate dehydrogenase (EC 1.2.1.43) activity was determined as described by Ljungdahl and Andreeson (7). Hydrogenase (EC 1.18.99.1) activity was determined as described by Drake (4), in stoppered cuvettes with a 3-ml reaction mix and 5.2-ml gas phase.

Isolation of metabolites from culture supernatants. Strain G41 was grown in 50-ml cultures in basal medium containing 50 mM sodium crotonate and 5 mM sodium gallate, but with no formate or hydrogen. After the culture reached stationary phase, the cells were removed by centrifugation. Culture supernatants were extracted three times with equal volumes of ethyl acetate. The culture fluids were then acidified with HCl to pH 2 to 3 and extracted three more times with ethyl acetate. Metabolites were isolated from the neutral extract by preparative thin-layer chromatography (TLC) on cellulose plates with 20% aqueous KCl as the mobile phase. They were then identified by GC-MS and PMR.

Identification of intermediates formed after ring cleavage. To identify the products of dihydrophloroglucinol metabolism, a 10 mM solution of dihydrophloroglucinol was prepared anaerobically with 1 mM dithiothreitol in 100 mM potassium phosphate, pH 7.0. To 400 ml of reaction mixture, 3.5 ml of dialyzed cell extract (31.2 mg of protein) was added, and the mixture was incubated at 37°C until the A_{278} (λ_{max} of dihydrophloroglucinol at pH 7.0) had decreased to 10% of its original value. No decrease was observed with boiled cell extract, and the reaction proceeded much more slowly when dithiothreitol was omitted. The reaction mixtures were acidified, and precipitated protein was removed by centrifugation. A portion (100 ml) of the supernatant was lyophilized and methylated with diazomethane. The methylated derivative of the major product was purified by preparative TLC on silica gel with toluene-ethyl acetate-formic acid (5:4:1).

A reaction mixture for the coenzyme A (CoA) transferase reaction containing a CoA donor and acceptor was used to determine the fate of the six-carbon ring cleavage product. The reaction mixture contained the major product of dihydrophloroglucinol metabolism (10 mM), acetyl- or butyryl-CoA (1 mM), potassium phosphate buffer (pH 7.0) (50 mM), and protein (about 800 μg/ml of reaction mixture). After a 1-h incubation at 37°C, 0.1 ml of a neutral solution of hydroxylamine was added, and the mixture was incubated for another 5 min. The reaction was stopped by addition of an equal volume of 95% ethanol followed by freezing. The hydroxamate product was identified by using two TLC systems with which the product was chromatographed alongside authentic hydroxamates. Cellulose plates were run with 2-butanol–formic acid–water (75:13:12), and silica gel plates were run with toluene-methanol-acetic acid (45:20:4). Hydroxamates were detected by spraying the plate with a 1% methanolic $FeCl_3$ solution.

Chemicals. Dihydrophloroglucinol was synthesized by a modification of the method of Patel et al. (8). Phloroglucinol was reduced with $NaBH_4$ to dihydrophloroglucinol, and after the destruction of $NaBH_4$ with acid, the crude reaction mixture was used in experiments to identify the reaction product. For the assay of the enzyme, dihydrophloroglucinol was purified by precipitation from a methanolic solution as the disodium salt. The identity of dihydrophologlucinol was confirmed by GC-MS. Hydroxamic acids, other than acetohydroxamic acid, were prepared from free acids as described by Sherma and Zweig (13). All other chemicals were from high-purity commercial sources.

RESULTS

Isolation and identification of intermediates. Strain G41 grows on trihydroxybenzenoids only when formate or H_2 is included in the medium (5). This organisms grows best on crotonate when formate or H_2 is deleted. The first attempts at isolation of intermediates in gallate catabolism involved the growth of strain G41 in basal medium containing 5 mM gallate and 50 mM crotonate. The intention was to accumulate intermediates before the formate-requiring step. Both acidic and neutral ethyl acetate extracts were analyzed by TLC. The neutral extract was found to contain compounds with R_f values corresponding to those of phloroglucinol and pyrogallol. The spots were scraped from preparative TLC plates, and the identities were confirmed by PMR and GC-MS. The results were confirmed by using authentic pyrogallol and phloroglucinol. Only gallic and crotonic acids were detectable in the acidic fraction by TLC. Both acetate and butyrate were most likely present as well, since they are the products of crotonate catabolism (5).

Accumulation of intermediates by nongrowing cells and cell extracts. To determine the sequence of reactions with pyrogallol and phloroglucinol and of further metabolic reactions, studies with cell extracts and washed cells were undertaken. The strain G41 cells for these studies were grown on either gallate-formate or crotonate as indicated. Several enzymes involved in ring metabolism were identified on the basis of their substrate and the reaction type. In each case, the major product of the reaction was identified.

On anaerobic incubation of cell extracts with gallate, pyrogallol was the major product. The enzyme catalyzing this reaction, gallate decarboxylase, exhibited some measurable activity (Table 1). No activity was detected with any of the other carboxylic acids tested. When cell extracts were incubated with pyrogallol, no change was observed. When gallate-grown log-phase cells were washed by centrifugation and incubated in phosphate buffer (788 μg of protein per ml of reaction mixture) with pyrogallol (5 mM), phloroglucinol was produced and accumulated. In the presence of NADPH,

TABLE 1. Specific activities of enzymes involved in a.omatic catabolism[a]

Enzyme	Sp act (μmol/min per mg of protein)	
	Gallate-formate-grown cells	Crotonate-grown cells
Gallate decarboxylase	0.095	0.082
Phloroglucinol reductase	4.18	2.08
Dihydrophloroglucinol hydrolase	0.886	0.244
Formate dehydrogenase	0.407	0.512
Hydrogenase (NADP)[b]	4.67	ND[c]
Hydrogenase (methyl viologen)[b]	31.6	ND

[a] Formate dehydrogenase, hydrogenase, and phloroglucinol reductase activities were NADP(H) dependent, with less than 1% of NADP(H) activity expressed with NAD(H) as substrate.
[b] The compound in parentheses was used as a cosubstrate with H_2 for determinations of hydrogenase activity.
[c] ND, Not determined.

phloroglucinol was converted to dihydrophloroglucinol by the phloroglucinol reductase in the cell extract (Table 1).

When a crude preparation of dihydrophloroglucinol was incubated with dialyzed cell extracts, a new product was obtained. The methylated product had a molecular ion, as determined by high-resolution field desorption MS, corresponding to a formula of $C_7H_{12}O_4$ (160.0735, Δ0 mmu). The PMR spectra ($CDCl_3$) showed 2.18 (s, 3H, 6-CH_3), 2.51 (d, 2H, 2-H), 2.66 (d, 2H, 4-H), 3.38 (s, 1H, OH), 3.69 (s, 3H, OCH_3), and 4.47 (m, 1H, 5-H). These data correspond to the structure for the methyl ester of 3-hydroxy-5-oxohexanoic acid (HOHN). The enzyme catalyzing this reaction will be called a dihydrophloroglucinol hydrolase as it hydrolyzed the ring to the noncyclic product.

Enzymes of β-oxidation and substrate-level phosphorylation. All of the enzymes of β-oxidation and substrate-level phosphorylation were present (Table 2). The only enzyme that probably does not play a significant role is butyrate kinase (EC 2.7.2.7). The reaction mixture for this assay contained 400 mM butyrate. When this was reduced to 50 mM, which is the amount of acetate used for the acetate kinase (EC 2.7.2.1) assay, no activity was detectable. Also, the activity of butyrate kinase is obviously low in comparison with acetate kinase.

TABLE 2. Specific activities of enzymes involved in β-oxidation and substrate-level phosphorylation

Enzyme	Sp act (μmol/min per mg of protein)		Reference for method
	Gallate-formate-grown cells	Crotonate-grown cells	
β-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35)	4.62[a]	5.06[a]	20
CoA transferase	37.5	45.4	18
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	0.871	0.760	22
Acetyl-CoA acetyltransferase (EC 2.3.1.9)	0.485	0.945	3
Enoyl-CoA hydrase (EC 4.2.1.17)	14.77	15.39	20
Phosphate acetyltransferase (EC 2.3.1.8)	5.12	4.18	14
Acetate kinase (EC 2.7.2.1)	6.69	3.99	9
Butyrate kinase (EC 2.7.2.7)	0.0068	ND[b]	19

[a] The reported activity is NAD(H) dependent; less than 1% of that activity was observed with NADP(H).
[b] ND, Not determined.

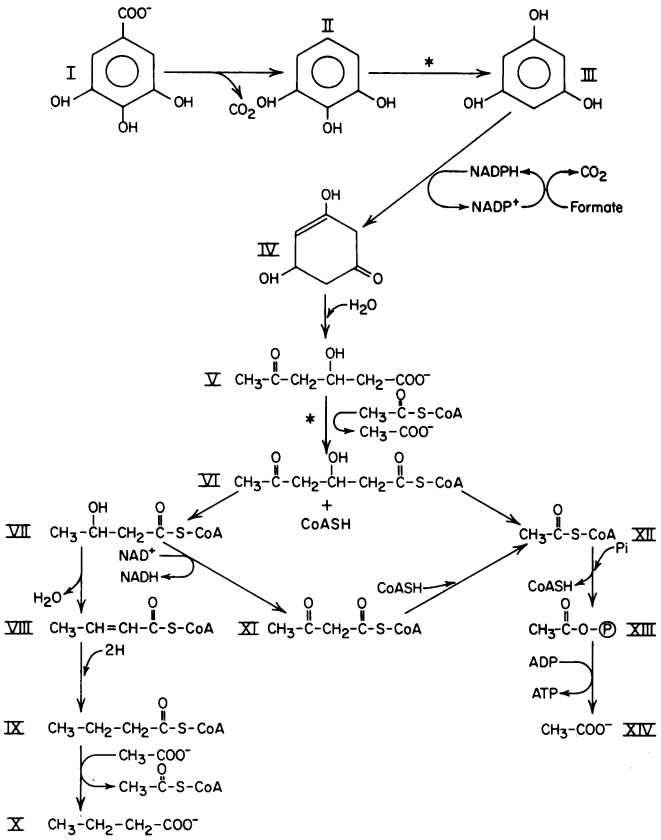

FIG. 1. Proposed pathway for energy metabolism of gallate and phloroglucinol by *E. oxidoreducens*. *, Steps that are still not well understood. The compounds represented are gallate (I), pyrogallol (II), phloroglucinol (III), dihydrophloroglucinol (IV), HOHN (V), 3-hydroxy-5-oxohexanoyl-CoA (VI), 3-hydroxybutyryl-CoA (VII), crotonyl-CoA (VIII), butyryl-CoA (IX), butyrate (X), acetoacetyl-CoA (XI), acetyl-CoA (XII), acetyl phosphate (XIII), and acetate (XIV).

Butyrate-CoA ligase (acyl-CoA synthetase, EC 6.2.1.2) was assayed as described by Blakley (2) with crotonate or enzymatically prepared HOHN as the substrate. No significant activity was detectable.

Conversion of HOHN to an intermediate in β-oxidation. After the incubation of cell extracts with acetyl-CoA and HOHN and the subsequent conversion of CoA derivatives to hydroxamic acids, only one hydroxamate was recovered. This was identified as 3-hydroxybutyrohydroxamic acid formed from the accumulation of 3-hydroxybutryl-CoA during the reaction. No remaining acetyl-CoA was detected. When butyryl-CoA replaced acetyl-CoA as the CoA donor, only butyrohydroxamic acid was observed.

DISCUSSION

The initial step in the degradation of gallate (Fig. 1) is the decarboxylation of the ring. This step likely results in the CO_2 which is observed as an end product of growth on gallate, but which is not observed for growth on other substrates (5). The activity observed for gallate decarboxylase was low in comparison with those of the other

enzymes involved in aromatic metabolism. This low activity may stem from the oxygen sensitivity of the enzyme or from other factors which may contribute to its lability. The enzyme(s) involved in this reaction is specific for gallate. This enzyme as well as the other enzymes assayed were produced constitutively when strain G41 was grown on crotonate, the only nontrihydroxybenzenoid substrate on which it could grow (Table 1). Gallate decarboxylation has been observed to occur under anaerobic nitrate-respiring conditions by a soil bacterium identified as a *Citrobacter* sp. (23). The *Citrobacter* enzyme was not as specific as the *Eubacterium* enzyme(s) and allowed the organism to grow on a number of phenolic compounds. Resting cells of *P. acidigallici* also showed nonspecificity, decarboxylating protocatechuate to catechol as well as decarboxylating gallate to pyrogallol (10).

The conversion of pyrogallol to phloroglucinol (Fig. 1) by washed cells is an interesting reaction and will be the subject of further study. The mechanism of this reaction is unclear. More than one enzyme may be responsible for the reaction; however, no other intermediates were detected in our GC studies. On observation of phloroglucinol reductase activity in gallate-grown *P. acidigallici*, Schink and Pfennig (11) proposed that a similar conversion of pyrogallol to phloroglucinol occurs.

The next step in our proposed pathway (Fig. 1), the reduction of phloroglucinol to dihydrophloroglucinol in conjunction with oxidation of NADPH, is known to occur in several anaerobic organisms (8, 10, 11, 21), as well as in some aerobic species (Y. D. Hang, Ph.D. thesis, McGill University, Montreal, Canada, 1968). Patel et al. (8) have partially purified a phloroglucinol reductase from a *Coprococcus* sp. The enzyme has a molecular weight of 130,000, and it oxidizes NADH at only 4% of the rate that it oxidizes NADPH. Crude cell extracts of *P. acidigallici* oxidize NADPH in the presence of phloroglucinol, resorcinol, or phloroglucinol carboxylate (10). The phloroglucinol reductase in a *Penicillium* sp. also oxidizes NADPH in the presence of resorcinol or phloroglucinol (D. K. Mathur, Ph.D. thesis, McGill University, Montreal, Canada, 1971).

In *E. oxidoreducens*, formate dehydrogenase, hydrogenase, and phloroglucinol reductase were NADP linked. The other oxidoreductases that we assayed were all NAD linked (Table 2). On the basis of this information, it seems likely that the oxidation of formate or hydrogen is coupled indirectly to the reduction of phloroglucinol. Other evidence for this phenomenon was that in the absence of formate, cells growing on crotonate and gallate accumulated phloroglucinol. Washed cells incubated without formate in the presence of pyrogallol also accumulated phloroglucinol.

The formate or hydrogen requirement of *E. oxidoreducens* differentiates it from the other anaerobic gallate-degrading bacteria. The indirect linkage of the two oxidoreductases, formate dehydrogenase and phloroglucinol reductase, may be favorable in an environment in which formate or hydrogen is plentiful, such as in the rumen. *E. oxidoreducens* apparently sacrifices energy at the point of substrate-level phosphorylation, rather than transferring reducing equivalents from NADH to NADP. This latter reaction most likely occurs in the gallate degrader, *P. acidigallici*.

Dihydrophloroglucinol hydrolase catalyzes the hydrolysis of dihydrophloroglucinol to HOHN. Although not common in aerobic systems, hydrolysis of the ring at a keto group is the most commonly proposed method of ring cleavage in anaerobic systems. For example, anaerobic black mud enrichments catabolize benzoate to pimelate via 2-oxocyclohexanecarboxylate (12), and phenol is broken down by a methanogenic consortium via cyclohexanone (1). This type of mechanism has also been proposed for the anaerobic metabolism of resorcinol (17). During catabolism, resorcinol may first be reduced and then be hydrolyzed to a six-carbon keto acid.

The product of the hydrolysis of dihydrophloroglucinol is HOHN, which is a good candidate for β-oxidation (Fig. 1). Since all of the enzymes required for β-oxidation of a compound such as HOHN were present in cell-free systems (Table 2), it seems likely that HOHN was converted to acetate and butyrate by a process similar to β-oxidation. It was not possible to determine the nature of the first steps for catabolism of HOHN. Since a high level of CoA transferase and virtually no acyl-CoA synthetase activities were detected, it seems likely that the CoA transferase is the predominant means of producing the first CoA derivative in the pathway.

The formation of 3-hydroxybutyryl-CoA after the incubation of acetyl-CoA with HOHN and cell extract provides evidence that HOHN may first be activated to its CoA derivative. This derivative could then be thiolytically cleaved to acetyl-CoA and 3-hydroxybutyryl-CoA. Acetyl-CoA was not detected as a product, possibly because some of it was reused to activate HOHN or was converted to acetyl phosphate by the phosphate acetyltransferase (EC 2.3.1.8) enzyme. It is unlikely that the HOHN is cleaved first to 3-hydroxybutyrate, because if this were to occur, 3-hydroxybutyrate would accumulate after incubation of dihydrophloroglucinol with cell extract. Once the 3-hydroxybutyryl-CoA is formed, it can be converted to acetate and butyrate via acetoacetyl-CoA and crotonyl-CoA, respectively, as is the case in *Clostridium kluyveri* (15). If crotonate is used as the substrate after activation to crotonyl-CoA, it can be broken down by the same set of enzymes (Table 2).

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