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Involvement of the Protocatechuate Pathway in the Metabolism of Mandelic Acid by *Aspergillus niger*

M. JAMALUDDIN, P. V. SUBBA RAO, AND C. S. VAIDYANATHAN
Department of Biochemistry, Indian Institute of Science, Bangalore-12, India

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Cell-free extracts of *Aspergillus niger* UBC 814 grown in the presence of DL-mandelate oxidized both D(−)- and L(+)-mandelate via benzoylformate and benzaldehyde to benzoate. DL-*p*-Hydroxymandelate was oxidized, presumably through a parallel pathway, to *p*-hydroxybenzoate. A particulate D(−)-mandelate dehydrogenase and a supernatant fraction L(+)-mandelate dehydrogenase converted their respective substrates to benzoylformate. Both flavine adenine dinucleotide and flavine mononucleotide showed a stimulatory effect on the activity of the L(+)-mandelate dehydrogenase. Benzoylformate was decarboxylated to benzaldehyde by an enzyme requiring thiamine pyrophosphate for maximal activity. Two benzaldehyde dehydrogenases dependent on nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), respectively, for their activity dehydrogenated benzaldehyde to benzoate. In the presence of reduced NADP (NADPH), benzoate was oxidized via *p*-hydroxybenzoate and protocatechuate. Reduced NAD could not replace NADPH. Sensitive methods of assay for D(−)-mandelate dehydrogenase and benzoylformate decarboxylase are described. The fungal pathway is compared with these systems in bacteria.

The mandelate pathway in *Pseudomonas putida* has been extensively investigated by Stanier and his colleagues (2–6, 13) and by Mandelstam and his co-workers (11, 14), and some aspects of the metabolism of mandelic acid by bacterium N.C.I.B. 8250 have been investigated by Kennedy and Fewson (7, 8). The overall pathway operating in the two groups of bacteria is the same except for the inability of bacterium N.C.I.B. 8250 to utilize D(−)-mandelic acid. It was of interest to investigate the pathway for the metabolism of mandelic acid by a fungus and compare it with that operative in bacteria. Preliminary studies in our laboratory have shown that *p*-hydroxybenzoic acid and protocatechuic acid, but not catechol, are the metabolites that accumulate in the culture filtrates of a strain (UBC 814) of *Aspergillus niger* grown in presence of mandelic acid. This observation led us to a more detailed investigation of the mandelate pathway in *A. niger*, the results of which are reported in the present paper.

MATERIALS AND METHODS

Organism and method of cultivation. *A. niger* UBC 814, obtained from R. J. Bandoni, Department of Botany, University of British Columbia, Canada, was grown for 45 hr at 28 C on a synthetic medium (1) supplemented with 0.1% (w/v) DL-mandelic acid. The final *p*H of the medium was adjusted to about 5.5 with dilute NaOH. Stock cultures were maintained on slopes of the same medium solidified with agar (2%, w/v).

In vivo experiments. The in vivo studies were carried out with mycelial mats washed aseptically three times with distilled water and replaced with a solution of 25 mg of benzoic acid in 50 ml of 0.01 M sodium phosphate buffer, *p*H 6 (9). This culture will hereafter be referred to as replacement culture and the medium as replacement medium.

Assay of benzoic acid oxidation in replacement studies. A sample of the replacement medium removed immediately after addition served as a reference. The replacement culture was shaken on a rotary shaker at 28 C, and 1-ml samples were withdrawn at 5-min intervals. The samples were extracted with 2 ml of peroxide-free diethyl ether. Suitable samples from the ether layer were removed, and the disappearance of benzoic acid was followed spectrophotometrically by measuring the absorbancy at 229 nm after taking the residue in 2 ml of 95% ethyl alcohol.

Analysis of products of benzoic acid oxidation. After shaking the replacement culture for 30 min to 1 hr, the medium was removed by filtration and the mycelium was washed twice with 50-ml portions of

distilled water. The medium and the washings were combined, acidified to *p*H 2 with 3 N HCl, and extracted with diethyl ether. The ether layer after drying with anhydrous sodium sulfate was evaporated in vacuo, and the residue was dissolved in a small volume of ethyl acetate. Suitable samples from the ethyl acetate solution were analyzed by two-dimensional paper chromatography on Whatman no. 1 filter paper in two solvent systems, solvent A [Benzene-acetic acid-water (10:7:3, upper phase)] and solvent B [2% (w/v) aqueous formic acid (12)], by the ascending method at room temperature (25 C). For the spectrophotometric examination, the spot corresponding to the R_F of the authentic compound run on the same chromatogram with the first solvent system, and located by spraying with diazotized *p*-nitroaniline followed by 1 N NaOH, was cut out and eluted with ether. The ether extract after evaporation to a small volume was subjected to two-dimensional chromatography. The spot corresponding to the position of the authentic compound was cut out and eluted with 95% ethyl alcohol, and the ultraviolet absorption spectrum was determined.

Preparation of cell-free extracts. The mycelium was washed three times with chilled distilled water, crushed with an equal weight of glass powder in a chilled porcelain mortar, and extracted twice with 0.025 M sodium phosphate buffer, *p*H 7 (4 ml/g of mycelium). For manometric studies with benzoate, cell-free preparations were obtained by extracting the mycelium with half this volume of buffer. The extract was squeezed through a cheesecloth, centrifuged at 4,000 × *g* for 30 min in a refrigerated centrifuge, and used without delay for manometric experiments.

Isolation of the product of mandelate oxidation by cell-free extracts. A reaction mixture containing 15 ml of 0.2 M sodium phosphate buffer (*p*H 7), 30 ml of the extract (7.35 mg of protein per ml), and 100 mg of sodium DL-mandelate was incubated for 3 hr at 30 C with mechanical agitation. After the specified time, the reaction mixture was acidified to *p*H 2 with H_2SO_4, clarified by centrifugation, and extracted repeatedly with peroxide-free diethyl ether. The organic layer was taken to dryness at room temperature after shaking with anhydrous sodium sulfate. The residue was sublimed in vacuo. The white crystalline solid obtained (30 mg) was recrystallized twice from water.

Preparation of particulate and supernatant fractions. The homogenate obtained after squeezing through a cheesecloth (50 ml) was centrifuged at 12,000 × *g* for 15 min, and the supernatant fluid was centrifuged at 100,000 × *g* for 1 hr in the no. 40 rotor of a Spinco model L preparative ultracentrifuge. The cream-colored pellet obtained was drained free from the supernatant fluid, resuspended in 10 ml of 0.025 M sodium phosphate buffer (*p*H 7), and centrifuged at 22,000 × *g* for 15 min. The residue obtained was dispersed in 5 ml of 0.025 M sodium phosphate buffer (*p*H 7) by squeezing through a fine hypodermic syringe. This preparation will be referred to as the particulate fraction and the 100,000 × *g* supernatant fluid as the supernatant fraction.

Manometry. The conventional Warburg technique (15) was used to measure oxygen consumption.

Enzyme assays. Enzyme assays were carried out at 30 C unless otherwise stated. One unit of enzyme activity is defined as the amount that catalyzes the transformation of 1 μmole of the substrate or the formation of 1 μmole of the product per min under the conditions of the assay. Specific activity is expressed as milliunits of enzyme per milligram of protein.

D(−)-Mandelate dehydrogenase: (D(−)-mandelate: (acceptor) oxidoreductase). The enzyme was assayed in the particulate fraction by estimating the benzoylformic acid, the product of the reaction, as its 2,4-dinitrophenylhydrazone in alkali.

The assay system contained 50 μmoles of sodium phosphate buffer (*p*H 7), enzyme, and 1 μmole of sodium D(−)-mandelate in a total volume of 1 ml. The reaction was stopped by the addition of 0.5 ml of a 0.1% (w/v) solution of 2,4-dinitrophenylhydrazine in 2 N HCl. The reaction mixture was kept in a boiling-water bath for 5 min, cooled to room temperature, and centrifuged to remove the denatured protein. A 0.5-ml amount of the clear supernatant fluid was pipetted out and made to 1 ml with water, and 1 ml of a 10% (w/v) aqueous sodium hydroxide was added; the color in the blank and that in the experiment were measured at 470 nm against a reagent blank consisting of 1 ml of water similarly treated. The amount of benzoylformic acid formed was determined from a standard graph.

L(+)-Mandelate dehydrogenase: (L(+)-mandelate: (acceptor) oxidoreductase). The enzyme was routinely assayed in the 100,000 × *g* supernatant fluid by the method of Hegeman (4) with the following modification. Since reduction of dye took place even in the absence of added substrate, a sample of the enzyme preparation was treated immediately before the assay, with the dye, until a slight color persisted. The assay was carried out in a total volume of 1 ml in 1-cm cuvettes at room temperature (25 C). In addition to the enzyme, the reaction mixture contained 50 μmoles of sodium phosphate buffer (*p*H 7), 10 μmoles of sodium L(+)-mandelate, and 0.1 μmole of 2,6-dichlorophenol indophenol. The reaction was started by adding the substrate. The reference cuvette contained water instead of substrate solution. A decrease of 20.6 absorbancy units at 600 nm corresponds to the oxidation of 1 μmole of substrate to benzoylformate.

Preparation of L(+)-mandelate dehydrogenase for testing the effect of cofactors. The cell-free extract was treated with solid $(NH_4)_2SO_4$ to 0.45 saturation. The precipitate formed within 15 min was removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant fluid was treated with 4.9 g of solid $(NH_4)_2SO_4$ for every 100 ml. The precipitate formed within 30 min was collected by centrifugation at 12,000 × *g* for 15 min and dissolved in 20 ml of 0.025 M sodium phosphate buffer (*p*H 7). The assay was as described above with the inclusion of 10 nmoles of the cofactor [flavine adenine dinucleotide (FAD) or flavine mononucleotide (FMN)] in the assay system.

Benzoylformate decarboxylase: (EC 4.1.1.7,

benzoylformate carboxy-lyase). The enzyme was assayed in the 100,000 × *g* supernatant fluid as follows. The reaction mixture contained in a total of 1.2 ml, 50 μmoles of sodium phosphate buffer (*p*H 6), enzyme, 50 μg of thiamine pyrophosphate chloride, and 1 μmole of benzoylformic acid. Substrate was added to the blank after stopping the reaction with 0.2 ml of 2 N HCl. The blank and experiment were shaken with 2 ml of carbon tetrachloride in which benzaldehyde, the product of the reaction, is completely soluble and benzoylformic acid is insoluble. The reaction mixture was centrifuged at low speed to separate the two layers, and 0.1 ml of the aqueous layer was pipetted out into a test tube and made up to 1 ml with water. A 0.1% (w/v) solution of 2,4-dinitrophenylhydrazine in 2 N HCl (0.25 ml) was added to these samples and kept in a boiling-water bath for 5 min. After cooling to room temperature, 1 ml of a 10% aqueous sodium hydroxide was added, and the color in the blank and that in the experiment were measured at 470 nm against a reagent blank consisting of 1 ml of water subjected to the same treatment. The benzoylformic acid which disappeared was determined from a standard graph.

Preparation and assay of nicotinamide adenine dinucleotide (NAD)-benzaldehyde dehydrogenase (EC 1.2.1.6, benzaldehyde: NAD oxidoreductase) and nicotinamide adenine dinucleotide phosphate (NADP)-benzaldehyde dehydrogenase (EC 1.2.1.7, benzaldehyde: NADP oxidoreductase). These enzymes could not be assayed in crude preparations by following the reduction of NAD or NADP spectrophotometrically because of the presence of powerful reduced NAD (NADH) and reduced NADP (NADPH) oxidizing systems insensitive to cyanide. They were prepared and assayed as follows.

NAD-benzaldehyde dehydrogenase. The cell-free extracts (25 ml) were treated with solid $(NH_4)_2SO_4$ to 0.45 saturation, and the precipitate formed within 15 min was removed by centrifugation at 12,000 × *g* for 10 min. The supernatant fluid was again treated with solid $(NH_4)_2SO_4$ to 0.6 saturation, and the precipitate formed in 30 min was collected by centrifugation at 12,000 × *g* for 10 min and dissolved in 10 ml of 0.025 M sodium phosphate buffer (*p*H 7). This preparation still contained NADPH-oxidizing activity.

The NAD-benzaldehyde dehydrogenase was assayed in the preparation by following the reduction of NAD spectrophotometrically at 340 nm. The assay system contained 50 μmoles of tris(hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (*p*H 8), enzyme, and 50 μg of NAD in a total volume of 1 ml. The reaction was started by the addition of benzaldehyde (0.94 μmole) and allowed to proceed at room temperature (25 C). The change in absorbance at 340 nm was measured at 1-min intervals for a period of 5 min against a blank containing water instead of benzaldehyde. An increase in absorbancy of 6.21 units corresponds to the oxidation of 1 μmole of substrate to benzoate.

NADP benzaldehyde dehydrogenase. The cell-free extract was adjusted to *p*H 9 by dropwise addition of chilled 6 N ammonium hydroxide, and the precipitate formed was removed by centrifugation at 17,000 × *g* for 5 min. The supernatant fluid was brought to *p*H 6.8 with chilled 3 N acetic acid, and 1 ml of a 2% (w/v) aqueous protamine sulfate was added with stirring for every 15 ml of the extract. The precipitate formed was removed after 5 min by centrifugation at 17,000 × *g* for 5 min, and the supernatant fluid was used as the enzyme.

The method of assay was the same as that for NAD-benzaldehyde dehydrogenase with NADP instead of NAD.

Protein. The method of Lowry et al. (10) was used for the determination of protein. Bovine serum albumin was the standard.

Spectrophotometry. All spectrophotometric measurements were made in a Beckman DB recording spectrophotometer.

Chemicals. DL-Mandelic acid and benzoylformic acid were purchased from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals used were from Sigma Chemical Co., St. Louis, Mo.

Enzymes. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) type VI and alcohol dehydrogenase (EC 1.1.1.1) stock no. 340-26 were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Cell-free extracts of *A. niger* UBC 814 grown in the presence of mandelic acid oxidized both D(−)- and L(+)-mandelates, consuming about 1 mole of oxygen per mole of substrate in the absence of any added cofactors (Fig. 1). The product of mandelate oxidation was identified as benzoic acid. The isolated product melted at 121.6 C (uncorrected), and the melting point was not depressed by admixture with synthetic benzoic acid. Both the isolated sample and the synthetic material showed λ_{max} at 226 nm (ϵ_{max} 10,000) in water, at 229 nm (ϵ_{max} 10,400) in 95% ethyl alcohol, and at 224 nm (ϵ_{max} 8,700) and 268 nm (ϵ_{max} 562) in 0.02 N NaOH.

D(−)-Mandelate was oxidized at a slightly faster rate than the L(+)-isomer, and the rate of oxidation of the DL-mixture at the same concentration was also slightly greater than that of the L(+)-isomer. On the other hand, when attempts were made to assay these enzymes by 2,6-dichlorophenol indophenol reduction, the L(+)-isomer alone was found to be oxidized. The reason for this difference was found to be due to the presence, in cell-free extracts of *A. niger*, of two distinct dehydrogenases acting on their respective stereoisomers. The D(−)-mandelate dehydrogenase was associated with the particulate fraction (specific activity, 0.81) and the L(+)-mandelate dehydrogenase was in the supernatant fraction (specific activity, 0.45). The particulate fraction did not show any L(+)-mandelate dehydrogenase activity (Table 1) and

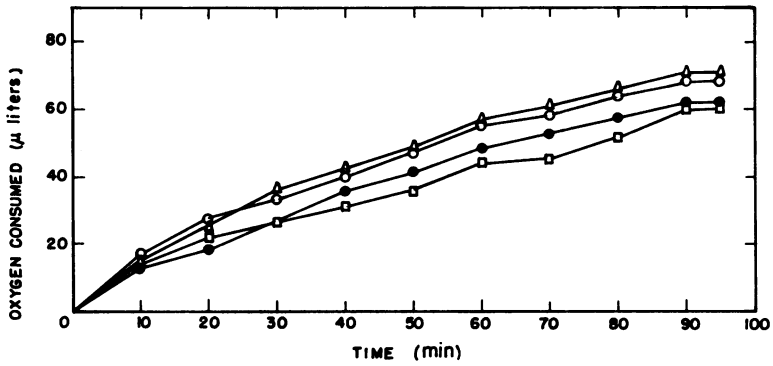

FIG. 1. *Oxygen uptake by cell-free extracts of Aspergillus niger grown in the presence of mandelate. Each Warburg flask contained in, a total volume of 3.2 ml, 1.5 ml of 0.1 M sodium phosphate buffer (pH 7), 1.2 ml of the cell-free extract (7.35 mg of protein per ml), and 3 μmoles of the substrate in the side arm. The central well contained 0.2 ml of 20% KOH. The substrate was tipped in at zero time. Incubation was at 30 C in an air atmosphere. There was no endogenous oxygen consumption. Symbols:* ●, L(+)-*mandelate;* ○, D(−)-*mandelate;* △, DL-*mandelate;* □, DL-*p-hydroxymandelate.*

did not reduce 2,6-dichlorophenol indophenol in presence of D(−)-mandelate. If the particulate fraction was prepared directly from the cell-free extract omitting the centrifugation at 12,000 × *g*, it readily consumed oxygen on addition of D(−)-mandelate, but if it was prepared after that step there was little oxygen consumption, showing that a fraction removed by centrifugation at 12,000 × *g* was necessary for the transfer of electrons to oxygen. D(−)-Mandelate dehydrogenase was comparatively unstable and its activity was rapidly lost by repeated squeezing through a hypodermic syringe. The enzyme did not show any requirement for added cofactors. The activity of L(+)-mandelate dehydrogenase, on the other hand, was stimulated by both FAD and FMN after being precipitated with $(NH_4)_2SO_4$ (Fig. 2).

The product of the D(−)-mandelate dehydrogenase reaction was identified as benzoylformic acid by preparing its 2,4-dinitrophenylhydrazone. The hydrazone melted at 186 C (uncorrected). The melting point was not depressed by admixture with the 2,4-dinitrophenylhydrazone of synthetic benzoylformic acid which also melted at the same temperature. The 2,4-dinitrophenylhydrazones of the isolated product and the synthetic benzoylformic acid gave a deep wine-red color in aqueous sodium hydroxide and showed an absorption maximum at 470 nm with ϵ_{max} 7150 and 7212, respectively.

Benzoylformate decarboxylase of *A. niger* UBC 814 required thiamine pyrophosphate for maximal activity. No stimulatory effect could be observed with added Mg^{2+} ions. The reaction product was separated from benzoylformic acid by extraction into CCl_4 and identified as benzaldehyde by comparing its properties with those

TABLE 1. *Activity of a particulate fraction towards* D(−)-*mandelate and* L(+)-*mandelate*[a]

Time (min)	Amt (nmoles) of benzoylformic acid formed from	
	D(−)-Mandelate	L(+)-Mandelate
5	5.3	0.0
10	10.2	0.0
20	20.0	0.0

[a] Assay conditions were standard. A 0.3-ml amount of the particulate fraction (4.3 mg of protein per ml) was used. L(+)-Mandelate dehydrogenase was also assayed by the same method as was used for D(−)-mandelate dehydrogenase by using L(+)-mandelate in place of D(−)-mandelate.

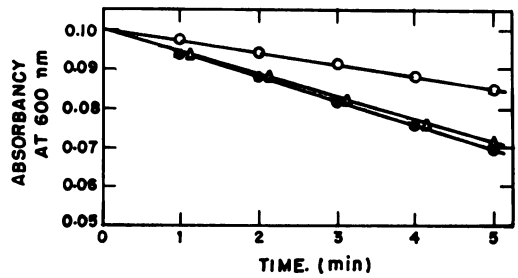

FIG. 2. *Effect of flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN) on the activity of* L(+)-*mandelate dehydrogenase of Aspergillus niger. Symbols:* ○, *no addition;* △, *10 nmoles of FMN;* ●, *10 nmoles of FAD. Conditions of assay were as described in the text. A 0.2-ml amount of the* $(NH_4)_2SO_4$ *fraction (2.5 mg of protein per ml) was used.*

of an authentic sample. The reaction product and benzaldehyde showed a sharp maximum at 280 nm and a shoulder at 276 nm in CCl_4. The 2,4-dinitrophenyl hydrazone of the enzymatic product was indistinguishable from the 2,4-dinitrophenylhydrazone of the synthetic material [melting point, 236.8 (uncorrected)]. The specific activity of the enzyme in the supernatant fraction was 6.

Cell-free extracts of *A. niger* oxidized benzaldehyde in the presence of either NAD or NADP, the rate of oxidation as well as the total uptake of oxygen being higher with the latter cofactor (Fig. 3). The quantity of oxygen consumed with NAD as the cofactor was about 0.5 mole per mole of benzaldehyde. The addition of NADP along with NAD resulted in a more than a twofold increase in the rate of oxygen uptake. The total consumption of oxygen also increased more than twofold compared to that with NAD alone. The doubling of the rate suggested the presence, in cell-free extracts of *A. niger*, of two benzaldehyde dehydrogenases dependent on NAD and NADP, respectively, for their activity. This was further confirmed by separating the two activities. The NADP-benzaldehyde dehydrogenase preparation, which was free from NADH-oxidizing activity, was inactive with NAD (Fig. 4). The reason for the greater consumption of oxygen with NADP was that the NADPH produced in the NADP-benzaldehyde dehydrogenase reaction supported the oxidation of endogenous substrates and of benzoate, another product of the reaction. This was shown in experiments with benzoate and an NADPH-regenerating system (Fig. 5). During the first 1 hr, the rate of oxygen uptake was only a little above that rate supported by endogenous substrates, the nature of which is unknown. After 1 hr, when oxygen consumption for endogenous oxidation completely ceased, the rate of oxygen uptake steadily increased and eventually the total consumption reached 3 moles per mole of benzoate. There was no oxygen consumption in excess of the endogenous with an NADH-regenerating system.

The capacity of *A. niger* grown in the presence of mandelate to oxidize benzoic acid was also shown in experiments with whole mycelia. Mycelia grown in the presence of mandelate were adapted for the immediate and rapid oxidation of benzoate, whereas mycelia grown on glucose alone were not (Fig. 6). The immediate products of benzoate oxidation were identified in these experiments. If the replacement culture was shaken for 30 min, the major phenolic product was 4-hydroxybenzoic acid. The oxidation product of benzoic acid and authentic 4-hydroxybenzoic acid moved with R_F values of 0.41 and 0.54 in solvents A and B, respectively. Color reactions of the product on chromatograms with spray reagents like diazotized *p*-nitroaniline and diazotized sulfanilic acid were identical with those of 4-hydroxybenzoic acid. The ultraviolet absorption spectrum of the compound isolated from the replacement medium and that of the authentic 4-hydroxybenzoic acid showed a peak at 257 nm and a trough at 230 nm in 95% ethyl alcohol.

Shaking the replacement culture for longer periods resulted in the appearance of another phenolic product on the chromatograms, which was identified as 3,4-dihydroxybenzoic acid by two-dimensional paper chromatography (R_F of 0.1 in solvent A and 0.43 in solvent B).

DISCUSSION

The foregoing results are consistent with the following scheme for mandelate oxidation by *A. niger* UBC 814 (Fig. 7).

A. niger utilizes D(−)-mandelate by means of

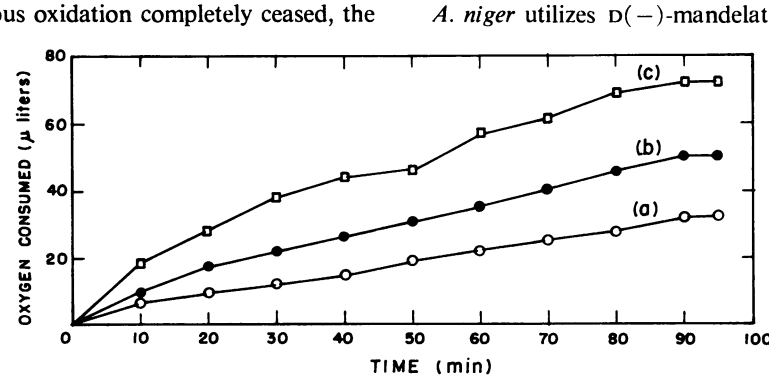

FIG. 3. *Oxidation of benzaldehyde by cell-free extracts of Aspergillus niger grown in the presence of mandelate. Each Warburg flask contained, in a total volume of 3.2 ml, 0.1 M sodium phosphate buffer, pH 7 (1.3 ml); cell-free extract (1.2 ml, 7.35 mg of protein per ml); NAD or NADP (100 μg) or NAD (50 μg) plus NADP (50 μg); and benzaldehyde (3 μmoles) in the side arm. The central well contained 0.2 ml of 20% KOH. The substrate was tipped in at zero time. Incubation was at 30 C in an air atmosphere. Oxidation in the presence of NAD (a), NADP (b), and NAD plus NADP (c). There was no endogenous oxygen consumption.*

a distinct dehydrogenase. In *P. putida*, on the other hand, a racemase mediates the utilization of D(−)-mandelate (3). Another bacterial species, namely, bacterium N.C.I.B. 8250, cannot utilize D(−)-mandelate at all (7). The rest of the fungal pathway up to the formation of benzoate is comparable to the pathway employed by *P. putida*, inasmuch as both organisms employ NAD- and NADP-dependent enzymes for the dehydrogenation of benzaldehyde. Bacterium N.C.I.B. 8250 does not possess an NADP-dependent enzyme (7). In contrast to the L(+)-mandelate dehydrogenases of *P. putida* and bacterium N.C.I.B. 8250, which are particulate (13) and membrane-bound (7), respectively, the *A. niger* enzyme

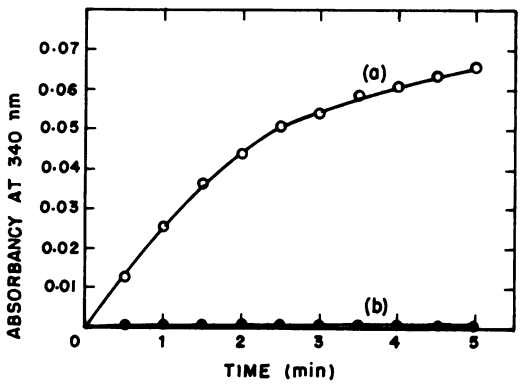

FIG. 4. *Reduction of pyridine nucleotides by an NADP-dependent benzaldehyde dehydrogenase preparation from Aspergillus niger. (a) NADP (50 μg); (b) NAD (50 μg). Conditions of assay were as described in the text. Readings were taken at 0.5-min intervals. A 0.3-ml amount of the enzyme preparation (2.5 mg of protein per ml) was used.*

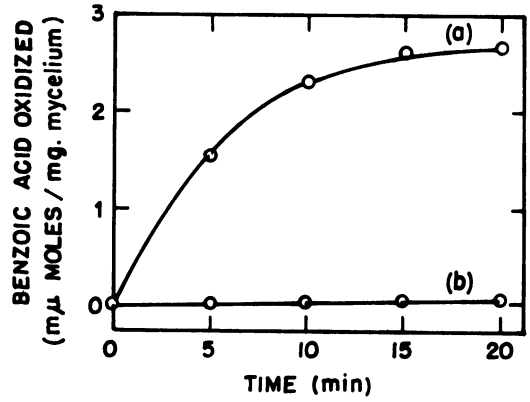

FIG. 6. *Oxidation of benzoic acid by Aspergillus niger. The oxidation studies were carried out with mycelium grown in presence of mandelate (a) or on glucose alone (b) and assayed as described in Materials and Methods.*

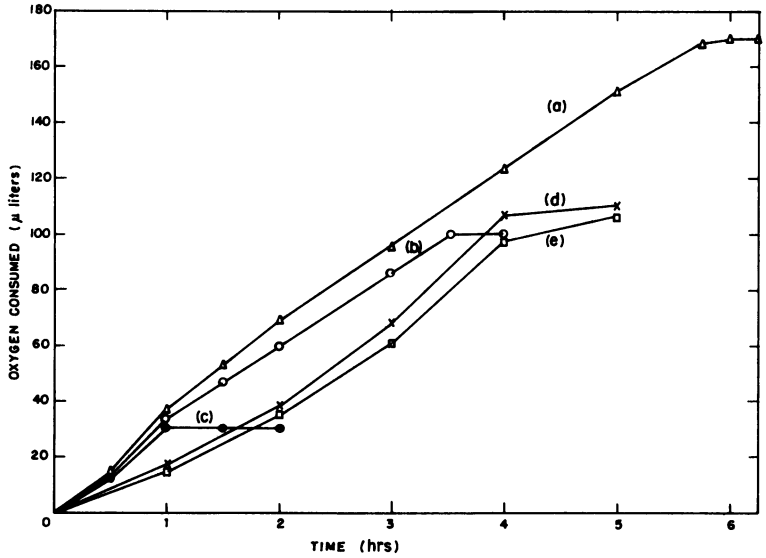

FIG. 5. *Oxidation of benzoate by cell-free extracts of Aspergillus niger grown in the presence of mandelate. Each Warburg flask contained 0.1 M sodium phosphate buffer, pH 7 (1.9 ml); cell-free extract (0.7 ml, 14.0 mg of protein per ml); NADP (0.4 μmole); glucose-6-phosphate (0.5 μmole); and glucose-6-phosphate dehydrogenase (0.02 units) in a total volume of 3.2 ml. The central well contained 0.2 ml of 20% KOH. Substrate [2 μmoles (a) or 1 μmole (b)] was tipped in from the side arm at zero time. Incubation was at 30 C in an air atmosphere. (c) Endogenous oxygen consumption with an NADPH regenerating system. (d) An NADH-regenerating system (NAD, 0.4 μmole; ethyl alcohol, 0.5 μmole; and alcohol dehydrogenase, 0.02 units) was used to study its effect on the oxidation of benzoate (2 μmoles). (e) Endogenous oxygen consumption with an NADH regenerating system.*

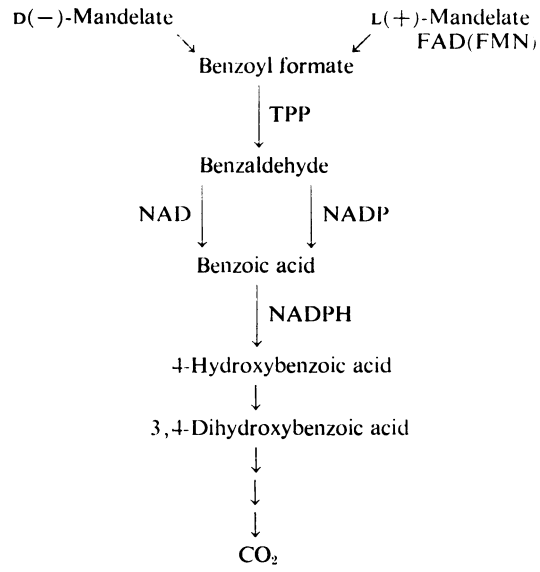

FIG. 7. *Scheme for mandelate oxidation by Aspergillus niger UBC 814.*

appears to be soluble. Unlike the bacterial enzyme which does not require any cofactor, the activity of the fungal dehydrogenase was considerably stimulated by FAD or FMN (Fig. 2).

The mandelate pathway in *A. niger* deviates completely from the bacterial pathway at the benzoate level. In the fungus, benzoate is metabolized via the protocatechuate pathway, whereas in bacteria catechol is the terminal aromatic compound derived from mandelate, which undergoes ring cleavage.

The pattern of oxygen uptake by cell-free extracts of *A. niger* UBC 814, with benzoate and an NADPH-regenerating system and the identification of 4-hydroxybenzoate and 3,4-dihydroxybenzoate as intermediates in the oxidation of benzoate by whole mycelia, would indicate that ring cleavage is preceded by two successive hydroxylation reactions requiring NADPH and oxygen, one hydroxylating benzoate at the 4-position and another oxidizing 4-hydroxybenzoate to 3,4-dihydroxy benzoate. Attempts to purify benzoate:NADPH oxidoreductase (4-hydroxylating) were unsuccessful.

Cell-free extracts of *A. niger* grown in the presence of mandelate oxidized DL-*p*-hydroxymandelate, consuming about 1 mole of oxygen per mole of substrate (Fig. 1). 4-Hydroxybenzoic acid was identified as the product of the reaction. Thus, 4-hydroxybenzoate is the converging point in the metabolism of mandelate and *p*-hydroxymandelate, both of which are dissimilated through the protocatechuate pathway by *A. niger*. This, again, is in sharp contrast to the bacterial systems, in which mandelate is degraded through the catechol pathway and *p*-hydroxymandelate is degraded through the protocatechuate pathway, the two pathways converging at *p*-keto adipic acid (14).

It is presumed, largely on the evidence of kinetic analyses, that the same group of enzymes degrades mandelate and *p*-hydroxymandelate to benzoate and *p*-hydroxybenzoate, respectively, in *P. putida* (14). The specificity of the enzymes degrading the side chain of mandelate in bacterium N.C.I.B. 8250 appears to be much broader (7). However, a definite statement can be made only after an examination of the substrate specificities of the purified enzymes. With this end in view, attempts at purification of the enzymes of the mandelate pathway in *A. niger* UBC 814 are in progress.

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