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Phthalate Pathway of Phenanthrene Metabolism: Formation of 2'-Carboxybenzalpyruvate

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The metabolism of phenanthrene by a gram-negative organism able to use this compound as a sole source of carbon and energy has been examined. 1-Hydroxy-2-naphthoic acid was oxidized by oxygen in a reaction catalyzed by a dioxygenase which was activated by ferrous ions. The stoichiometry of the oxidation and the UV spectrum of the product were consistent with the identification of the product as 2'-carboxybenzalpyruvate. This was confirmed by cleaving the product with a partially purified aldolase to yield 2-carboxybenzaldehyde and pyruvate. A number of enzymes for the metabolism of 1-hydroxy-2-naphthoic acid were induced by growth on phthalate or (less well) by growth on protocatechuate. The latter supported only a slow rate of growth, and this and poor induction may have been due to a slow rate of entry into the cell.

Two metabolic pathways have been described (4, 7) for the metabolism of phenanthrene (Fig. 1, compound I). Both pathways involve the formation of 1-hydroxy-2-naphthoic acid (Fig. 1, compound II), which in the more recently described pathway (7) is converted successively into 2-carboxybenzaldehyde (Fig. 1, compound V), phthalic acid (Fig. 1, compound VI), and protocatechuic acid. It was proposed that 2'-carboxybenzalpyruvate (Fig. 1, compound III) is an intermediate in the pathway. This paper provides evidence for the chemical structure of this intermediate and describes the properties of the enzymes isolated from a gram-negative coccus which catalyze its formation (1-hydroxy-2-naphthoic acid dioxygenase) and degradation (2'-carboxybenzalpyruvate aldolase). It is also shown that in this organism the enzyme which catalyzes the oxidation of compound II to compound III and later enzymes of the pathway are induced during growth on phthalate.

MATERIALS AND METHODS

Bacteria, cultures, and cell extracts. A gram-negative coccus, strain B156, isolated from local soil and able to grow on phenanthrene as a sole source of carbon and energy, was used. Cultures were grown at 25°C in mineral salts solution containing 0.01% nutrient broth and phenanthrene (0.2%) or other sterile filtered carbon sources (5 mM) (1a). Cultures (250 ml) were filtered through a Whatman no. 1 filter, harvested by centrifugation, washed twice with 0.05 M phosphate (pH 7.0), and suspended in phosphate (2 ml). Cells were disrupted by sonication (four 30-s bursts at 50 W with intervening cooling on ice) with a Sonifier cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Disrupted cells were centrifuged at 15,600 × *g* for 10 min at 5°C, and the supernatant was used as a source of enzymes.

Chemicals. Phenanthrene (BDH Chemicals, Toronto, Ontario, Canada) in hot ethanolic solution was treated with activated charcoal. The phenanthrene which crystallized after filtration was separated and vacuum dried. Potassium hydrogen *ortho*-phthalate was purchased from BDH Chemicals, 2-carboxybenzaldehyde from Aldrich Chemical Co., Milwaukee, Wis., and 1-hydroxy-2-naphthoic acid from Sigma Chemical Co., St. Louis, Mo. This last was recrystallized from benzene.

Partial purification of 1-hydroxy-2-naphthoic acid oxygenase and 2'-carboxybenzalpyruvate aldolase. The supernatant (1 ml) from extracts of cells (see above) grown either on phenanthrene or on phthalate was passed through a column (34 by 2-cm diameter) of Sephacryl S-200 superfine (Pharmacia Ltd., Dorval, Quebec, Canada) equilibrated with 0.05 M phosphate (pH 7.0). Eluate fractions were tested for the presence of each enzyme (see below), and appropriate fractions were combined. The oxygenase eluted close to the void volume and had a molecular weight greater than catalase (237,000). The aldolase eluted in a volume corresponding to a molecular weight of 71,000.

Measurement of enzymatic activities. (i) 1-Hydroxy-2-naphthoic acid dioxygenase was activated with ferrous ions immediately before measurement (11). The reaction mixture contained 0.026 mM 1-hydroxy-2-naphthoic acid in 0.05 M phosphate (pH 7.0) at 25°C. The reaction was started by the addition of enzyme, and the rate of increase of the absorbance at 300 nm was measured. The rate was calculated with an extinction coefficient of 15.4 mM^{-1} cm^{-1}, the difference between that of 2'-carboxybenzalpyruvate (19.2; see below) and 1-hydroxy-2-naphthoate (3.8). The substrate concentrations were saturating. The K_m values for O_2 and 1-hydroxy-2-naphthoic acid were very low

and have not been measured. (ii) 2'-Carboxybenzalpyruvate aldolase was measured in 0.05 M phosphate (pH 7.3) containing 0.0495 mM 2'-carboxybenzalpyruvate at 25°C. The reaction was started by the addition of enzyme, and the rate of decrease in absorbance at 300 nm was measured. The rate was calculated with an extinction coefficient of 17.6 mM^{-1} cm^{-1}, the difference between that of the substrate (19.2) and that of 2-carboxybenzaldehyde (1.6). The initial concentration of substrate required careful control because the K_m for 2'-carboxybenzalpyruvate was 0.047 mM. The concentration used gave an initial absorbance of 0.95. (iii) 2-Carboxybenzaldehyde dehydrogenase was measured by a published method (7), except that 0.05 M phosphate (pH 7.8), 2 mM NAD^+, and 0.33 mM 2-carboxybenzaldehyde were used. The rate was calculated with an extinction coefficient of 6.18 mM^{-1} cm^{-1}, the difference between that of NADH (6.22) and 2-carboxybenzaldehyde (0.04) at 340 nm. (iv) Protocatechuate-3,4-oxygenase was measured by a published method (5).

Cell respiration measurement. Rates of cell respiration were measured polarographically in 0.05 M phosphate (pH 7.0) at 25°C. After measurement of the endogenous rate, the increase due to added substrate was recorded. Phenanthrene was added as 10 μl of a 10 mM solution in dimethyl sulfoxide; acids were added as 20 μl of a 20 mM solution of their potassium salts, except for protocatechuate, which was added as 20 μl of 0.1 M solution.

Protein measurement. Protein was measured by the method of Lowry et al. (9) with bovine serum albumin as the standard. Cell suspensions and extracts were heated for 15 min at 100°C in 5% trichloroacetic acid, cooled, and centrifuged, and the sediments were dissolved in 0.1 M NaOH for analysis.

Preparation of 2'-carboxybenzalpyruvate and measurement of its extinction coefficient. 1-Hydroxy-2-naphthoic acid was added to phosphate (pH 7.0), and oxidation by dissolved oxygen was initiated by adding partially purified and reactivated 1-hydroxy-2-naphthoic acid dioxygenase. For measurement of the extinction coefficient, concentrations of the acid up to 0.10 mM were used, and the increase in absorbance at 300 nm was measured. For preparative purposes 0.2 mM 1-hydroxy-2-napthoic acid was used, and in all cases enough enzyme was used to complete the reaction in 10 to 20 min. At the end of the reaction, the absorbance at 300 nm was steady. The reaction product was isolated by two methods. Filtration through a column of Sephadex G-25 equilibrated with phosphate (pH 7.3) gave a product which could be used directly for the measurement of aldolase activity. Alternatively, the reaction medium was adjusted to pH 1.5 with concentrated HCl and extracted with an equal volume of ethyl acetate, which extracts about 80% of the product (Fig. 1, compound IV). To obtain the product for use as a substrate, the ethyl acetate solution was shaken with 0.05 M K_2HPO_4, which extracts the anionic form of compound IV from the organic solvent and catalyzes its conversion to the dianion of 2'-carboxybenzalpyruvate (Fig. 1, compound III). The aqueous extract was then adjusted to pH 7.3 by the very slow infusion of dilute HCl into the stirred solution. This must be done with care because compound IV is formed very rapidly in acid solution and can form in significant quantities in regions of local acidity during titration to pH 7.3. The cyclic compound IV does revert to the dianion of compound III at pH 7.3, but the reaction is slow. It can be speeded by heating.

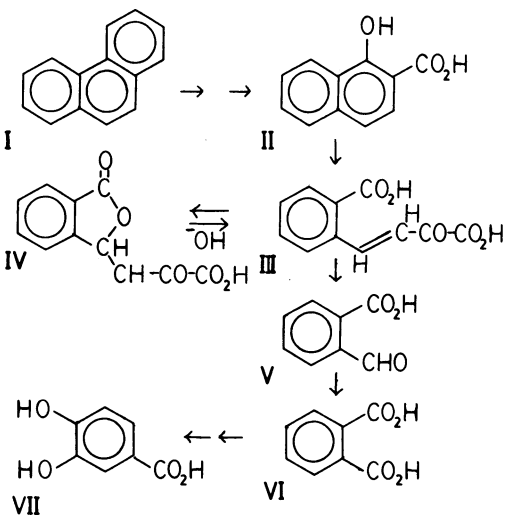

FIG. 1. Metabolic pathway for the degradation of phenanthrene (I). Double arrows represent two or more steps. For details, see text. II, 1-Hydroxy-2-naphthoic acid; III, 2'-carboxybenzalpyruvate; V, 2-carboxybenzaldehyde; VI, phthalic acid.

Acid production and oxygen consumption in the oxidation of 1-hydroxy-2-naphthoic acid. Limiting quantities of the acid were added to phosphate (0.05 M, pH 7.0) containing partially purified oxygenase, and the ensuing uptake of oxygen was measured polarographically at 25°C. Acid produced in the reaction was measured separately by the change in absorbance at 615 nm of bromothymol blue in 2 mM phosphate (pH 7.0). This procedure was similar to that described previously (1).

Formation of 2-carboxybenzaldehyde and pyruvate from 2'-carboxybenzalpyruvate. The reaction catalyzed by partially purified aldolase was monitored at 300 nm (see above) and was stopped by adding the predetermined volume of M-$NaHCO_3$-NaOH (pH 10.5) required to bring the pH of the reaction mixture to 10.0. NADH (0.16 mM) was added, and the decrease in absorbance at 340 nm caused by adding lactic dehydrogenase was measured. The concentration of NADH oxidized, equivalent to the pyruvate formed in the aldolase reaction, was calculated with an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The 2'-carboxybenzalpyruvate cleaved in the reaction was calculated from the decrease in absorbance at 300 nm of the original reaction mixture (extinction coefficient, 17.6; see above). The chemical nature of the products of the aldolase reaction was confirmed by allowing the reaction to proceed to completion and then filtering the reaction mixture through a column of Sephadex G-25 equilibrated with 0.05 M phosphate. The absorption spectrum of the low-molecular-weight fraction was identical with that of authentic 2-carboxybenzaldehyde. The solution was then treated with acid diphenylhydrazine solution, and hydrazones were extracted

TABLE 1. Oxidation of compounds added to suspension of strain B156

Carbon source for growth	Respiration rate[a] with:				
	Phenanthrene	1-Hydroxy-2-naphthoic-acid	2-Carboxybenzaldehyde	Phthalate	Protocatechuate
Phenanthrene	0.50	0.051	0.11	0.068	0.015
Phthalate	0.001	0.52	0.22	0.29	0.059
Protocatechuate	<0.001	0.057	0.008	<0.001	0.033
Succinate	<0.001	<0.001	<0.001	<0.001	<0.001

[a] Micromoles of O_2 taken up per minute per milligram of total protein, corrected for endogenous respiration.

with ether and compared by thin-layer chromatography with the derivatives made from pyruvic acid and from 2-carboxybenzaldehyde. These experiments used Silica Gel G plates, benzene-ether (1:1 [vol/vol]; solvent 1), and methanol-acetic acid (49:1 [vol/vol]; solvent 2).

RESULTS AND DISCUSSION

The organism used in this work was a gram-negative coccus selected for its ability to grow on phenanthrene as a sole source of carbon and energy (1a). It grew on phthalate but did not grow on nephthalene or salicylate, and cells grown on phenanthrene oxidized 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, and phthalic acid very rapidly (Table 1). It seemed likely that this organism used the pathway for phenanthrene degradation described by Kiyohara and Nagao (7). Those authors (6) clearly showed that the oxidation of 1-hydroxy-2-naphthoic acid is accompanied by the formation of a compound which absorbs strongly at 300 nm. This compound was not characterized but it was assumed to be 2'-carboxybenzalpyruvate (Fig. 1, compound III). Direct evidence for this has now been produced.

An enzyme which catalyzed the oxygen-dependent oxidation of 1-hydroxy-2-naphthoic acid was partially purified by filtration through Sephacryl S-200 of extracts of strain B156 grown on either phthalate or phenanthrene. Comparison of the elution volume with that of standards showed that it had a molecular weight greater than that of catalase (237,000). Activity was not detected in column effluents unless the enzyme was activated with Fe^{2+}. Other metal ions (Mn^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}) could not replace Fe^{2+}. Fe^{3+} was inhibitory. The enzyme was not stable, and, after 24 h at 0°C, more than 90% of its activity was lost and was not regained by incubation with Fe^{2+}. The enzyme was not stabilized by the presence of ethanol, acetone, or glycerol (concentrations up to 10% [vol/vol]), nor was it stabilized by mercaptoethanol or mixtures of these reagents. Consequently, the enzyme has not been fully characterized. It was, however, pure enough to demonstrate the nature of the oxidation product of 1-hydroxy-2-naphthoic acid and the stoichiometry of the reaction (see below). These observations and its activation by Fe^{2+} suggest that it is a dioxygenase which catalyzes a reaction analogous to that catalyzed by gentisate 1,2-dioxygenase (2, 8).

When limiting quantities of 1-hydroxy-2-naphthoic acid were oxidized in the presence of partially purified 1-hydroxy-2-naphthoic acid dioxygenase, there was an uptake of 0.95 ± 0.03 molecular proportions of O_2 and a release of 1.01 ± 0.03 molecular proportions of H^+. These values are consistent with the reaction shown in Fig. 2. The change in absorption spectrum observed during the reaction is shown in Fig. 3. The large absorption at 300 nm is consistent with the presence of double bonds in the substituent conjugated with the aromatic ring. The carbon-carbon double bond is assumed to have the *cis* configuration.

Direct evidence for the structure of the product was obtained by identifying and measuring the quantities of products released during treatment with an aldolase separated from cell extracts. Reaction of the enzymatic reaction products with 2,4-dinitrophenylhydrazine gave two hydrazones which were chromatographically identical with those prepared from authentic pyruvic acid and 2-carboxybenzaldehyde (R_f, 0.04 and 0.15, respectively, in solvent 1 and 0.60 and 0.70, respectively, in solvent 2). The quantity of pyruvate produced during the reaction was 0.79 ± 0.06 mol/mol of 2'-carboxybenzalpyruvate which had disappeared. On completion of the reaction, the absorption at 300 nm corresponded to the presence of 0.98 ± 0.05 molecular proportions of 2-carboxybenzaldehyde. These observations are consistent with the reaction shown in Fig. 4.

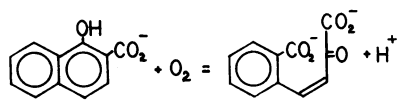

FIG. 2. Oxidation of 1-hydroxy-2-naphthoic acid in the presence of partially purified 1-hydroxy-2-naphthoic acid dioxygenase.

TABLE 2. Enzymatic activities in extracts of strain B156 after growth on different carbon sources

Carbon source for growth	Enzymatic activity[a]			
	1-Hydroxy-2-naphthoic-acid	Aldolase	2-Carboxybenzaldehyde dehydrogenase	Protocatechuate 3,4-dioxygenase
Phenanthrene	0.11	<0.008	<0.029	0.040
Phthalate	0.49	0.038	0.12	0.16
Protocatechuate	0.016	0.002	0.029	0.014
Succinate	<0.001	<0.001	<0.001	<0.001

[a] Micromoles per minute per milligram of protein.

The direct isolation of 2'-carboxybenzalpyruvate (Fig. 1, compound III) was not possible, except as an aqueous solution of its salt obtained by chromatography with Sephadex G-25. When a solution of compound III was acidified, the absorption at 300 nm decreased very rapidly (Fig. 3). The original spectrum could be obtained by making the solution alkaline or by boiling an aqueous solution at pH 7. The product obtained was spectrally identical with the original compound produced by the oxidation of 1-hydroxy-2-naphthoic acid and was cleaved by the aldolase with K_m and maximum velocity unchanged. It is probable that in acid solution compound III is converted to compound IV, but reconversion to compound III may give a product with *trans* configuration.

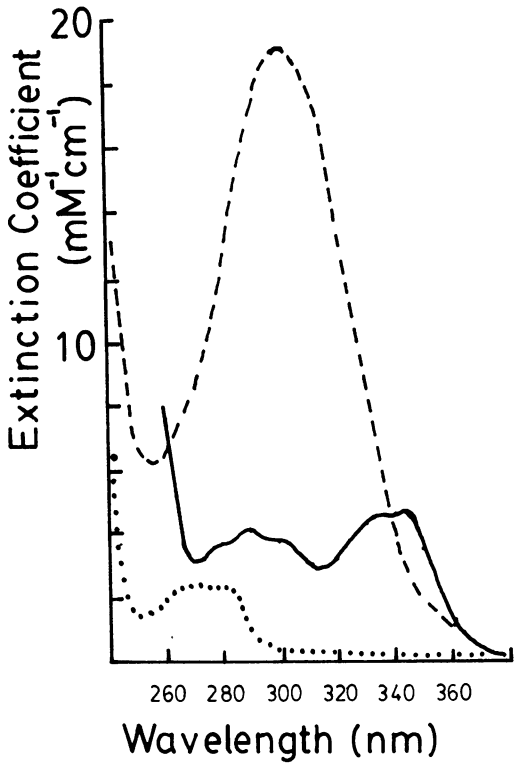

FIG. 3. Absorption spectra in aqueous solutions. Solid line, 1-Hydroxy-2-naphthoic acid (Fig. 1, II) in phosphate (pH 7.0); dashed line, 2'-carboxybenzalpyruvate (Fig. 1, III) in phosphate (pH 7.0); dotted line, the same chemical in dilute acid. The spectrum in dilute acid is due to compound IV.

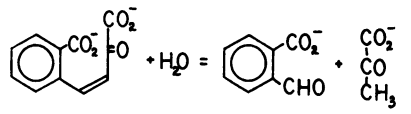

FIG. 4. Identification of reaction product from Fig. 2.

The growth of strain B156 on phenanthrene is accompanied by the induction of enzymes which catalyze the oxidation of 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, phthalic acid, and protocatechuic acid (Table 1). In cell extracts, the activities of 1-hydroxy-2-naphthoic acid dioxygenase, 2'-carboxybenzalpyruvate aldolase, 2-carboxybenzaldehyde dehydrogenase, and protocatechuate 3,4-dioxygenase were also increased (Table 2). Although enzymatic pathways for the conversion of phthalate to protocatechuate have been delineated in other organisms (3, 10, 12), I was not able to detect the activity of these enzymes in extracts of strain B156. It was observed (Tables 1 and 2) that growth on phthalate induced some metabolic activities very strongly and that growth on protocatechuate induced them rather weakly. An unequivocal conclusion about the nature of the inducer of 1-hydroxy-2-naphthoic acid dehydrogenase and later enzymes cannot be made. Growth on protocatechuate was slow compared with growth on phthalate (doubling times, 14 and 5.2 h, respectively). The low induction with protocatechuate may have been due to the slow entry of the carbon source limiting induction and growth rate.

ACKNOWLEDGMENTS

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