JOURNAL OF BACTERIOLOGY, Mar. 1976, p. 975-984
Vol. 125, No. 3

Printed in U.S.A.

Metabolism of Resorcinylic Compounds by Bacteria: Orcinol Pathway in *Pseudomonas putida*

PETER J. CHAPMAN AND DOUGLAS W. RIBBONS*

*Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108, and Department of Biochemistry, University of Miami School of Medicine, and Howard Hughes Medical Institute, Miami, Florida 33152**

Received for publication 6 October 1975

Enrichment cultures yielded two strains of *Pseudomonas putida* capable of growth with orcinol (3,5-dihydroxytoluene) as the sole source of carbon. Experiments with cell suspensions and cell extracts indicate that orcinol is metabolized by hydroxylation of the benzene ring followed successively by ring cleavage and hydrolyses to give 2 mol of acetate and 1 mol of pyruvate per mol of orcinol as shown: orcinol → 2,3,5-trihydroxytoluene → 2,4,6-trioxoheptanoate → acetate + acetylpyruvate → acetate + pyruvate. Evidence for this pathway is based on: (i) high respiratory activities of orcinol-grown cells towards 2,3,5-trihydroxytoluene; (ii) transient accumulation of a quinone, probably 2-hydroxy-6-methyl-1,4-benzoquinone, during growth with orcinol; (iii) formation of pyruvate and acetate from orcinol, 2,3,5-trihydroxytoluene, and acetylpyruvate catalyzed by extracts of orcinol, but not by succinate-grown cells; (iv) characterization of the product of oxidation of 3-methylcatechol (an analogue of 2,3,5-trihydroxytoluene) showing that oxygenative cleavage occurs between carbons bearing methyl and hydroxyl substituents; (v) transient appearance of a compound having spectral properties similar to those of acetylpyruvate during 2,3,5-trihydroxytoluene oxidation by extracts of orcinol-grown cells. Orcinol hydroxylase exhibits catalytic activity when resorcinol or *m*-cresol is substituted for orcinol; hydroxyquinol and 3-methylcatechol are substrates for the ring cleavage enzyme 2,3,5-trihydroxytoluene-1,2-oxygenase. The enzymes of this pathway are induced by growth with orcinol but not with glucose or succinate.

Although 1,3-dihydroxy-derivatives of benzenoid and more complex aromatic ring systems (resorcinylic compounds) are widespread products of plant and microbial metabolism (17), the catabolism of even the simplest of these compounds has received little attention. Resorcinol and 2-, 4-, and 5-substituted resorcinols are detoxicated in animal bodies by conjugation with glucuronate or sulphate (11, 12).

The ability of resorcinol to support the growth of microorganisms as the sole source of carbon is not surprising since large amounts (up to 1 g per liter) are present in gas liquors and are removed by biological purification before discharge into rivers (33). Despite reports of its utilization by *Azotobacter vinelandii* (6), *Trichosporon cutaneum* (30), and by a species of *Pseudomonas* (P. Larway and W. C. Evans, Biochem. J. 95:52P, 1965), few studies of its catabolism have been reported. Thus enzymes have been extracted from *Piricularia oryzae* and *Polyporus versicolor*, which oxidize 1,3-hydroxy compounds, in particular resorcinol and orcinol (28). Four products were obtained from each substrate, but none of these was completely characterized (44). Larway and Evans described a soil pseudomonad capable of growth on resorcinol as sole carbon source. Extracts from these cells oxidized resorcinol in the presence of reduced nicotinamide nucleotides. Hydroxyquinol, which was isolated from culture filtrates, was also oxidized by crude extracts with an equimolar consumption of oxygen to give an unsaturated oxo acid with spectral properties identical with those of maleylacetic acid. Whole cells oxidized this acid and fumarylacetate, maleate and fumarate. The data indicated a catabolic pathway: resorcinol → hydroxyquinol → maleylacetate →→ fumarate + acetate. Resorcinol has also been shown to arise as a product of 2,4-dihydroxybenzoic acid metabolism by *Aspergillus* sp. (16).

We have isolated two strains of *Pseudomonas putida* capable of growth with orcinol (3,5-dihydroxytoluene) as the sole source of carbon. Orcinol was selected for study because it is a simple resorcinylic compound that occurs naturally and because of our continuing interest in

the low specificity of enzymes induced in bacteria for the metabolism of alkylphenols (4, 20, 35, 36). This paper presents the evidence for a pathway of orcinol oxidation suggested in a preliminary report (D. W. Ribbons and P. J. Chapman, Biochem. J. 106:44–45P, 1967).

MATERIALS AND METHODS

Organisms. Both strains used in this study are gram-negative motile rods isolated from stagnant pond mud and river water in Kingston-upon-Hull, Yorkshire, England, by enrichment in a mineral-salts medium containing orcinol (0.1%) as the sole source of carbon. Two strains, isolated independently, were both identified as *P. putida* by the nutritional and biochemical criteria described by Stanier et al. (42) and are designated O1 and ORC.

Growth of bacteria. Organisms were maintained on nutrient agar slants, and induced cells were obtained by growth in the mineral salts media described earlier (35) supplemented with 0.1% orcinol as the sole carbon source. The sequence of transfers for large batches of cells was as follows. Nutrient broth (80 ml) was inoculated from a slant, and the culture was grown for approximately 16 h at 30 C without shaking; this was used in toto to inoculate 1 liter of mineral salts-orcinol medium in 4-liter Erlenmeyer flasks. These were shaken for 16 to 24 h and then added to 10 liters of the same medium in 14-liter fermenters (New Brunswick Scientific Co., New Brunswick, N.J.). Additions of orcinol (in sterile aqueous solution) were made periodically as the carbon source was depleted. Growth was followed turbidimetrically at 660 nm. Cultures were harvested in a Sharples supercentrifuge (Sharples Corp., Philadelphia, Pa.) at 60,000 × *g* and at 26 to 33 C. The cell paste was either used directly or stored at 0 C for up to 48 h. *P. aeruginosa* T1 was grown as previously described and used as a source of 2-hydroxy-6-keto-2,4-heptadienoate hydrolase (35).

Cell-free extracts. Cell-free extracts were prepared from suspensions of harvested cells in 50 mM KH_2PO_4-NaOH buffer, pH 6.8 (1 g [wet weight] of cells per 2.0 ml of buffer) either by sonic disruption using a Branson S-110 Sonifier (Branson Instruments, Inc., Stanford, Conn.) at maximum output for 1 min or by a single passage through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 5 to 10 C. After centrifugation at 20,000 × *g* for 15 min the resulting crude extract was centrifuged at 100,000 × *g* for 1 h to give a high-speed supernatant. Such extracts contained 25 to 30 mg of protein per ml.

Materials. Hydroxyquinol (1,2,4-trihydroxybenzene) was obtained by hydrolysis of its triacetate (18) and was purified by filtering its ethereal solution through decolorizing carbon and crystallizing the product from ether-petroleum to give off-white crystals (melting point, 141 C; decomposition). Clemmenson reduction of 2,4-dihydroxybenzaldehyde gave 4-methylresorcinol (2). A sample of 2-hydroxy-5-methyl-1,4-benzoquinone was kindly provided by J. F. Corbett (Gillette Research Laboratories, Reading, England) and was also synthesized from 2-methyl-1,4-benzoquinone as described by Woodward et al. (46). Synthesis of 2-hydroxy-6-methyl-1,4-benzoquinone was accomplished by oxidizing orcinol with freshly prepared potassium nitrosodisulfonate (7) essentially as described by Musso (27). W. C. Evans (University College of North Wales, U.K.) kindly provided a sample of 2,3,5-trihydroxytoluene. Acetylpyruvic acid (2,4-dioxovaleric acid) as its disodium salt was obtained by alkaline hydrolysis of its methyl ester (25, 39) and was also purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were available from commercial sources.

Analytical procedures. Spectrophotometric determinations were made either with a fully automated Unicam SP800 or a Perkin-Elmer model 124 ultraviolet-visible spectrophotometer. Polarographic measurements of oxygen consumption were made with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Simultaneous measurements of absorbance and oxygen were made in a cuvette as described previously (37). Manometric measurements were made in conventional constant-volume Warburg manometers (Braun, Melsungen, West Germany).

Protein was estimated by the method of Lowry et al. (24). Pyruvate was estimated both by the Friedemann and Haugen method (10) and with a lactate dehydrogenase kit (Sigma Chemical Co., St. Louis, Mo.). Acetate was identified and determined by liquid-liquid chromatography on silica gel and quantitated by base titration (8). Gas chromatography of acetic acid on Porapak Q (Water Associates, Framingham, Mass.) was as described previously (9); detection was by flame ionization.

Chromatography. Pyruvate was identified by paper and thin-layer chromatography of its 2,4-dinitrophenylhydrazone derivatives in the solvent systems of Dagley and Gibson (8). Phenols were chromatographed on Whatman no. 4 paper in benzene-acetic acid (80:20 by volume, saturated with water) and butanol-acetic acid-water (4:5:1 by volume) and on silica gel layers (Eastman Organic Chemicals, Rochester, N.Y.) in petroleum ether-ethyl acetate (7:3 and 9:1 by volume) and detected by the use of Gibbs spray reagent (34). Procedures for the formation of trimethylsilyl derivatives and their examination by gas chromatography and mass spectrometry were as previously described (41).

RESULTS

Growth experiments. The mean generation time of *P. putida* O1 at 30 C was 53 to 58 min in 0.1% orcinol mineral salts media. Growth was limited in this medium by orcinol, which is toxic in concentrations greater than 0.15%. The yield of cells was approximately 1.75 g (wet weight)/liter per 1 g of orcinol supplied. Early in the growth period in fermenters, the medium turned brick red, but this color later disappeared. The material responsible for this color (λ_{max} = 485 nm) appeared to be a quinone, since it disappeared on treatment with sodium dithi-

onite. Its visible absorption spectrum was identical with that of 2-hydroxy-6-methyl-1,4-benzoquinone in neutral aqueous solution. Under the same conditions 2-hydroxy-5-methyl-1,4-benzoquinone also gave a brick-red solution (λ_{max} = 485 nm). Both quinones were readily reduced with dithionite. When 1 liter of colored culture filtrate was reduced by the addition of solid sodium dithionite and extracted with diethyl ether the dried extract yielded a dark-brown oil. Treatment of this oil with hexamethyl disilazane and trimethylchlorosilane in anhydrous tetrahydrofuran allowed the detection by gas chromatography-mass spectrometry of small amounts of a compound having a parent ion (electronic mass, 356) readily distinguishable from that of the trimethylsilyl derivative of orcinol (electron mass, 268). Neutral aqueous solutions of 2-hydroxy-6-methyl-1,4-benzoquinone could be similarly reduced and extracted to give a trimethylsilyl derivative having an electron mass of 356 with a mass spectrum indistinguishable from that of the above derivative. These observations are consistent with the presence of 2-hydroxy-6-methyl-1,4-benzoquinone in orcinol-grown cultures arising as a result of autooxidation of 2,3,5-trihydroxytoluene. Separate experiments with 2,3,5-trihydroxytoluene have shown that it is readily autooxidized to a brick-red product with λ_{max} = 485 nm in neutral aqueous solution.

Respiratory activities of P. putida after growth on orcinol. Both orcinol and resorcinol were oxidized without lag after growth of *P. putida* O1 on orcinol (Fig. 1A). Similar results were obtained with orcinol-grown *P. putida* ORC. The difference in the amount of oxygen consumed (5.35 mol of O_2/mol of orcinol and 3.6 mol of O_2/mol of resorcinol) corresponded to that required for the oxidation of a methylene fragment to carbon dioxide and water. Neither strain oxidized 3,5-dihydroxybenzoate, nor was it a growth substrate. Extensive oxidation of 2-methyl-, 4-methyl-, 4-ethyl-, 4-chloro-, or 4-bromoresorcinol by either strain grown with orcinol or resorcinol was not observed. A lag period of approximately 40 min was observed, before oxidation of orcinol or resorcinol occurred, with succinate-grown cells of *P. putida* ORC.

Oxidations catalyzed by extracts. Since both strains showed similar oxidative activities, only those data obtained with *P. putida* O1 are presented. Table 1 shows the distribution of reduced nicotinamide adenine dinucleotide (NADH) oxidase activity measured in the presence and absence of orcinol. NADH oxidation was markedly stimulated by orcinol in the 109,000 × *g* supernatant, compared with the 20,000 × *g* supernatant, due to the removal of the particulate NADH oxidase activity. When extracts of cells were supplemented with NADH, orcinol and resorcinol were oxidized, but 3,5-dihydroxybenzoate was not (Fig. 1B). Although not shown, NADPH was similarly effective. This suggested that the methyl group remained intact and that orcinol was first hydroxylated in the benzene ring by a soluble monooxygenase. 3,5-Dihydroxybenzoate is further excluded as an intermediate, since it did not accumulate as a product of orcinol oxidation

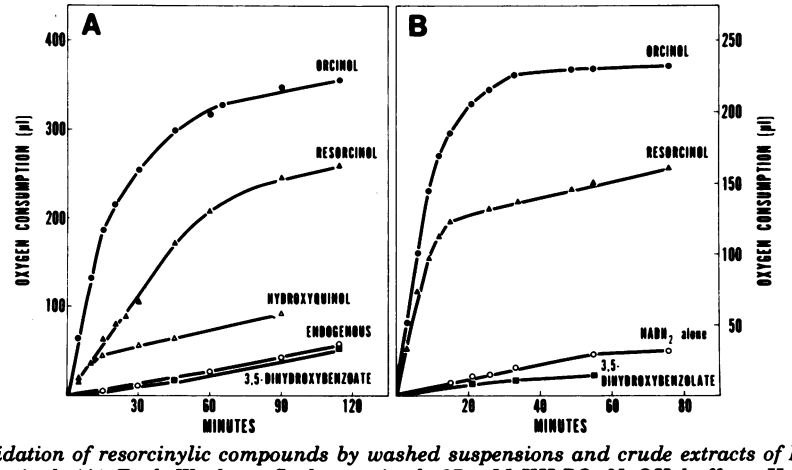

FIG. 1. *Oxidation of resorcinylic compounds by washed suspensions and crude extracts of P. putida O1 grown with orcinol. (A) Each Warburg flask contained: 67 mM KH_2PO_4-NaOH buffer, pH 7.1 (1.7 ml); washed cell suspension in buffer (1 ml, approximately 2 mg [dry weight] of cells); 25 mM substrate or water in side arm (0.1 ml); and 20% KOH in the center well (0.2 ml). (B) Each Warburg flask contained: 67 mM KH_2PO_4-NaOH buffer, pH 7.1 (1.2 ml); 25 mM substrate or water (0.2 ml); 25 mM NADH (0.2 ml); cell-free extract, 100,000 × g supernatant in side arm (0.2 ml); and 20% KOH in the center well (0.2 ml). Temperature, 30 C.*

TABLE 1. *Distribution of NADH oxidase and orcinol hydroxylase activities in extracts of P. putida O1*[a]

Fraction	NADH oxidation (μmol/min per mg of protein)	
	− Orcinol	+ Orcinol
20,000 × *g* supernatant	0.25	0.42
109,000 × *g* supernatant	0.05	0.32
109,000 × *g* debris	0.19	0.20

[a] NADH oxidase activities and orcinol-stimulated NADH oxidase activities were assayed by absorbance changes at 340 nm measured against reactions lacking NADH. Reaction mixtures contained 50 mM KH_2PO_4-NaOH buffer, pH 7.1 (2.8 ml); enzyme fraction (50 μl); 25 mM NADH (20 μl); and 25 mM orcinol (20 μl) as indicated. The 109,000 × *g* debris was resuspended in 50 mM KH_2PO_4-NaOH to give approximately the same volume from which it was derived. Temperature, 30 C. Similar results have been obtained by measurement of O_2 consumption, which is a better indication of "NADH oxidase" activities than the absorbance changes. In the absence of NADH, O_2 consumption is negligible ± orcinol added as substrate.

after the consumption of 2 mol of O_2 per mol of orcinol. Figure 2 shows a simultaneous assay of O_2 consumption and NADH disappearance at 366 nm. The stoichiometric relationships in this experiment and similar experiments using resorcinol as aromatic substrate are given in Table 2. The results for orcinol and resorcinol oxidation support the following stoichiometry: orcinol + $2O_2$ + NADH + H^+ → products + NAD^+ + H_2O; resorcinol + 1-$2O_2$ + NADH + H^+ → products + NAD^+ + H_2O. Similar experiments with *m*-cresol as a substrate were not reproducible; this is attributed to relatively low activity towards this substrate, although NADH and O_2 consumptions were equimolar. Chromatographic analysis of the deproteinized reaction mixtures showed that orcinol and resorcinol had disappeared but that *m*-cresol remained.

Ring cleavage enzymes in extracts. Since the initial enzyme of orcinol metabolism appeared to be a mono-oxygenase, and the benzene ring was absent after the consumption of 2 mol of O_2 per mol of orcinol, we tested 2,3,5-trihydroxytoluene as a substrate for a ring cleavage enzyme. This compound was rapidly oxidized with the consumption of 1 mol of oxygen per mol supplied by extracts of orcinol-grown cells but not by extracts of glucose-grown cells, with the transient appearance of spectral species absorbing at 320 nm (Fig. 3). Details of the spectral changes that occurred during the oxidation of 2,3,5-trihydroxytoluene are shown in Fig. 4. The interpretation of the spectral species observed is complicated by the various keto-enol tantomers (and their hydrates) as described by Guthrie (15). Similarly, hydroxyquinol and 3-methylcatechol (potential products of hydroxylation of the growth substrate analogues resorcinol and *m*-cresol) were also readily oxidized, but 1,2,3-trihydroxyben-

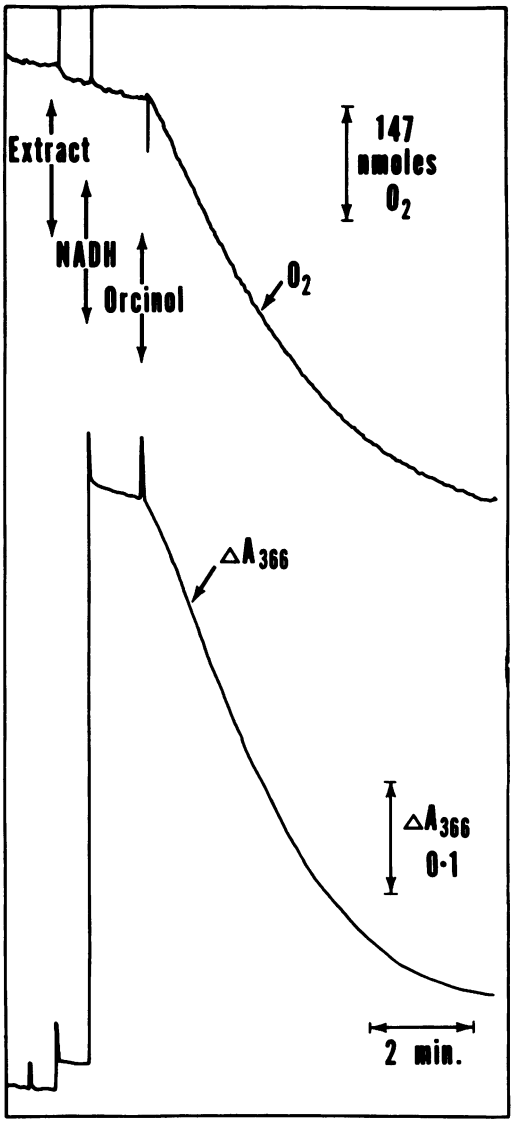

FIG. 2. *Simultaneous assay of oxygen consumption and NADH oxidation during orcinol oxidation by cell-free extracts of P. putida. The reaction mixture contained: 67 mM KH_2PO_4-NaOH buffer, pH 7.1 (3.3 ml); cell-free extract, 100,000 × g supernatant (0.1 ml); 25 mM NADH (20 μl); and 25 mM orcinol (10 μl) as indicated. Temperature, 24 C.*

TABLE 2. *Stoichiometry of orcinol and resorcinol oxidations by crude extracts of P. putida O1*[a]

Substrate	Amt of substrate supplied (μmol)	NADH supplied (μmol)	Oxygen consumption		
			μmol	μmol of O_2 per μmol of NADH supplied	μmol of O_2 per μmol of "phenol" supplied
Manometric assay					
	0	3.7	0.7[b]	–[c]	–
Orcinol	5	3.7	7.15	1.95	–
Resorcinol	5	3.7	4.54	1.23	–
Orcinol	5	7.4	9.8	–	1.96
Resorcinol	5	7.4	5.8	–	1.17
3,5-Dihydroxybenzoate	5	3.7	0.65[b]	0.18	–
Polarographic assay					
Orcinol	0.25	1.0	0.53	–	2.1
Resorcinol	0.25	1.0	0.36	–	1.4
Orcinol	0.25	0.2	0.49	2.45	–
Resorcinol	0.25	0.2	0.31	1.55	–

[a] Each reaction mixture contained: 50 mM KH_2PO_4-NaOH buffer, pH 7.1 (2.8 ml); cell extract (25 to 100 μl); aromatic substrates and NADH as indicated; and 20% KOH in the center well for manometric experiments. Final reaction volumes, 3 ml. Temperature, 30 C.

[b] Incomplete oxidations.

[c] –, Not relevant.

zene (pyrogallol), catechol, 4-methylcatechol, and 2,3-dihydroxybenzoate were not (Table 3).

Since 3-methylcatechol is a substrate for a ring cleavage enzyme induced during growth on orcinol, it was possible to establish whether 2,3-cleavage or 1,6-cleavage of this benzene nucleus occurred. When 3-methylcatechol was oxidized by these extracts, the reaction mixtures turned yellow, and 1 mol of O_2 was consumed per mol of 3-methylcatechol supplied (Table 3). There was no further degradation of this yellow reaction product even on incubation for as long as 1 h. The absorption spectra of the product in acid and base were identical to those obtained when 3-methylcatechol was a substrate for catechol 2,3-oxygenase from *P. aeruginosa* T1 (λ_{max}pH1, 315 nm; λ_{max}pH12, 388 nm) (35). Furthermore, the presumed product, 2-hydroxy-6-keto-2,4-heptadienoic acid, was rapidly degraded by extracts of *P. aeruginosa* T1 to acetate and pyruvate, as is 3-methylcatechol (1, 35). These experiments indicated that oxidative fission of 3-methylcatechol occurred between carbon atoms 2 and 3 to give 2-hydroxy-6-keto-2,4-heptadienoic acid. These data suggested that the site of ring cleavage of 2,3,5-trihydroxytoluene is between carbon atoms 1 and 2 and not 3 and 4, i.e., between carbon atoms substituted by methyl and hydroxyl groups.

Formation of oxo acids. Pyruvate was identified as a product of orcinol oxidation using these extracts, by formation of its 2,4-dinitrophenylhydrazone by the specific method of Friedemann and Haugen (10). It was chromatographed in three solvent systems, and its two geometric isomers co-chromatographed with authentic derivatives. The time course of pyruvate formation from orcinol is shown in Table 4. Independent assays of pyruvate using lactate dehydrogenase also demonstrated that 1 mol of pyruvate is formed per mol of orcinol, and that pyruvate is not metabolized further by these extracts. Pyruvate was also shown to be formed from 2,3,5-trihydroxytoluene by extracts of *P. putida* O1 and *P. putida* ORC.

Formation of acetate. The equimolar formation of pyruvate from orcinol suggested that the other four carbon atoms might appear as acetate or acetoacetate. The formation of acetate was shown, although we were unable to recover the expected quantities from any of the incubations. Table 5 shows the formation and recovery of acetate from incubations of extracts of orcinol-grown cells with orcinol and NADH, acetylpyruvate, acetoacetate, or acetate, and, as can be seen, recovery of acetate from control incubations was similarly low.

Hydrolysis of acetylpyruvate by extracts. Figure 5 shows the disappearance of acetylpyruvate when incubated with extracts of orcinol-grown and glucose-grown cells. The initial increase in absorbance at 275 nm can be attributed to the establishment of an equilibrium mixture of the known keto and enol tautomers and their hydrates (15). The products of this hydrolysis were shown to be acetate (Table 5)

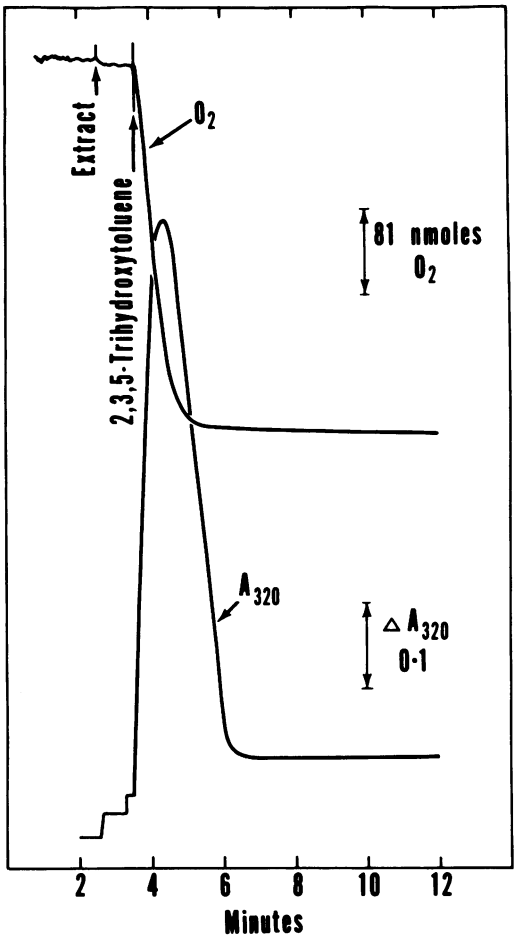

FIG. 3. *Simultaneous assay of oxygen consumption and absorbance changes at 320 nm during the oxidation of 2,3,5-trihydroxytoluene by cell-free extracts of P. putida. The reaction mixture contained: 67 mM KH_2PO_4-NaOH buffer, pH 6.5 (3.2 ml); cell-free extract (50 μl); and 25 mM 2,3,5-trihydroxytoluene (15 μl) as indicated. Temperature, 24 C.*

and pyruvate. In two separate experiments, 4 μmol of pyruvate was formed from 4 μmol of acetylpyruvate, respectively. Acetylpyruvate was not hydrolyzed by extracts of glucose-grown cells (Fig. 5). Acetoacetate hydrolysis was not observed by spectral assays, and only small amounts of acetate were formed in larger incubations (Table 5).

DISCUSSION

The results presented here suggest that orcinol is metabolized in *P. putida* O1 and *P. putida* ORC by hydroxylation to give 2,3,5-trihydroxytoluene, oxygenative ring cleavage between carbon atoms 1 and 2, and successive hydrolyses to yield 2 mol of acetate and 1 mol of pyruvate, via the intermediate acetylpyruvate (Fig. 6).

The evidence for this pathway is partly circumstantial. Several enzymic activities are expressed after growth of both strains of *P. putida* with orcinol: (i) the reduced nicotinamide nucleotide-dependent oxidation of orcinol, with the consumption of 2 mol of O_2 and the formation of acetate and pyruvate; (ii) the oxidation of 2,3,5-trihydroxytoluene and the analogues 3-methylcatechol and hydroxyquinol with the consumption of 1 mol of O_2 per mol of "catechol" supplied; (iii) the identification of the product of 3-methylcatechol oxidation as 2-hydroxy-6-keto-2,4-heptadienoate, showing that ring cleavage occurs between carbon atoms 2 and 3; (iv) the appearance of a spectral species similar to acetylpyruvate during the enzymic oxidation of 2,3,5-trihydroxytoluene; (v) the hydrolysis of acetylpyruvate to acetate and pyruvate. These activities are not observed in glucose- or in succinate-grown cells. The metabolic pathway is also consistent with evidence obtained of a negative nature. Thus, potential intermediates of orcinol metabolism which are possible on structural grounds, such as 3,5-dihydroxybenzoate and acetoacetate, were not attacked by extracts of orcinol-grown cells. Failure to observe acetoacetate disappearance may be due to a lack of cofactors for formation of its coenzyme A derivative (19, 45). Further support for the pathway proposed is derived from studies of the purified enzymes of the pathway such as orcinol hydroxylase (31, 32), 2,3,5-trihydroxytoluene-1,2-dioxygenase (Y. Ohta and D. W. Ribbons, Bacteriol. Proc., p. 124, 1970), and acetylpyruvate hydrolase (9). The position of hydroxylation of orcinol is suggested from the identification of hydroxyquinol as a product of resorcinol hydroxylation by crystalline orcinol hydroxylase (31). Furthermore, the transient accumulation of a hydroxyquinone, tentatively identified as 2-hydroxy-6-methyl-1,4-benzoquinone (hydroxytoluoquinone), during growth with orcinol suggests that it results from autooxidation of an early catabolite, 2,3,5-trihydroxytoluene.

The pathway of resorcinol metabolism by *Pseudomonas* sp. elucidated by Larway and Evans (Biochem. J. 95:52P, 1965) and the pathway of orcinol metabolism proposed from these studies suggest that a necessary condition for the degradation of 1,3-dihydroxybenzene compounds is the formation of 1,2,4-trihydroxy derivatives which are then substrates for ring fission enzymes. This conclusion is also supported by the data of Jeffrey et al. (22, 23), who have shown that the resorcinylic ring of taxi-

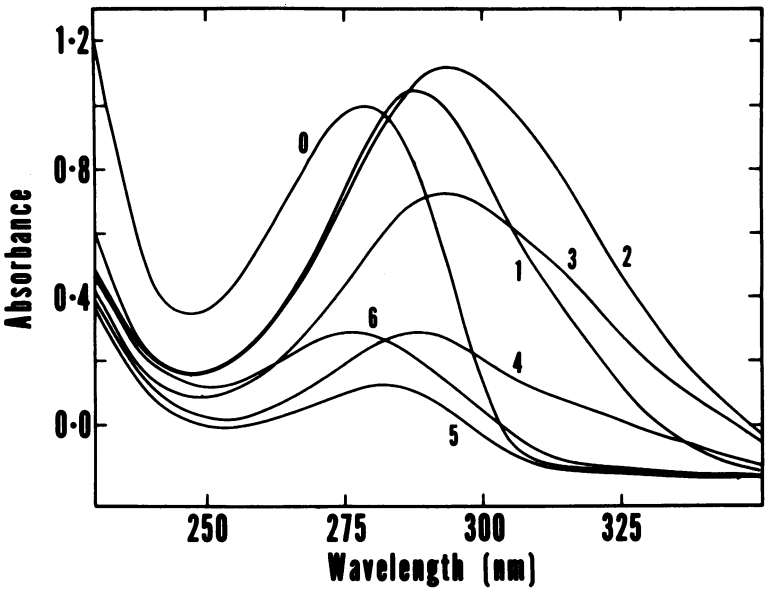

FIG. 4. *Spectral changes occurring during the oxidation of 2,3,5-trihydroxytoluene by cell-free extracts of P. putida. The reaction mixture contained: 67 mM KH_2PO_4-NaOH buffer, pH 6.5 (2.5 ml) and 25 mM 2,3,5-trihydroxytoluene (25 μl) (curve 0). Cell-free extract (100,000 × g supernatant) (10 μl) was added and the spectrum was scanned immediately (curve 1). Curves 2, 3, 4, 5, and 6 represent spectral scans made 30, 60, 90, 120, 150 and 300 s, respectively, after the addition of the extract. Temperature, 30 C.*

TABLE 3. *Oxidation of potential substrates of ring cleavage by extracts of P. putida O1*[a]

Substrate	Amt of substrate supplied (μmol)	Oxygen consumed	
		μmol	μmol/μmol of substrate supplied
2,3,5-Trihydroxytoluene	5[b]	4.3	0.86
	0.25[c]	0.257	1.03
	0.5[c]	0.46	0.92
3-Methylcatechol	5[b]	4.5	0.9
	0.25[c]	0.24	0.96
	0.5[c]	0.42	0.84
Hydroxyquinol	0.25	0.21	0.84
	0.375	0.33	0.88
Catechol	5[b]	0.4	0.08
	0.25	0	0

[a] Each reaction mixture contained: 50 mM KH_2PO_4-NaOH, pH 6.8 (2.8 ml); cell extract (10 to 100 μl); aromatic substrates as indicated; and 20% KOH in the center well for manometry. Final reaction volumes, 3 ml. Temperature, 30 C.

[b] Manometric assay.

[c] Polarographic assay.

folin is further hydroxylated to give a hydroxyquinol derivative before cleavage of the ring can occur. Schultz et al. (40) have also shown this phenomenon during quercetin catabolism. Usually, benzenoid compounds are metabolized to either *o*-dihydroxy- or *p*-dihydroxyderivatives, and these are the substrates for ring cleavage (4). This is not always the case, however, since Chamberlain and Dagley (3) showed that the pathway of thymol metabolism by *Pseudomonas* sp. proceeds via successive hydroxylations to give the trihydroxy compound 3-hydroxythymoquinol (3-methyl-6-isopropyl-1,2,4-trihydroxybenzene), which is the substrate for the ring cleavage enzyme. The

TABLE 4. *Pyruvate formation from orcinol*[a]

Time (min)	Pyruvate formed (μmol)	
	Expt A	Expt B
0.5	12.3	15.8
5	19.0	26.2
15	22.0	46.4
20	25.1	54.9
30	27.7	64.0
50	27.2	54.9

[a] Reaction mixtures contained 20 mM KH_2PO_4-NaOH buffer, pH 6.8 (8 ml); cell extract, 100,000 × *g* (0.2 ml); 25 mM NADH (2 ml); and 25 mM orcinol (A, 1 ml; B, 2 ml). Samples (1 ml) were taken at the times indicated and pipetted into 10% trichloroacetic acid (0.5 ml) and centrifuged. Suitable volumes of the supernatant were used for pyruvate estimation by the method of Friedemann and Haugen (10). Temperature of incubation, 30 C.

TABLE 5. *Acetate formation catalyzed by extracts of P. putida*[a]

Expt no.	Substrate	Amt of substrate supplied (μmol)	Incubation period (min)	Acetate recovered	
				μmol	mol/μmol of substrate supplied
1	Orcinol + NADH	150	30	40.2	0.27
2	Orcinol + NADH	5	120	6.0	1.2
3	Acetoacetate	100	40	4.3	0.04
4	Acetylpyruvate	100	40	53	0.53
5	Acetylpyruvate	5	120	3.5	0.7
6	Acetate	99	120	60	0.61

[a] Reaction mixture 1 contained: 20 mM KH_2PO_4-NaOH buffer, pH 6.8 (24 ml); cell extract (6 ml); 25 mM orcinol (6 ml); 25 mM NADH (6 ml). After shaking for 30 min at 30 C, 2 N H_2SO_4 (4 ml) was added to the reaction mixture, and the precipitated protein was centrifuged. The supernatant was extracted with equal volumes of ether (4×), and the pooled ether extracts were evaporated to 0.5 ml in a stream of N_2. The acetic acid formed was chromatographed on silicic acid and estimated by titration as described by Dagley and Gibson (8). Reaction mixtures 3, 4, and 6 differed only in the amounts of the various substrates supplied. Reaction mixtures 2 and 5, in Warburg flasks, contained: 20 mM KH_2PO_4-NaOH buffer, pH 6.8 (0.4 ml); cell extract (0.15 ml); 25 mM substrates as indicated (0.2 ml of each); and water to a final volume of 1 ml. Acidified samples were analyzed by gas chromatography for acetic acid formation.

transient formation of its highly colored 1,4-quinone was also observed in cultures (3). It has also been shown previously that 2,3,5-trihydroxytoluene is a metabolite of the herbicide 3,5-dinitro-*o*-cresol and that it is the substrate for ring cleavage (44).

All the ring cleavage dioxygenases which are known to act on substituted 1,2,4-trihydroxybenzene rings are of the *meta* or extradiol type, whereas that which cleaves hydroxyquinol is of the intradiol or *ortho* category. Evidence is presented elsewhere (5), however, to show that in certain strains of *P. putida* hydroxyquinol may undergo both *ortho* and *meta* cleavage.

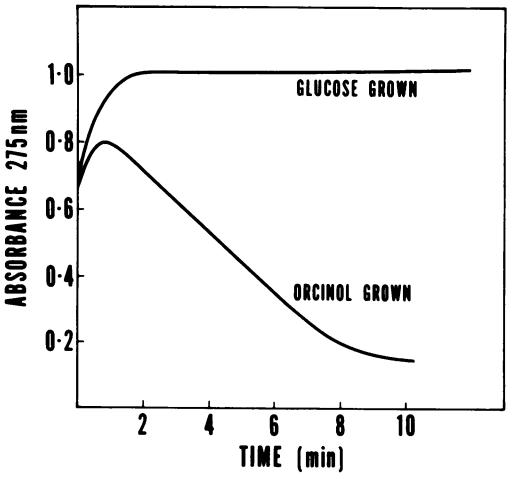

FIG. 5. *Spectral assay of acetylpyruvate hydrolysis by extracts of orcinol-grown and glucose-grown P. putida. The reaction mixture contained: 50 mM KH_2PO_4-NaOH buffer, pH 6.8 (2.9 ml) and extract (0.1 ml). Reactions were initiated by addition of 25 mM acetylpyruvate (20 μl) and assayed at 275 nm. Temperature, 26 C.*

Much of the evidence for 2,4,5-trioxoheptanoate as an orcinol metabolite is indirect. It is based on (i) the 2,3-cleavage of the analogue 3-methylcatechol by crude extracts of orcinol-grown cells, (ii) the spectral changes at longer wavelengths observed during the oxidation of 2,3,5-trihydroxytoluene, (iii) the formation of acetate and pyruvate as end products of reactions catalyzed by extracts, and (iv) the presence of these activities in orcinol- but not glucose-grown cells.

The participation of acetylpyruvate in the metabolic pathway is supported by the appearance of a compound with spectral properties similar to those of this oxoacid during 2,3,5-trihydroxytoluene oxidation by extracts and by the presence of acetylpyruvate hydrolase in orcinol-grown but not glucose-grown cells. Acetylpyruvate was earlier suggested as an intermediate on the *meta* fission pathway of catechol (30), although no evidence for its hydrolysis was obtained. More detailed studies of acetylpyruvate hydrolase from *P. putida* (9) and of an enzyme from beef liver catalyzing the same reaction (21) have been reported.

It was concluded initially that orcinol, resorcinol, and *m*-cresol were substrates for the hydroxylase reaction. The expected hydroxylated products (2,3,5-trihydroxytoluene, hydroxyquinol, and 3-methylcatechol, respectively) were shown to be substrates for a ring cleavage enzyme induced during growth on orcinol. It was therefore proposed that a single sequence of enzymes of low specificity had been synthesized which could oxidize these analogous sub-

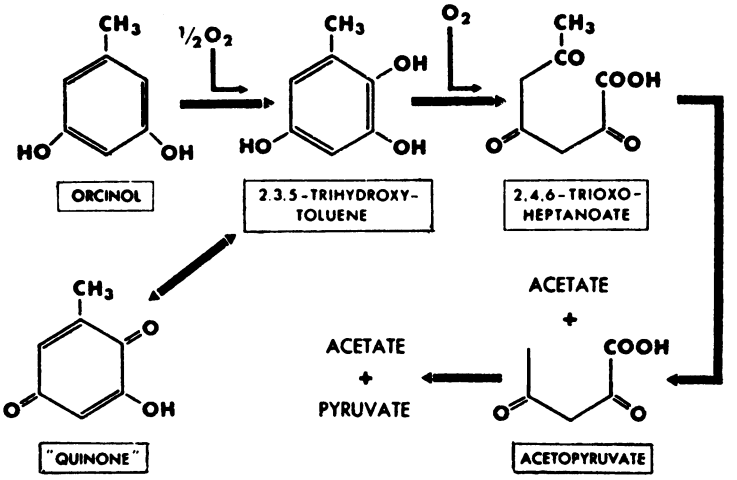

FIG. 6. *Pathway proposed for orcinol catabolism by P. putida O1 and ORC.*

strates into common cellular metabolites. This type of situation has been well documented for the bacterial oxidation of the cresols and xylenols (1, 20), certain reactions of camphor dissimilation (14), and latterly for alkyl-substituted benzoic acids (26). Similarly, the early enzymes of aromatic hydrocarbon oxidation show low specificity (13). However, it is now clear that this conclusion was erroneous for *m*-cresol at least; data are now available to show that *m*-cresol is not transformed to 3-methylcatechol by these extracts, but that this analogue completely uncouples the flow of electrons from NADH to oxygen from the hydroxylation reaction (38). This uncoupling is responsible for the observed oxygen consumption with *m*-cresol. It still seems possible that resorcinol may be completely catabolized by the enzymes of the orcinol pathway in one of these strains, *P. putida* O1. Data to support this conclusion are presented in the accompanying communication, but they also show that *P. putida* ORC utilizes another sequence of enzymes for growth on resorcinol (5).

ACKNOWLEDGMENTS

We are grateful to Edye E. Groseclose, John L. Michalover, Yoshiyuki Ohta, and Placida Venegas for help with these experiments, and to Tom Krick for skilled technical assistance in the operation of the mass spectrometry facilities provided and maintained by the Minnesota Agricultural Experiment Station.

This research was supported by grant no. GB44228 from the National Science Foundation to P.J.C. and Public Health Service grant no. GM 20172 from the National Institute of General Medical Sciences to D.W.R. D.W.R. was a Howard Hughes Medical Institute Investigator.

LITERATURE CITED

1. **Bayly, R. C., S. Dagley, and D. T. Gibson.** 1966. The metabolism of cresols by species of *Pseudomonas*. Biochem. J. 101:293-301.
2. **Bell, J. C., W. Bridge, and A. Robertson.** 1937. Constituents of the bark of *Zanthoxylum americanum* (Mill). IV. The constitution of xanthyletin. J. Chem. Soc., p.1542-1545.
3. **Chamberlain, E. M., and S. Dagley.** 1968. The metabolism of thymol by a *Pseudomonas*. Biochem. J. 110:755-763.
4. **Chapman, P. J.** 1972. An outline of reaction sequences used for the bacterial degradation of phenolic compounds, p. 17-55. *In* Degradation of synthetic organic molecules in the biosphere. Printing and Publishing Office, National Academy of Sciences, Washington, D. C.
5. **Chapman, P. J., and D. W. Ribbons.** 1976. Metabolism of resorcinylic compounds by bacteria: alternative pathways for resorcinol catabolism in *Pseudomonas putida*. J. Bacteriol. 125:985-998.
6. **Claus, D., and W. Hempel.** 1970. Specific substrates for isolation and differentiation of *Azotobacter vinelandii*. Arch. Mikrobiol. 73:90-96.
7. **Cram, D. J., and R. A. Reeves.** 1958. Macro rings. XIV. Substitution studies in the [4.4]paracyclophane system. J. Am. Chem. Soc. 80:3094-3103.
8. **Dagley, S., and D. T. Gibson.** 1965. The bacterial degradation of catechol. Biochem. J. 95:466-474.
9. **Davey, J. F., and D. W. Ribbons.** 1975. Metabolism of resorcinylic compounds by bacteria. Purification and properties of acetylpyruvate hydrolase. J. Biol. Chem. 250:3826-3830.
10. **Friedemann, T. E., and G. E. Haugen.** 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. 147:415-442.
11. **Garton, G. A., and R. T. Williams.** 1948. Studies in detoxication. 26. The fates of phenol, phenylsulfuric acid and phenylglucuronide in the rabbit, in relation to the metabolism of benzene. Biochem. J. 43:206-213.
12. **Garton, G. A., and R. T. Williams.** 1949. Studies in detoxication. 21. The fates of quinol and resorcinol in the rabbit in relation to the metabolism of benzene. Biochem. J. 44:234-238.
13. **Gibson, D. T.** 1972. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. 1:199-224.
14. **Gunsalus, I. C., P. J. Chapman, and W. J. Kuo.** 1965. Control of catabolic specificity and metabolism. Biochem. Biophys. Res. Commun. 18:924-931.
15. **Guthrie, J. P.** 1972. Acetopyruvic acid. Rate and equilibrium constants for hydration and enolization. J. Am. Chem. Soc. 94:7020-7024.

16. **Halvorson, H.** 1963. Formation of resorcinol as an intermediate in the metabolism of 2,4-dihydroxybenzoic acid and its corresponding aldehyde. Biochem. Biophys. Res. Commun. 10:440–443.
17. **Harborne, J. B., and N. W. Simmonds.** 1964. The natural distribution of phenolic aglycones, p. 77–128. *In* J. B. Harborne (ed.), Biochemistry of phenolic compounds. Academic Press Inc., London.
18. **Healey, M., and R. Robinson.** 1934. Some derivatives of hydroxyquinol including a synthesis of pyrylium salts of anthocyanidin type. J. Chem. Soc., p.1625–1631.
19. **Himes, R. H., and F. Lynen.** 1964. Metabolism of acetoacetate by *Achromobacter* IV S. Biochem. Z. 340:206–212.
20. **Hopper, D. J., and P. J. Chapman.** 1971. Gentisic acid and its 3- and 4-methyl substituted homologues as intermediates in the bacterial degradation of *m*-cresol, 3,5-xylenol and 2,5 xylenol. Biochem. J. 122:19–28.
21. **Hsiang, H. H., S. S. Sim, D. T. Mahuran, and D. E. Schmidt.** 1972. Purification and properties of a diketo acid hydrolase from beef liver. Biochemistry 11:2098–2102.
22. **Jeffrey, A. M., D. M. Jerina, R. Self, and W. C. Evans.** 1972. The bacterial degradation of flavonoids. Oxidative fission of the A-ring of dihydrogossypetin by a *Pseudomonas* sp. Biochem. J. 130:383–390.
23. **Jeffrey, A. M., M. Knight, and W. C. Evans.** 1972. The bacterial degradation of flavonoids. Hydroxylation of the A-ring of taxifolin by a soil pseudomonad. Biochem. J. 130:373–381.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
25. **Meister, A., and J. P. Greenstein.** 1948. Enzymatic hydrolysis of 2,4-diketo acids. J. Biol. Chem. 175:573–588.
26. **Murray, K., C. J. Duggleby, J. M. Sala-Trepat, and P. A. Williams.** 1972. The metabolism of benzoate and methylbenzoates via the meta-cleavage pathway by *Pseudomonas arvilla* mt-2. Eur. J. Biochem. 28:301–310.
27. **Musso, H.** 1958. Synthese, Konstitution und Lichtabsorption des Henrichschen Chinons. Chem. Ber. 91:349–363.
28. **Neufeld, H. A., F. M. Latterell, L. F. Green, and R. L. Weintraub.** 1958. Oxidation of meta-polyhydroxyphenols by enzymes from *Piricularia oryzae* and *Polyporus versicolor*. Arch. Biochem. Biophys. 76:317–327.
29. **Neujahr, H. Y., and J. M. Varga.** 1970. Degradation of phenols by intact cells and cell-free preparations of *Trichosporon cutaneum*. Eur. J. Biochem. 13:37–44.
30. **Nishizuka, Y., A. Ichiyama, S. Nakamura, and O. Hayaishi.** 1962. A new metabolic pathway of catechol. J. Biol. Chem. 237:268–270.
31. **Ohta, Y., I. J. Higgins, and D. W. Ribbons.** 1975. Metabolism of resorcinylic compounds by bacteria. Purification and properties of orcinol hydroxylase from *Pseudomonas putida* O1. J. Biol. Chem. 250:3814–3825.
32. **Ohta, Y., and D. W. Ribbons.** 1970. Crystallization of orcinol hydroxylase from *Pseudomonas putida*. FEBS Lett. 11:189–192.
33. **Pankhurst, E. S.** 1959. The biological oxidation of spent gas liquor. J. Appl. Bacteriol. 22:202–215.
34. **Reio, L.** 1958. A method for the paper-chromatographic separation and identification of phenol derivatives, mould metabolites and related compounds of biochemical interest, using a "reference system." J. Chromatogr. 1:338–373.
35. **Ribbons, D. W.** 1966. Metabolism of *o*-cresol by *Pseudomonas aeruginosa* strain T1. J. Gen. Microbiol. 44:221–231.
36. **Ribbons, D. W.** 1970. Specificity of monohydric phenol oxidations by meta cleavage pathways in *Pseudomonas aeruginosa* T1. Arch. Mikrobiol. 74:103–115.
37. **Ribbons, D. W., A. J. W. Hewitt, and F. A. Smith.** 1968. Apparatus for simultaneous spectrophotometric and polarographic measurements. Biotechnol. Bioeng. 10:238–242.
38. **Ribbons, D. W., and Y. Ohta.** 1970. Uncoupling of electron transport from oxygenation in the monooxygenase, orcinol hydroxylase. FEBS Lett. 12:105–108.
39. **Royals, E. E.** 1945. The use of sodium methoxide in the Claisen reaction. J. Am. Chem. Soc. 67:1508–1509.
40. **Schultz, E., F. E. Engle, and J. M. Wood.** 1974. New oxygenases in the degradation of flavones and flavanones by *Pseudomonas putida*. Biochemistry 13:1768–1776.
41. **Sparnins, V. L., P. J. Chapman, and S. Dagley.** 1974. Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. J. Bacteriol. 120:159–167.
42. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic Pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159–271.
43. **Stephayne, D.** 1961. Oxidation of metadihydroxyphenols by enzymes of *Piricularia oryzae*. J. Bacteriol. 82:617–618.
44. **Tewfik, M. S. and W. C. Evans.** 1966. The metabolism of 3,5-dinitro-*o*-cresol (DNOC) by soil micro-organisms. Biochem. J. 99:31–32.
45. **Winnacker, E. L., and H. A. Barker.** 1971. Aerobic metabolism of β-amino-n-butyric acid by *Pseudomonas putida*. Biochim. Biophys. Acta 237:284–292.
46. **Woodward, R. B., F. Sondheimer, D. Taub, K. Hensler, and W. M. McLamore.** 1952. The total synthesis of steroids. J. Am. Chem. Soc. 74:4223–4251.