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Phthalate Metabolism in *Pseudomonas testosteroni*: Accumulation of 4,5-Dihydroxyphthalate by a Mutant Strain

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A mutant strain of *Pseudomonas testosteroni* blocked in phthalate catabolism converted phthalate into 4,5-dihydroxyphthalate. The latter compound was isolated, and its physical properties were determined. A stoichiometric conversion of the compound to protocatechuate was demonstrated spectrophotometrically with crude extracts of a protocatechuate 4,5-dioxygenase-deficient mutant. Therefore, phthalate is metabolized through 4,5-dihydroxyphthalate and protocatechuate, which is further degraded by protocatechuate 4,5-dioxygenase in *P. testosteroni*. By using several mutants blocked in phthalate catabolism, 4,5-dihydroxyphthalate decarboxylase was shown to be induced by phthalate. A simple spectrophotometric assay for the enzyme is also reported.

Phthalate esters, commonly used as plasticizers and produced in large quantities, are known pollutants of our environment (8). In recent years, considerable attention has been directed to the toxicity of these compounds (1), with little information available on microbial degradation. Engelhardt et al. (5) reported that several microorganisms were able to utilize di-*n*-butyl phthalate and related dialkyl phthalates as carbon sources, and phthalate was suggested as an intermediate in the biodegradation of phthalate esters to protocatechuate. Evans (Biochem. J. 61:x, 1955) identified the presence of 4,5-dihydroxyphthalate from early-log-phase cultures of a soil pseudomonad growing on phthalate. Ribbons and Evans (11) demonstrated an enzyme, 4,5-dihydroxyphthalate decarboxylase, that converts 4,5-dihydroxyphthalate to protocatechuate and carbon dioxide under anaerobic conditions. The subsequent oxidation of protocatechuate to β-ketoadipate suggested the operation of the *ortho*-cleavage pathway of protocatechuate in this organism.

According to a taxonomic study by Stanier et al. (12), the fluorescent group of *Pseudomonas* (*Pseudomonas aeruginosa*, *P. putida*, and *P. fluorescens*) as well as most other species of *Pseudomonas* have the *ortho*-cleavage pathway of protocatechuate. The two species of the acidovorans group, *Pseudomonas acidovorans* and *P. testosteroni*, are distinguished by the *meta*-cleavage pathway for the utilization of protocatechuate (3, 4, 12, 14). In addition to this and some other systematic biochemical pathways, the acidovorans group differs from other species in terms of deoxyribonucleic acid composition (7) and nutritional characters (12). Considering these facts, studies on phthalate metabolism were carried out to determine whether the compound is degraded through 4,5-dihydroxyphthalate and protocatechuate, as was reported in other species of *Pseudomonas* (11).

We shall describe here the isolation of a mutant strain of *P. testosteroni* that accumulates 4,5-dihydroxyphthalate after incubation with phthalate.

MATERIALS AND METHODS

Bacterial strains. A soil pseudomonad isolated by enrichment culture on phthalate and used for the purification of protocatechuate 4,5-dioxygenase (EC 1.13.11.8) by Ono et al. (9) was obtained from M. Nozaki of the Department of Biochemistry, Shiga Medical College, Shiga, Japan. The strain was tentatively identified as *P. testosteroni* according to the methods described by Stanier et al. (12) (see Results). The wild-type and mutant strains were given NH designations.

Chemicals. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Daiichi Pure Chemical Co. Ltd., Tokyo, Japan, and D-cycloserine and dithiothreitol were obtained from Nakarai Chemicals Ltd., Kyoto, Japan. Phthalic acid disodium salt, *p*-hydroxybenzoic acid sodium salt, *m*-hydroxybenzoic acid, and protocatechuic acid were obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan, and penicillin G potassium salt was obtained from Meiji Seika Co. Ltd., Tokyo, Japan. All other chemicals were of reagent grade and commercially available.

Media and culture conditions. Medium M9 (2) without carbon sources was used as a basal medium. Nutrient agar (Eiken Chemicals Co. Ltd., Tokyo, Japan) and medium M9 containing 1.5% agar (M9 agar) supplemented with L-glutamate or aromatic substrates were used as solid media. Basal medium containing 20 mM L-glutamate (medium G) was used where indicated. Cells used for enzyme sources

were grown in medium G containing 0.05% yeast extract (Difco Laboratories, Detroit, Mich.) (medium GY). All the incubations were carried out at 27°C.

Isolation of mutants. Mutant strains of *P. testosteroni* unable to grow on aromatic substrates were prepared by nitrosoguanidine mutagenesis followed by penicillin-cycloserine screening. An overnight culture of the wild type, NH1000, in 20 ml of medium G was treated with 300 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in 0.1 M citrate buffer (pH 5.5) for 60 min without shaking, and the cells were washed twice with medium M9. The cells were suspended in 10 ml of medium G and incubated for 2 h with shaking, followed by washing with medium M9. These cells were resuspended in 100 ml of medium M9 containing 5 mM phthalate and incubated for 4 h before the addition of 10 mg of D-cycloserine and 10^6 U of penicillin G. After incubation for 14 h with shaking to allow cell lysis, the remaining cells were collected and washed with and suspended in medium M9. In one experiment, cells were suspended in medium G and incubated for 3 h, followed by a second treatment with penicillin-cycloserine. The cell suspensions were appropriately diluted and spread on M9 agar containing 20 mM L-glutamate. After incubation for 24 h, the resulting colonies were transferred to nutrient agar and replicated on M9 agar containing 10 mM phthalate, *p*-hydroxybenzoate, *m*-hydroxybenzoate, or 5 mM protocatechuate. Among 3,000 colonies examined, 40 clones showing negative growth on either of these plates were obtained. All the mutants used in this study reverted spontaneously to the wild type at frequencies of 10^{-6} to 10^{-9}.

Analytical methods. Ultraviolet spectra were determined with a Union SM-401 high-sensitivity recording spectrophotometer equipped with a repeat scan programmer. Mass spectra were determined with a Hitachi RMU-6MG mass spectrometer, using a direct insertion probe (performed at the Hitachi Factory, Hachioji, Japan). Nuclear magnetic resonance spectra were recorded on a JEOL JNM-4H-1000 spectrometer. Absorptions were assigned δ values at the midpoint of half-height, with tetramethylsilane as the reference.

Isolation of compound I. A mutant strain, NH1024, was grown in 1 liter of medium GY containing 5 mM phthalate. Cells were harvested, washed with 50 mM tris(hydroxymethyl)aminomethane (Tris)-acetate (pH 7.5), and resuspended at a density of 2×10^9 per ml in 500 ml of the same buffer containing 10 mM phthalate. The accumulation of compound I in the reaction mixture was monitored by measuring ultraviolet spectra of samples diluted 50-fold with the same buffer and filtered through membrane filters (type HA, 0.45 μm; Millipore Corp., Bedford, Mass.) (see Results). After complete conversion of phthalate to compound I, cells were removed by centrifugation followed by filtration through a membrane filter. The filtrate was applied on a column (4 by 10 cm) of Dowex 1 (Cl^- form, 100 to 200 mesh). The column was washed extensively with distilled water and eluted with a linear gradient of 1,000 ml of NH_4Cl, pH 8.0, from 0.1 to 1.0 M. Ultraviolet spectra of the eluted fractions were determined, and those containing compound I were pooled. A small amount of remaining phthalate in the reaction mixture appeared just before the bulk of compound I. No other compounds of ultraviolet-absorbing material were detected. The pooled fractions containing compound I (290 ml) were adjusted to pH 2.0 with HCl and extracted repeatedly with ethyl ether. The organic extract was dried over anhydrous sodium sulfate, and the solvent was removed by flash evaporation at 32°C, leaving a brownish-white solid. The yield was approximately 50%, based on the number of moles of phthalic acid added. Paper chromatography of the obtained material, carried out according to the method described by Ribbons and Evans (11), gave a single spot with an R_f value similar to that reported for 4,5-dihydroxyphthalic acid.

A solution of compound I dissolved in ethyl ether was treated with diazomethane to give methylated compound I, which was obtained in a crystalline form.

Preparation of crude extracts. Cells grown in medium GY in the absence or presence of inducer were harvested by centrifugation and washed with medium M9. For a source of protocatechuate 4,5-dioxygenase, cells were suspended in 50 mM potassium phosphate (pH 7.5) containing 10% ethanol (buffer A) to prevent inactivation of the enzyme (9). For the preparation of extracts used for the assay of 4,5-dihydroxyphthalate decarboxylase, cells were suspended in 50 mM Tris-acetate (pH 7.5) containing 0.2 mM dithiothreitol and 0.2 mM ethylenediaminetetraacetate (buffer B). Cells were disintegrated for 1 min in an ice bath with a 20-kc probe-type Tomy sonic oscillator, model UR 105P (Tomy Seiko Co. Ltd., Tokyo, Japan). The preparations were centrifuged at $12,000 \times g$ for 30 min at 2°C to remove whole cells and cell debris and used immediately for the enzyme assays.

Enzyme assays. The activity of protocatechuate 4,5-dioxygenase was determined by measuring the rate of increase of α-hydroxy-γ-carboxymuconic semialdehyde according to the method of Ono et al. (9). The reaction mixture contained, in a total volume of 3.0 ml, 150 μmol of Tris-acetate (pH 9.0), 1.0 μmol of protocatechuic acid, and an adequate amount of enzyme in a cuvette with a 10-mm light path. The reaction was started by the addition of enzyme, and the initial rate of increase at 410 nm was measured at 27°C with a Union SM-401 high-sensitivity recording spectrophotometer. Based on the molar extinction coefficient of the product (9), the formation of 1 μmol of the product corresponded to an increase of 3.73 optical density units at 410 nm.

The activity of 4,5-dihydroxyphthalate decarboxylase was assayed at 27°C by measuring the rate of decrease in absorbance at 230 nm, due to the conversion of 4,5-dihydroxyphthalate to protocatechuate (see Results). The reaction mixture, in a cuvette with a 10-mm light path, contained 0.25 μmol of 4,5-dihydroxyphthalate, 125 μmol of Tris-acetate (pH 7.5), and the enzyme (less than 150 μg of protein) in a total volume of 2.5 ml. The reference cuvette contained the substrate and the buffer. The reaction

was started by the addition of enzyme, and the initial rate of decrease at 230 nm was recorded. The conversion of 1 μmol of the substrate to the product corresponded to a decrease of 2.2 optical density units at 230 nm.

The unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate into product per min at 27°C. Protein was determined by the biuret method (6), with bovine serum albumin as a standard. The specific activity of enzyme was defined as units of enzyme activity per milligram of protein.

RESULTS

Identification of the organism. The organism used for the purification of protocatechuate 4,5-dioxygenase was previously identified as a species of *Pseudomonas* (9). Further identification has been carried out according to the method described by Stanier et al. (12). The organism had polar multitrichous flagella, produced no pigment, and did not require any growth factors. It conformed perfectly to the ideal phenotype for the acidovorans group, i.e., accumulation of poly-β-hydroxybutyrate as cellular reserve material, utilization of adipate, pimelate, glycolate, levulinate, itaconate, *m*-hydroxybenzoate, and norleucine, and non-utilization of L-arabinose, D-glucose, D-galactose, α-ketogluconate, pelargonate, and putrescine. Based on the organism's ability to utilize benzoate and testosterone as well as the inability to utilize D-fructose, mannitol, malonate, maleate, L-tartrate, quinate, ethanol, phenylacetate, D-tryptophan, L-tryptophan, β-alanine, DL-α-aminobutyrate, γ-aminobutyrate, δ-aminovalerate, and acetamide, the organism, designated NH1000 in this study, was tentatively identified as *P. testosteroni*.

Phthalate metabolism in *P. testosteroni* NH1000. *P. testosteroni* dissimilates *m*- and *p*-hydroxybenzoate through protocatechuate and α-hydroxy-γ-carboxymuconic semialdehyde (14). Other pathways such as the *ortho*-cleavage pathway of protocatechuate and the catechol dissimilation pathways, widely distributed and commonly used for the degradation of many aromatic compounds in *Pseudomonas*, are absent from this species. Therefore, it was expected that degradation of phthalate in the test strain, NH1000, would be by the *meta*-cleavage pathway of protocatechuate. In fact, a mutant strain, NH1027, unable to utilize protocatechuate showed a pleiotropic effect on the utilization of phthalate and *m*- and *p*-hydroxybenzoate (Table 1). Other mutants listed in Table 1 were unable to grow on either phthalate, *m*-hydroxybenzoate, or *p*-hydroxybenzoate, but were able to grow on protocatechuate. Therefore, the conversion of phthalate to protocatechuate appears not to involve either of the two hydroxybenzoates.

Table 2 shows the protocatechuate 4,5-dioxygenase activity of *P. testosteroni* NH1000 and derived mutants grown in the absence or presence of inducers. The enzyme of the wild type, NH1000, was increased by 5- and 10-fold by growth with phthalate and protocatechuate, respectively, whereas that of a protocatechuate-nonutilizing mutant, NH1027, was not induced at all by the inducers. A spontaneous revertant, NH1027R, obtained by spreading a thick cell suspension of NH1027 on phthalate-minimal agar, showed the same levels of the enzyme as those of the wild type. In a phthalate-nondegrading mutant, NH1024, the enzyme was not induced by phthalate but was induced by its substrate, protocatechuate. These results further confirmed the above findings that phthalate is degraded through protocatechuate and α-hydroxy-γ-carboxymuconic semialdehyde in *P. testosteroni* NH1000 and suggested that protocatechuate 4,5-dioxygenase is induced by the substrate, protocatechuate.

Accumulation of compound I by strain NH1024. Phthalate shows an ultraviolet spectrum with an absorption peak at 223 nm and a broad absorption band around 280 nm in 50 mM Tris-acetate (pH 7.5). When washed cells of

TABLE 1. *Growth response of P. testosteroni NH1000 and derived mutants*

Strain	Growth substrate phenotype[a]			
	Pht	Mhb	Phb	Pro
NH1000 (wild type)	+	+	+	+
NH1027	−	−	−	−
NH1024	−	+	+	+
NH1028	+	−	+	+
NH1032	+	+	−	+

[a] +, Growth; −, no growth. Phenotype abbreviations and concentrations of compounds in M9 agar. Pht, phthalate, 10 mM; Mhb, *m*-hydroxybenzoate, 10 mM; Phb, *p*-hydroxybenzoate, 10 mM; Pro, protocatechuate, 5 mM.

TABLE 2. *Specific activity of protocatechuate 4,5-dioxygenase in P. testosteroni and derived mutants grown in the absence or presence of phthalate (5 mM) or protocatechuate (2.5 mM)*

Strain	Sp act[a]		
	Noninduced	Phthalate-induced	Protocatechuate-induced
NH1000	0.057	0.254	0.633
NH1027	0.053	0.047	0.064
NH1027R	0.071	0.187	0.516
NH1024	0.055	0.043	0.303

[a] For definition of specific activity, see the text.

NH1024 grown in the presence of phthalate were incubated with phthalate, the spectrum changed, suggesting the formation of a new compound (Fig. 1). After the reaction was completed, the reaction product(s) was purified by passing it through a Dowex 1 column and obtained as a brownish-white solid (compound I).

Identification of compound I and its methyl derivative. The ultraviolet spectrum of compound I in 50 mM Tris-acetate (pH 7.5) showed λ_{max} = 225 nm (ϵ = 5,700) with shoulders at 250 and 290 nm (Fig. 2). The mass spectrum of compound I showed that the molecular ion has a mass of 198 (Fig. 3). Analyses were also performed with methylated compound I, which had a melting point of 85 to 87°C. Chemical analysis of the latter compound was: $C_{12}H_{14}O_6$; calculated: C, 56.69; H, 5.55; found: C, 57.00; H, 5.55. This observation was confirmed by its nuclear magnetic resonance spectrum, which showed signals at: 7.18; 2H, singlet (two equivalent protons); 3.93, 6H, singlet (two methyl group protons); 3.88, 6H, singlet (the other two methyl group protons) (Fig. 4). The above results established the structure of compound I as 4,5-dihydroxyphthalate.

Enzymatic conversion of 4,5-dihydroxyphthalate to protocatechuate. When 4,5-dihydroxyphthalate (0.1 mM) in Tris-acetate (pH

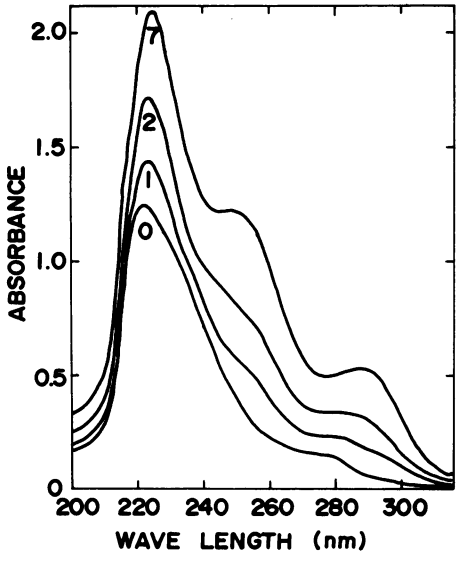

FIG. 1. *Spectral changes of phthalate incubated with cells of NH1024. Incubation mixture contained 10 mM phthalate, 50 mM Tris-acetate (pH 7.5), and freshly harvested cells of NH1024 (2 × 10⁹ cells per ml). At the times indicated (in hours) absorption spectra were recorded with a sample (0.5 ml) diluted with 25 ml of 50 mM Tris-acetate, pH 7.5, and filtered through a membrane filter.*

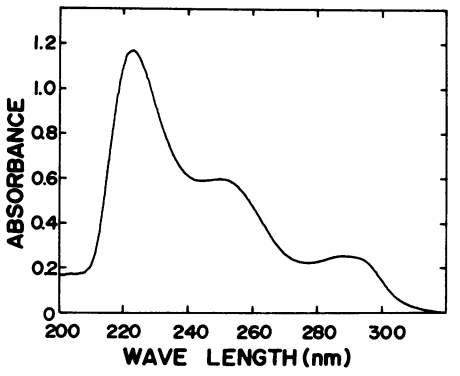

FIG. 2. *Ultraviolet spectrum of 4,5-dihydroxyphthalate. The sample was dissolved in 50 mM Tris-acetate (pH 7.5) at a concentration of 0.1 mM.*

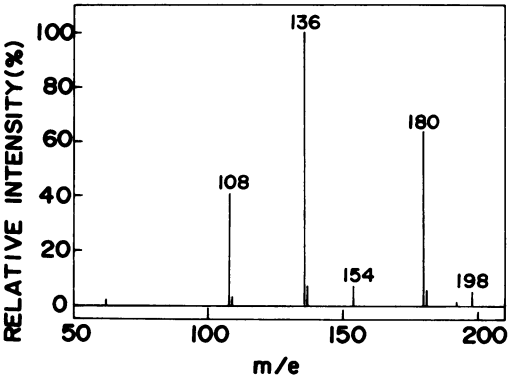

FIG. 3. *Mass spectrum of 4,5-dihydroxyphthalic acid.*

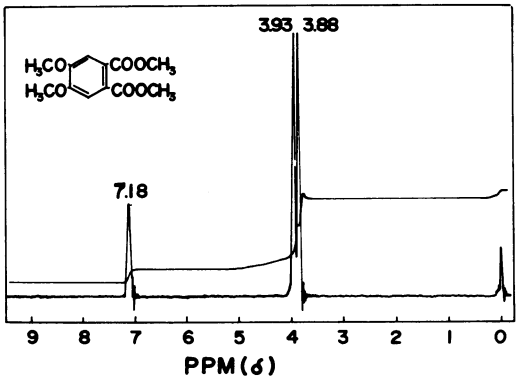

FIG. 4. *Nuclear magnetic resonance spectrum of 4,5-dimethoxyphthalic acid dimethyl ester. The sample was dissolved in $CdCl_3$, and the spectrum was recorded at 100 MHz. Tetramethylsilane was used as the internal standard.*

7.5) was incubated with the crude extract of NH1027 cells grown in the presence of phthalate, the ultraviolet spectra of the reaction mixture changed with time, resulting in a final spectrum with the absorption maxima at 219, 245, and 288 nm (Fig. 5). The final spectrum was identical to that of 0.1 mM protocatechuate in the same buffer, indicating a stoichiometric conversion of 4,5-dihydroxyphthalate to protocatechuate. Since the crude extract used in this experiment was prepared in the absence of ethanol, the residual activity of protocatechuate 4,5-dioxygenase (Table 2) was no longer stable (14) and evidently insufficient to remove protocatechuate. When protocatechuate 4,5-dioxygenase-containing extract of NH1024 (170 μg of protein) grown in the presence of protocatechuate and prepared in buffer A was introduced to the reaction mixture, protocatechuate was oxygenated to α-hydroxy-γ-carboxymuconic semialdehyde, as evidenced by the formation of a characteristic absorption peak at 410 nm in alkaline conditions (9). These results indicated that 4,5-dihydroxyphthalate was stoichiometrically converted to protocatechuate by the action of 4,5-dihydroxyphthalate decarboxylase present in the crude extract of NH1027 cells.

Spectrophotometric assay of 4,5-dihydroxyphthalate decarboxylase. From the difference in the optical densities at 230 nm between 4,5-dihydroxyphthalate and protocatechuate, the conversion of 1 μmol of the substrate to the product in the reaction mixture of 2.5 ml containing 125 μmol of Tris-acetate, pH 7.5, was calculated to give a decrease of 2.2 optical density units. The reaction velocity of 4,5-dihydroxyphthalate decarboxylase, determined by the rate of decrease in absorption at 230 nm, was linear with time as well as with the amount of the crude extract of NH1027 cells added (up to 126 μg of protein) (Fig. 6). Similar results were obtained with the crude extract of NH1000 cells, suggesting that further degradation of protocatechuate did not disturb this assay under these conditions. The optimal pH for the reaction was determined to be 7.5 to 8.0 and gave somewhat higher activities in 50 mM Tris-acetate buffer than were observed with the same concentration of potassium phosphate buffer. All these experiments were carried out with 0.1 mM substrate, and the reaction velocity was not increased by doubling the concentration of the substrate, but a decrease of about 50% in reaction velocity was observed with 0.01 mM substrate. The reaction was not stimulated by the addition of 0.1 mM $MgCl_2$ and/or 20 μM pyridoxal-5'-phosphate. Based upon these results, a standard reaction mixture for the spectrophotometric assay of 4,5-dihydroxyphthalate decarboxylase was established that contained 0.25 μmol of 4,5-dihydroxyphthalate, 125 μmol of Tris-acetate (pH 7.5), and enzyme in a total volume of 2.5 ml.

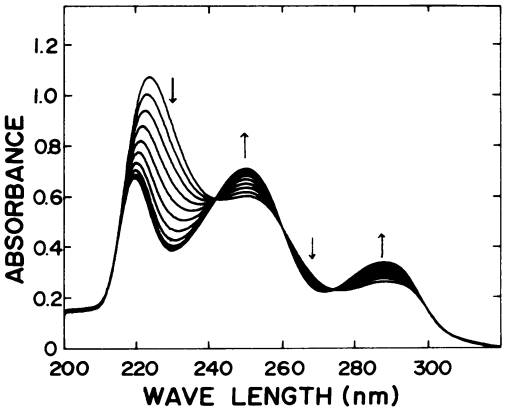

FIG. 5. *Absorption spectra of reaction mixture during the conversion of 4,5-dihydroxyphthalate into protocatechuate. The reaction mixture contained 0.1 mM 4,5-dihydroxyphthalate, 50 mM Tris-acetate (pH 7.5), and the crude extract (19 μg of protein) of NH1027 cells grown in the presence of phthalate and prepared in buffer B in a total volume of 2.5 ml. Spectra were recorded automatically at 2-min intervals at 27°C, using a repeat scan programmer. Arrows show a decrease (↓) or increase (↑) in absorption at the indicated wavelength.*

Induction of 4,5-dihydroxyphthalate decarboxylase. Table 3 shows the specific activities of 4,5-dihydroxyphthalate decarboxylase in noninduced or phthalate-induced cells of the wild type, NH1000, and various phthalate-nondegradative mutants. In NH1000 the enzyme

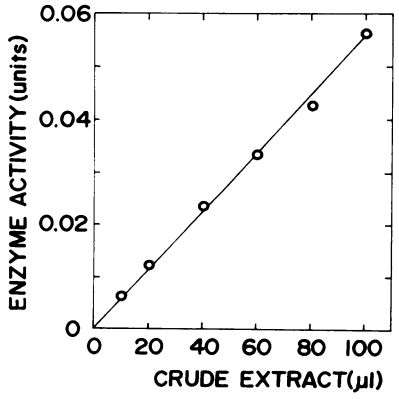

FIG. 6. *Effect of enzyme concentration on 4,5-dihydroxyphthalate decarboxylase activity. The reaction mixture was the same as in Fig. 5, except that the amount of the crude extract (1.26 mg of protein per ml) was changed.*

activity increased 100-fold when phthalate was added to the growth medium, whereas no activity was detected in NH1024 cells. The other phthalate-nondegradative mutants, NH1021 and NH1051, blocked in the conversion of phthalate to 4,5-dihydroxyphthalate as indicated by the ability to utilize 4,5-dihydroxyphthalate but not phthalate itself, produced high levels of the enzyme by growing in the presence of phthalate. One of the mutants, NH1021, did not cause any change in the ultraviolet spectrum of phthalate, whereas NH1051 accumulated a nonidentified intermediate during incubation with phthalate (data not shown). Since these mutants were not able to produce 4,5-dihydroxyphthalate but did produce high levels of enzyme comparable to the wild type or NH1027, it was concluded that 4,5-dihydroxyphthalate decarboxylase is induced by phthalate.

DISCUSSION

The dissimilatory pathway of phthalate was previously reported in a soil pseudomonad (11) that is probably different from *P. testosteroni*, as suggested by the operation of the *ortho*-cleavage pathway of protocatechuate. Although *P. testosteroni* degrades protocatechuate via the *meta*-cleavage pathway, it is evident from the present study that phthalate is also metabolized through 4,5-dihydroxyphthalate and protocatechuate in this species. Figure 7 shows a hypothetical reaction scheme of the dissimilatory pathway of phthalate in *P. testosteroni* where 2,6-cyclohexadiene-4,5-diol-1,2-dicarboxylic acid (compound II) is assumed to be an intermediate, as suggested previously (11) and indicated by recent communications concerning *P. fluorescens* (P. Keyser and D. W. Ribbons, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, I60, p. 126). The reaction sequence from phthalate to 4,5-dihydroxyphthalate is analogous to that of the dissimilatory pathway of benzoate, which is converted to catechol via 3,4-cyclohexadiene-1,2-diol-1-carboxylic acid (10). The latter pathway is initiated by the action of benzoate 1,2-dioxygenase (15), followed by dehydrogenation of the intermediate to form catechol. In addition to the mutant NH1024, which accumulated 4,5-dihydroxyphthalate from phthalate, some other mutants were isolated. One of them, NH1051, accumulated a nonidentified compound upon incubation with phthalate. This mutant was suggested to have a block in the conversion of phthalate to 4,5-dihydroxyphthalate, indicated by its ability to utilize 4,5-dihydroxyphthalate but not phthalate itself. The identification of the accumulated compound as well as the demonstration of phthalate oxygenase activity are now in progress in our laboratory.

TABLE 3. *Induction of 4,5-dihydroxyphthalate decarboxylase in P. testosteroni NH1000 and derived mutants*

Strain	Growth substrate phenotype[a]	Addition of inducer[b]	Sp act[c]
NH1000	Pht$^+$ Dhp$^+$ Pro$^+$	None	0.003
NH1000	Pht$^+$ Dhp$^+$ Pro$^+$	Phthalate	0.362
NH1024	Pht$^-$ Dhp$^-$ Pro$^+$	Phthalate	<0.001
NH1021	Pht$^-$ Dhp$^+$ Pro$^+$	Phthalate	0.420
NH1051	Pht$^-$ Dhp$^+$ Pro$^+$	Phthalate	0.149
NH1027	Pht$^-$ Dhp$^-$ Pro$^-$	Phthalate	0.447

[a] Phenotype abbreviations and concentrations of compounds in M9 agar: Dhp, 4,5-dihydroxyphthalate, 5 mM; for others, see footnote, Table 1.

[b] The concentration of the inducer added was 5 mM.

[c] For definition of specific activity, see the text.

Ribbons and Evans (11) reported a manometric assay of 4,5-dihydroxyphthalate decarboxylase by measuring the decarboxylation under anaerobic conditions. An alternative assay described in this study is based on the difference in ultraviolet absorption between 4,5-dihydroxyphthalate and protocatechuate. The assay was not disturbed by the subsequent reactions, thus providing a simple and sensitive method to determine the enzyme activity. By using this method we have recently purified 4,5-dihydroxyphthalate decarboxylase from NH1027 cells. It was revealed that no cofactor is required for the reaction, even with highly purified preparations (manuscript in preparation).

Phthalate was shown to be an inducer of 4,5-dihydroxyphthalate decarboxylase (Table 3).

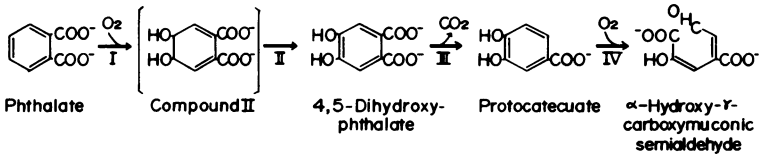

FIG. 7. *A tentative scheme of dissimilation of phthalate in P. testosteroni. I, phthalate oxygenase; II, 2,6-cyclohexadiene-4,5-diol-1,2-dicarboxylic acid dehydrogenase; III, 4,5-dihydroxyphthalate decarboxylase; IV, protocatechuate 4,5-dioxygenase.*

The possibility that the substrate is an inducer is unlikely, since the mutants NH1021 and NH1051, unable to form 4,5-dihydroxyphthalate, also produced high levels of the enzyme. It may be suggested that the enzymes catalyzing the conversion of phthalate to protocatechuate are coordinately induced by the primary substrate. On the other hand, the enzyme catalyzing the subsequent step, protocatechuate 4,5-dioxygenase, is induced by its own substrate (Table 2). Thus the dissimilatory pathway of phthalate to protocatechuate and the *meta*-cleavage pathway of protocatechuate may be controlled by different regulatory units. More than 20 mutants so far isolated as blocked mutants in phthalate catabolism reverted spontaneously to the wild type, suggesting that they are point mutants. In recent years, enzymes of dissimilatory pathways of toluic acid isomers, xylene isomers, *n*-octane, camphor, naphthalene, and salicylate have been reported to be controlled by degradative plasmids (13). There was no indication, however, that the dissimilatory pathway of phthalate in *P. testosteroni* is controlled by such a plasmid.

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