

# Metabolism of Aromatic Compounds in Bacteria

## PURIFICATION AND PROPERTIES OF THE CATECHOL-FORMING ENZYME, 3,5-CYCLOHEXADIENE-1,2-DIOL-1-CARBOXYLIC ACID (NAD<sup>+</sup>) OXIDOREDUCTASE (DECARBOXYLATING)

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### SUMMARY

The conversion of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (DHB) to catechol has been shown to be catalyzed by a single protein from the bacterium *Alcaligenes eutrophus*. The enzyme was purified by ultracentrifugation, ammonium sulfate fractionation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. Its molecular weight is approximately 94,600 (by sedimentation equilibrium) and the molecular weight of its subunits is approximately 24,000 (by sodium dodecyl sulfate gel electrophoresis). The proposed mechanism of the reaction is dehydrogenation of DHB to an unstable  $\beta$ -ketoacid which decarboxylates to form catechol.

benzene-methanol-acetic acid. ( $R_F$  for benzoic acid, 0.67.) [*Carboxyl*-<sup>14</sup>C]DHB was prepared biologically by modifying, as follows, the procedure for preparation of unlabeled DHB (5). Five microcuries of purified [*carboxyl*-<sup>14</sup>C]benzoic acid (56  $\mu$ Ci per  $\mu$ mole) was incubated with an aerated 1-ml culture of strain B9 ( $10^9$  cells per ml) for 15 min at 30°. The culture supernatant fluid was extracted with ethyl acetate, and the organic phase was concentrated under a stream of nitrogen. [<sup>14</sup>C]DHB was purified from this by thin layer chromatography in the solvent system described above. ( $R_F$  for DHB, 0.23.) *Cis*-3,5-cyclohexadiene-1,2-diol was a gift from Dr. D. T. Gibson. NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, ATP, yeast alcohol dehydrogenase, and DEAE-cellulose were from Sigma. Ammonium sulfate was ultrapure grade from Mann. Hyamine was from Nuclear Chicago. Serum caps and plastic center wells for measurement of <sup>14</sup>CO<sub>2</sub> release were from Kontes, Vineland, New Jersey.

*Growth of Bacteria*—*Alcaligenes eutrophus* strain 335 (5, 6) was grown aerobically at 30° in Hutner's mineral medium (7) supplemented with benzoic acid as sole carbon and energy source and was harvested in late log phase by centrifugation. The yield was 2.5 g of cells (wet weight) per liter of growth medium. Cells were stored at -20° for up to 2 months before use.

*Standard DHB Dehydrogenase Assay Based upon NADH Formation*—DHB dehydrogenase activity was assayed routinely by the DHB-dependent formation of NADH. Reactions were performed at 25° by adding enzyme to a cuvette containing 20  $\mu$ moles of Tris-HCl, pH 8.0, 0.4  $\mu$ mole of DHB, and 2  $\mu$ moles of NAD<sup>+</sup> in a volume of 1 ml, and measuring the increase in absorption at 340 nm. One unit of activity was defined as the formation of 1  $\mu$ mole of NADH per min under these assay conditions.

*Alternative DHB Dehydrogenase Assay Based upon <sup>14</sup>CO<sub>2</sub> Release*—DHB dehydrogenase was assayed alternatively by measuring the release of <sup>14</sup>CO<sub>2</sub> from [*carboxyl*-<sup>14</sup>C]DHB. Each reac-

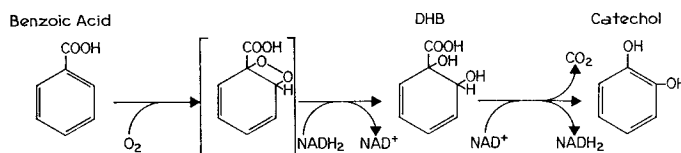


FIG. 1. The conversion of benzoic acid to catechol in bacteria (4) showing the chemical structure of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (DHB).

Benzoic acid as sole carbon and energy source can support growth of a variety of bacteria. The degradative pathway by which benzoic acid is converted to tricarboxylic acid cycle intermediates has been well characterized (1-3), with the exception of the first steps which involve the conversion of benzoic acid to catechol. We have recently demonstrated that a previously unknown compound, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid, is an intermediate in the conversion of benzoic acid to catechol in bacteria (4) (Fig. 1). This paper describes the purification and properties of the enzyme which catalyzes the conversion of DHB<sup>1</sup> to catechol. *Alcaligenes eutrophus*, used here as the source of the enzyme, was the bacterial species from which DHB originally was recognized and purified (5).

### EXPERIMENTAL PROCEDURE

*Materials*—DHB was prepared biologically as described previously (5) by using *Alcaligenes eutrophus* B9, a mutant strain which is blocked in the conversion of DHB to catechol and so accumulates DHB from benzoic acid. [*Carboxyl*-<sup>14</sup>C]benzoic acid (56  $\mu$ Ci per  $\mu$ mole) was purchased from Amersham-Searle and was purified by thin layer chromatography using Eastman 6060 type K301R silica gel sheets and a solvent system of 4:6:1

<sup>1</sup> The abbreviations used are: DHB, (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (derived from its trivial name, dihydrodihydroxybenzoate); SDS, sodium dodecyl sulfate.

TABLE I  
Purification of DHB dehydrogenase

Fraction	Volume	Protein	Enzyme	Specific activity
	ml	mg	units	units/mg
I. Extract.....	1,560	22,100	17,700	0.8
II. Supernatant fraction.....	1,400	18,000	18,800	1.0
III. Ammonium sulfate.....	102	1,185	11,400	9.6
IV. DEAE-cellulose.....	7	55	4,400	80.2
V. Sephadex G-200.....	7	14	2,090	150.0

tion took place in a 25-ml flask sealed by a serum cap. Through the cap hung a plastic center well containing 0.15 ml of 1 M Hyamine to trap released CO<sub>2</sub>. Reactions were performed at 25° by injecting DHB dehydrogenase through the serum cap into a 1-ml reaction mixture containing, in addition to the standard assay components described above, 10,000 cpm of [*carboxyl*-<sup>14</sup>C]DHB. Reactions were stopped by injecting 0.2 ml of 1% acetic acid. This rendered the medium sufficiently acidic to release CO<sub>2</sub> from solution but not to decompose the acid-labile DHB (5). The flasks then were shaken gently for 15 min at room temperature, after which the center well was placed directly into a vial containing Bray's scintillation fluid (8) for measurement of its radioactive content.

**Measurement of Catechol Formation**—Catechol formation was measured either by the method of Arnow (9), which is based on the reaction of certain hydroxyaromatic compounds with a nitrite-molybdate reagent, or spectrophotometrically by the increase in absorptivity at 220 nm. In the latter instance, pyridine nucleotide concentration was restricted to 0.05 mM or less, in order to permit sufficient transmittance at 220 nm. Conversion of NAD<sup>+</sup> to NADH does not affect transmittance at 220 nm (10).

**Ultracentrifugation Studies**—Sedimentation equilibrium and sedimentation velocity determinations on purified DHB dehydrogenase were carried out in a Spinco model E centrifuge, using Fraction V material which had been equilibrated with 0.1 M KCl in 0.02 M Tris-HCl, pH 8.0, by passage through a Sephadex G-25 column. A partial specific volume for DHB dehydrogenase of 0.73 was assumed for the calculations. Sedimentation equilibrium was carried out by the method of Yphantis (11) in a six-channel centerpiece. After centrifugation at 16,100 rpm at 19° for 44 hours, recordings of absorption at 280 nm *versus* radial distance were obtained by ultraviolet optics.

Sedimentation velocity data were obtained in the model E centrifuge by subjecting the enzyme prepared as described above to centrifugation at 56,700 rpm at 25°, during which time the resulting schlieren patterns were photographed at 8-min intervals. Sedimentation velocity data based on enzymatic activity were obtained by sucrose gradient centrifugation. Samples of 150 μl containing DHB (see text) and 1 μg of yeast alcohol dehydrogenase as a sedimentation standard (*S* = 7.61, Reference 12) were layered on 5-ml gradients of 5% to 20% sucrose in 0.02 M Tris-HCl, pH 8.0. Approximately 30 fractions were collected, and DHB dehydrogenase activity in each fraction was determined by the standard assay.

**Other Methods**—Protein concentrations were determined by the method of Lowry *et al.* (13) for DHB dehydrogenase Fractions I to III and by absorption at 280 nm for Fractions IV and V, assuming an absorption of 1.0 for a 1 mg per ml solution. Ultraviolet and visible spectra were obtained with a Cary model 14 recording spectrophotometer.

## RESULTS

### Purification of DHB Dehydrogenase

**Extract**—Approximately 500 g of cells were thawed and suspended in 2 liters of 0.02 M Tris-HCl, pH 8.0, at 0°. Cells were disrupted by exposure to sonic oscillation in 1-liter batches for two periods of 15 min each with a Bronwell Biosonic II system operating at maximum intensity. Unbroken cells were removed by centrifugation at 10,000 × *g* for 15 min, yielding a crude bacterial extract (Fraction I, Table I).

**Supernatant Fraction**—The extract was centrifuged at 4° at

100,000 × *g* for 90 min, and the supernatant fraction was retained as Fraction II.

**Ammonium Sulfate Fractionation**—Operations were performed between 0° and 4°. A 24% to 33% of saturation ammonium sulfate fraction was obtained as follows. 12.8 g of ammonium sulfate were added per 100 ml of Fraction II. After 20 min of stirring, the precipitate was removed by centrifugation at 10,000 × *g* and the supernatant was retained. An additional 5.4 g of ammonium sulfate per 100 ml of Fraction II were added to the supernatant, and after 20 min stirring, the precipitate was collected by centrifugation and resuspended in 0.02 M Tris-HCl, pH 8.0. This material was dialyzed against 0.02 M Tris-HCl, pH 8.0, and retained as Fraction III.

**Chromatography on DEAE-Cellulose**—A column of DEAE-cellulose (4.9 cm<sup>2</sup> × 70 cm) was prepared and equilibrated at 4° with 0.02 M Tris-HCl, pH 8.0. Fraction III was applied to the column, washed with 500 ml of 0.02 M Tris-HCl, pH 8.0, and then eluted with a linear ammonium sulfate gradient generated with a mixing chamber containing initially 700 ml of 0.02 M Tris-HCl, pH 8.0, and a reservoir containing initially 700 ml of 0.02 M Tris-HCl, pH 8.0, and 0.075 M ammonium sulfate. Fractions of 6.5 ml were collected every 6 min. A single peak of DHB dehydrogenase activity was obtained, and the 15 fractions containing the highest specific activities were combined and concentrated to 7 ml using a Diaflo apparatus with a UM-10 membrane (Fraction IV).

**Chromatography on Sephadex G-200**—A column of Sephadex G-200 (4.9 cm<sup>2</sup> × 100 cm) was equilibrated with 0.02 M Tris-HCl, pH 8.0. Fraction IV was passed through the column at room temperature by upward flow in 0.02 M Tris-HCl, pH 8.0. Fractions of 4.9 ml were collected every 6 min. A single peak of protein and of DHB dehydrogenase activity was obtained, and the 10 central fractions were combined and concentrated using a Diaflo apparatus with a UM-10 membrane (Fraction V).

### Properties of Purified Enzyme

**Molecular Weight Determination**—The molecular weight of the purified enzyme was estimated by equilibrium sedimentation following the method described under "Experimental Procedure." From the results obtained at two protein concentrations (Fig. 2), the molecular weight was estimated to be 94,600 ± 2,100.

**Sedimentation Velocity Determination**—The sedimentation velocity of DHB dehydrogenase was determined by centrifugation of Fraction V enzyme in the model E centrifuge, as described under "Experimental Procedure." A single, symmetric schlieren peak was observed to sediment at *S*<sub>20,H<sub>2</sub>O</sub> = 6.20. The sedimentation velocity of DHB dehydrogenase activity also was estimated by sucrose gradient centrifugation. Fraction I and Fraction V enzymes were sedimented as described under "Experimental Procedure." Fraction V enzyme was also sedimented in three additional gradients which contained 1 mM NAD<sup>+</sup>,



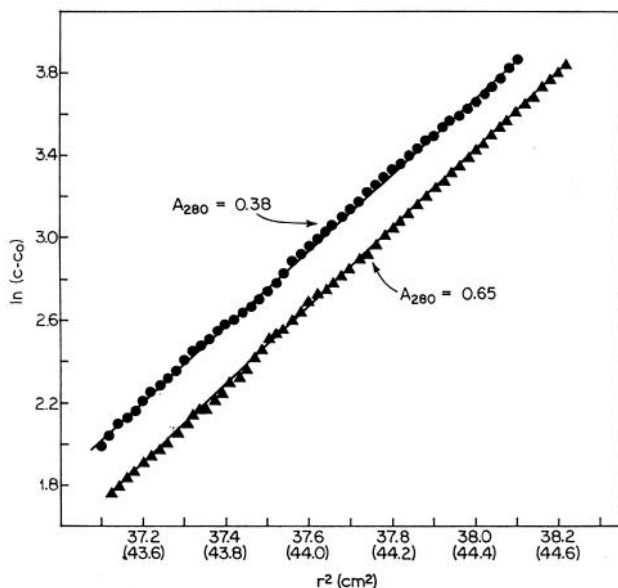


FIG. 2. Estimation of molecular weight of DHB dehydrogenase by equilibrium sedimentation. DHB dehydrogenase was subjected to ultracentrifugation at concentrations corresponding to absorptions at 280 nm of 0.65 and 0.38 for the lower and upper curves respectively, as described under "Experimental Procedure." The abscissa values of  $r^2$  in parentheses correspond to the data for the higher protein concentration.

NADH, or DHB. In each instance,  $S$  was found to be in the range 6.0 to 6.5.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis of Fraction V enzyme, in the presence and absence of SDS, showed essentially single protein bands (Fig. 3). By comparison with standard molecular weight markers in SDS polyacrylamide gel electrophoresis (Fig. 4), the subunit size of DHB dehydrogenase was estimated to be 24,000 daltons.

**Ultraviolet Spectrum**—The spectrum of purified DHB dehydrogenase is shown in Fig. 5. A shoulder is visible at 290 nm, presumably caused by absorption by tryptophan residues.

**Reaction Stoichiometry**—The stoichiometry of the reaction catalyzed by purified DHB dehydrogenase was investigated by varying the concentrations of DHB and  $\text{NAD}^+$  and measuring formation of three products, catechol, NADH, and  $\text{CO}_2$ . The results (Table II) show that 1 mole of each of the reactants is converted to 1 mole of each of these three products.

To confirm the identity of catechol, the reaction products were extracted at neutrality with ether. The ether was evaporated and the residues were resuspended in aqueous solutions at pH 1, pH 7, and pH 13. The ultraviolet spectra of these solutions were indistinguishable from those of authentic catechol at the same pH levels. A further confirmation was obtained using the nitrite-molybdate reagent which forms characteristic colored products with catechol and certain other hydroxyaromatic compounds (9). The visible spectra, at acid and basic pH, of the reaction products treated (9) with this reagent were indistinguishable from that of authentic catechol treated in the same way.

**Kinetics of Formation of NADH, Catechol, and  $\text{CO}_2$** —The kinetics of formation of each of the three reaction products was compared. As shown in Fig. 6a their formations follow approximately the same kinetics. NADH and catechol formations were more closely compared at reduced  $\text{NAD}^+$  concentration by measuring catechol formation continuously by ultraviolet spectrophotometry (see "Experimental Procedure"). These results are shown in Fig. 6b.

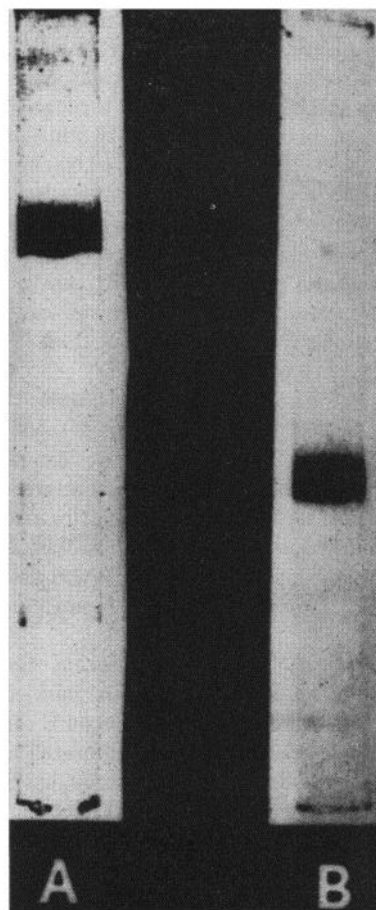


FIG. 3. Polyacrylamide gel electrophoresis of DHB dehydrogenase (Fraction V). Standard gels at 7% acrylamide were run according to the procedures described in the Canalco manual and were stained with Amido black. SDS gels at 10% acrylamide were run according to the procedures of Weber and Osborn (14), and were stained with Coomassie brilliant blue. Destaining was by transverse electrophoresis. Gel A, SDS gel, 20  $\mu\text{g}$  of protein; Gel B, standard gel, 20  $\mu\text{g}$  of protein.

**Pyridine Nucleotide Specificity**—DHB dehydrogenase activity, as measured either by pyridine nucleotide formation or by release of  $^{14}\text{CO}_2$  from [*carboxyl- $^{14}\text{C}$* ]DHB, depends strictly on  $\text{NAD}^+$ . No activity was detected when  $\text{NAD}^+$  was replaced by  $\text{NADP}^+$ .  $\text{NADP}^+$ , when present in excess over  $\text{NAD}^+$ , partially inhibits activity. These results are summarized in Table III.

**No Stimulation of Activity by Other Cofactors**—Other cofactors associated with decarboxylation or dehydrogenation reactions were tested for their possible effects on either Fraction I or Fraction V DHB dehydrogenase. Thiamine pyrophosphate, biotin, coenzyme A, pyridoxal phosphate,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ , and  $\text{Zn}^{++}$ , each tested at 0.1 mM and 1 mM under standard DHB dehydrogenase assay conditions, had no stimulatory effect on activity.

**Results with Substrates Other than DHB**—Benzoic acid, *cis*-3,5-cyclohexadiene-1,2-diol and malic acid were tested as possible substrates for DHB dehydrogenase. Reaction mixtures were those routinely used to assay DHB dehydrogenase, except that DHB was replaced by the test substrate at concentrations of 1 mM and 10 mM, and an excess of enzyme (1 unit) was present. No NADH formation was detected with these substrates. Neither was any enzyme-stimulated release of  $^{14}\text{CO}_2$  from [*carboxyl- $^{14}\text{C}$* ]benzoic acid detected. None of these compounds, when added at 1 mM to standard assay mixtures, was found to inhibit DHB dehydrogenase.

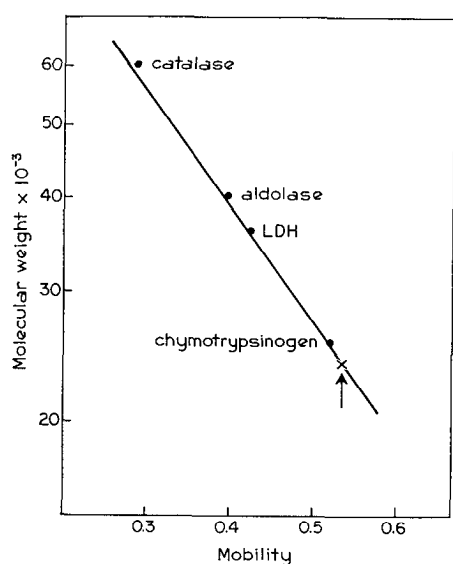


FIG. 4. Molecular weight determination of DHB dehydrogenase by SDS polyacrylamide gel electrophoresis. Samples of catalase, aldolase, lactate dehydrogenase, chymotrypsinogen, and DHB dehydrogenase (Fraction V) were denatured in SDS and run on SDS gels at 20  $\mu\text{g}$  of protein per gel according to standard procedures (14). Mobilities shown are the averages of duplicate gels for marker proteins and quadruplicate gels for DHB dehydrogenase. The arrow indicates the average mobility of the single band on DHB dehydrogenase gels and corresponds to a molecular weight of approximately 24,000.

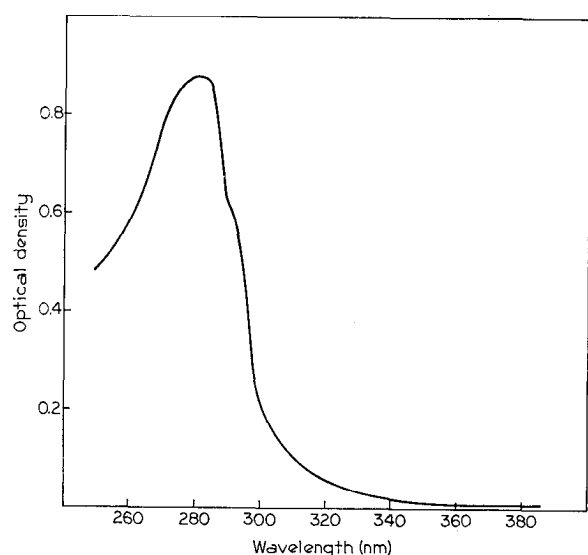


FIG. 5. Ultraviolet spectrum of purified DHB dehydrogenase. Enzyme (Fraction V) was in 0.02 M Tris-HCl, pH 8.0. A Cary model 14 spectrophotometer with 1-cm quartz cells was used.

Attempts were made to demonstrate the reverse of the dehydrogenase reaction by using catechol and NADH as substrates. With NADH at 1 mM, catechol at 0.1, 1.0, or 10 mM, and buffers at pH 8.0 (Tris-HCl), pH 7.0, or pH 6.0 ( $\text{NaHCO}_3$ ) no enzyme-dependent oxidation of NADH could be demonstrated. Catechol, when added at 1 mM to a standard assay mixture, did not inhibit DHB dehydrogenase.

*K<sub>m</sub> Values for DHB and NAD<sup>+</sup>*—The *K<sub>m</sub>* values for DHB and NAD<sup>+</sup> were determined by double-reciprocal plots to be 0.20 mM and 0.15 mM, respectively, for purified DHB dehydrogenase.

*Effects of Salt and of pH on DHB Dehydrogenase*—DHB dehydrogenase is sensitive to inhibition by salt. The inhibition de-

TABLE II

*Stoichiometry of reaction catalyzed by DHB dehydrogenase*

Reaction mixtures of 1 ml, containing 20  $\mu\text{moles}$  of Tris-HCl, pH 8.0, 0.5 unit of DHB dehydrogenase (Fraction V), and NAD<sup>+</sup> and DHB as stated, were incubated for 10 min at 25°. For each pair of substrate concentrations, two reaction mixtures were used. With one mixture, NADH was determined by absorption at 340 nm ( $\epsilon = 6,220$  liters mole<sup>-1</sup> cm<sup>-1</sup>) and catechol was determined by the method of Arnow (9). The other reaction contained, as an additional component, 10,000 cpm of [*carboxyl*-<sup>14</sup>C]-DHB and was performed in flasks which allowed <sup>14</sup>CO<sub>2</sub> release to be measured as described under "Experimental Procedure."

DHB added	NAD added	Catechol formed	NADH formed	CO <sub>2</sub> formed
$\mu\text{moles}$		$\mu\text{moles}$		
0.0	1.0	0.001	0.001	0.010
0.1	1.0	0.098	0.091	0.112
0.2	1.0	0.210	0.190	0.217
0.3	1.0	0.295	0.288	0.309
1.0	0.0	0.001	0.001	0.015
1.0	0.1	0.097	0.092	0.118
1.0	0.2	0.194	0.192	0.195
1.0	0.3	0.304	0.291	0.310

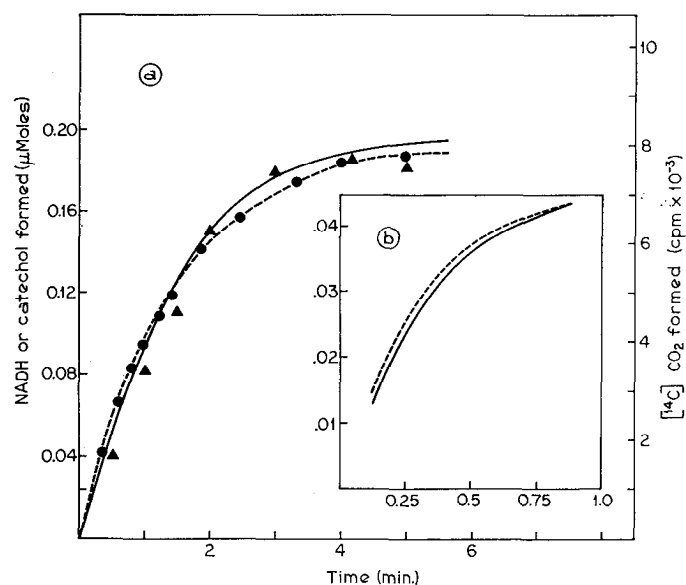


FIG. 6. Kinetics of formation of NADH, catechol, and CO<sub>2</sub> by DHB dehydrogenase. *a*, 10 separate reaction mixtures each containing 20  $\mu\text{moles}$  of Tris-HCl, pH 8.0, 1  $\mu\text{mole}$  of NAD<sup>+</sup>, and 0.2  $\mu\text{mole}$  of DHB in 1 ml were used. In one, NADH formation was measured by continuously recording the increase in absorption at 340 nm (—). From a second, samples were removed at intervals and the catechol concentration was measured by the method of Arnow (9) (●---●). The remaining eight mixtures contained, in addition, 10,000 cpm of [<sup>14</sup>C]DHB in flasks designed to measure <sup>14</sup>CO<sub>2</sub> release (see "Experimental Procedure") (▲). *b*, two identical reaction mixtures contained 20  $\mu\text{moles}$  of Tris-HCl, pH 8.0, 0.05  $\mu\text{mole}$  of NAD<sup>+</sup>, and 0.2  $\mu\text{mole}$  of DHB in 1 ml. NADH (—) and catechol (---) formation were measured by the increase in absorption at 340 nm and 220 nm, respectively.

pends markedly on the pH of the assay mixture and on the particular salt used. DHB dehydrogenase activity as a function of pH, in the presence and absence of 30 mM Na<sub>2</sub>SO<sub>4</sub>, is shown in Fig. 7. This salt dramatically inhibits activity at pH 8 or below (50% inhibition at 1 mM) but has little effect on activity at pH 9 and above. The effects of some other salts at pH 8.0 are shown in Table IV:



TABLE III

## Pyridine nucleotide specificity of DHB dehydrogenase

Reaction mixtures of 1 ml contained 20  $\mu$ moles of Tris-HCl, pH 8.0, 0.4  $\mu$ mole of DHB, 0.25 unit of DHB dehydrogenase (Fraction V), and pyridine nucleotide concentration as shown. Products were measured as in Table II.

Pyridine nucleotide	NADH or NADPH formed	$^{14}\text{CO}_2$ released in 4 min
$\mu$ moles	$\mu$ moles/min	cpm
0.5 NAD	0.201	3182
0.5 NADP <sup>+</sup>	<0.0004	61
2.5 NADP <sup>+</sup>	<0.0004	63
0.5 NAD <sup>+</sup> and 2.5 NADP <sup>+</sup>	0.153	2418
None	<0.0004	54

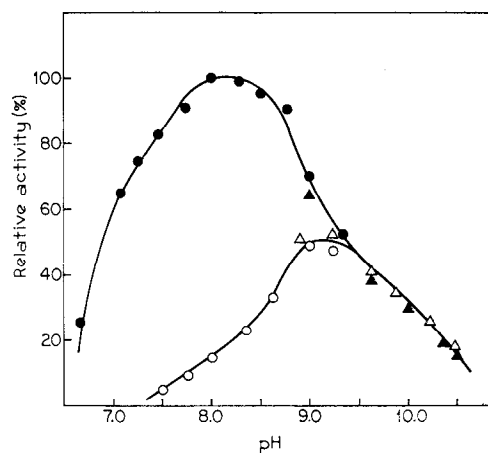


FIG. 7. The effect of pH on DHB dehydrogenase activity in the presence and absence of 30 mM  $\text{Na}_2\text{SO}_4$ . Activity was assayed under standard conditions, except that 0.02 M Tris, pH 8.0, was replaced by 0.02 M Tris at different pH levels or by 0.03 M glycine at different pH levels, and that  $\text{Na}_2\text{SO}_4$  was present where shown. ●, Tris buffer; ○, Tris buffer,  $\text{Na}_2\text{SO}_4$  added; ▲, glycine buffer; △, glycine buffer,  $\text{Na}_2\text{SO}_4$  added.

TABLE IV

## DHB dehydrogenase activity in the presence of inorganic salts

Assays were performed at standard conditions, except for the presence of salt.

Salt	Concentration	Activity
	mM	
None	0	100
$\text{Na}_2\text{SO}_4$	1	51
$\text{Na}_2\text{SO}_4$	2	25
$\text{Na}_2\text{SO}_4$	10	12
$(\text{NH}_4)_2\text{SO}_4$	4	30
$(\text{NH}_4)_2\text{SO}_4$	10	18
$\text{MgSO}_4$	10	31
$\text{NaNO}_2$	30	32
$\text{NaNO}_3$	30	33
$\text{NH}_4\text{Cl}$	60	55
$\text{Na}_2\text{HPO}_4$	30	61
$\text{Na}_2\text{succinate}$	80	75

Properties of DHB dehydrogenase were compared at pH 8.0 and pH 9.6. At pH 9.6, the  $K_m$  values for DHB and  $\text{NAD}^+$  each were 6-fold higher than at pH 8.0 (1.2 mM and 1.0 mM, respectively).  $V_{\text{max}}$  was the same at both pH values. At pH 9.6, neither the  $K_m$  values nor the  $V_{\text{max}}$  was affected by the presence of 30 mM  $\text{Na}_2\text{SO}_4$ . The sedimentation rate of DHB dehydro-

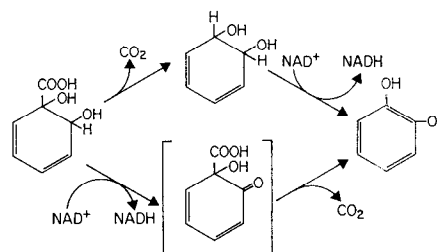


FIG. 8. Two possible schemes for the conversion of DHB to catechol by DHB dehydrogenase.

genase activity, determined by sucrose gradient sedimentation of Fraction V enzyme, was the same at pH 8.0 and pH 9.6 and was not affected at either pH by the presence of 30 mM  $\text{Na}_2\text{SO}_4$  in the gradients.

## DISCUSSION

The conversion of DHB to catechol, which involves both a dehydrogenation and a decarboxylation, has been shown to be catalyzed by a single protein which apparently is comprised of a single type of subunit. Two different schemes for the reaction, depending on the order of dehydrogenation and decarboxylation, are shown in Fig. 8. In the *upper scheme*, decarboxylation occurs first, with the formation of 3,5-cyclohexadiene-1,2-diol, a compound known to be an intermediate in the conversion of benzene to catechol in *Pseudomonas putida* (15). In the *lower scheme*, dehydrogenation occurs first, with the formation of an unstable  $\beta$ -ketoacid intermediate which then decarboxylates to catechol.

Two results presented here favor the *lower scheme*. 3,5-Cyclohexadiene-1,2-diol was neither a substrate for purified DHB dehydrogenase nor stimulated  $\text{NADH}$  formation in crude extracts. Decarboxylation of DHB was strictly dependent on the presence of  $\text{NAD}^+$ . The *lower scheme*, which is consistent with the well established mechanism of biochemical decarboxylation via  $\beta$ -ketoacids, is suggested as the route of catechol formation by DHB dehydrogenase. The proposed  $\alpha$ -hydroxy- $\beta$ -ketoacid intermediate would spontaneously decarboxylate to form catechol. Whether decarboxylation *in vivo* is spontaneous or DHB dehydrogenase-mediated is not known. No intermediate was detected by comparing the kinetics of catechol and  $\text{NADH}$  formation (Fig. 6). Although no requirement for catechol formation other than  $\text{NAD}^+$  was demonstrated, the presence of a divalent cation or another decarboxylation-facilitating cofactor tightly bound to DHB dehydrogenase has not been ruled out.

The presence of a stable enzyme which catalyzes the conversion of DHB to catechol indicates that the difficulties in characterizing the formation of catechol from benzoic acid in bacteria concern the step or steps involving the conversion of benzoic acid to DHB. This now remains as the one poorly characterized portion of the 8- to 9-step conversion of benzoic acid (and the 12- to 13-step conversion of mandelic acid) to tricarboxylic acid intermediates via  $\beta$ -ketoacid in bacteria (16-18).

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