populations have shown that H and L chains can reassociate into molecules with some of the properties of the parent immunoglobulin population, but which are characterized by a reduced binding activity. This residual activity could be due to (a) nonhomologous chain pairing, in which case the association of chains within the antibody population to form effective active sites would be relatively permissive; or (b) predominantly to the re-formation of original chain pairs, which alternatively suggests that the association of two *particular* chains is required. Mixtures of proteins 315 and 460, potential members of a heterogeneous anti-DNP population, fail to display either recovery of full binding activity or normal four-chain structure. This emphasizes that ligand binding activity depends upon effective chain–chain interactions even when the nonhomologous chains are derived from molecules with similar combining sites.

Acknowledgment

We thank Michael Potter for providing plasmacytomas MOPC-173, 315, and 460 and Martin Weigert, for S-176.

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Metabolism of Benzoic Acid by Bacteria. Accumulation of (−)-3,5-Cyclohexadiene-1,2-diol-1-carboxylic Acid by a Mutant Strain of *Alcaligenes eutrophus**

Albey M. Reiner† and George D. Hegeman

ABSTRACT: A mutant strain of *Alcaligenes eutrophus* blocked in benzoic acid catabolism converts benzoic acid into a previously unknown compound which was identified as 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid. The incorporation of two atoms of molecular oxygen was established by mass spectroscopic examination of the pattern of incorporation of ^{18}O from an $^{18}O_2$-enriched atmosphere during formation of the compound from benzoic acid by whole cells. This evidence, together with that favoring the cis configuration for the compound, suggests that it is formed by reduction of a peroxide produced from benzoic acid in a dioxygenase-mediated reaction.

Several monosubstituted benzoic acids are also converted into the corresponding substituted 3,5-cyclohexadiene-1,2-diol-1-carboxylic acids by benzoate-induced cell suspensions, indication that the enzyme(s) responsible for the conversion are relatively nonspecific. A scheme is presented which includes this previously unknown compound as an intermediate in the conversion of benzoic acid into catechol by bacteria.

The oxidation of benzoic acid by bacteria has been known for many years to proceed *via* catechol (Stanier, 1948). Molecular oxygen is known to participate, but little mechanistic detail has been learned about the conversion of benzoic acid into catechol, in part because it has been difficult to demonstrate appreciable oxidation of benzoic acid in cell-free extracts.

Because of the chemical complexity of the process it has been suggested that several enzymatic steps are involved in the

* From the Department of Bacteriology and Immunology, University of California, Berkeley, California 94720. *Received February 5, 1971.* This work was supported by a U. S. Public Health Service Postdoctoral Fellowship to A. M. R. and by U. S. Public Health Service Grants AI-1808 from the National Institute of Allergy and Infectious Disease and HD-2448 from the National Institute of Child Health and Human Development.

† Present address: Department of Microbiology, University of Massachusetts, Amherst, Mass. 01002.

formation of catechol, and the enzymes involved have been referred to as benzoate oxidase or the benzoate oxidase system.

In the oxidation of certain other aromatic compounds to dihydroxy aromatic compounds, nonaromatic dihydroxy intermediates have been isolated (Table I). Formation of these compounds can occur through either peroxide or oxide intermediates (Gibson, 1968). Here we report the isolation of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (I), which is produced by a mutant strain of *Alcaligenes eutrophus* blocked in the conversion of benzoic acid into catechol. Two atoms of molecular oxygen are incorporated into the compound suggesting that it is formed *via* a peroxide intermediate. The trivial name 1,2-dihydro-1,2-dihydroxy benzoic acid is suggested for this compound to emphasize its role in benzoic acid metabolism.

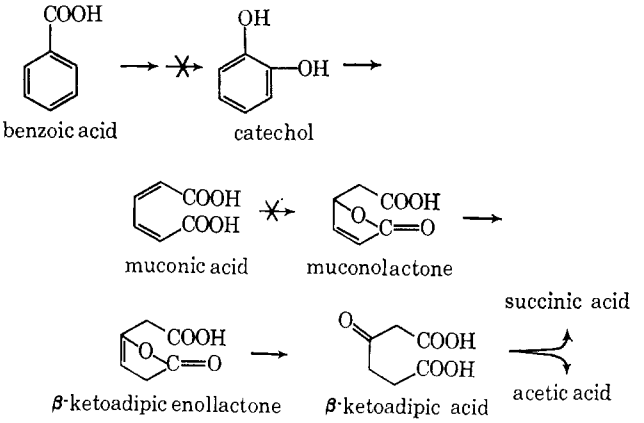


FIGURE 1: Benzoic acid metabolism in *A. eutrophus*. Crosses mark the two lesions of strain B9.

Materials and Methods

Biological Materials and Methods. A. eutrophus mutant strain B9 and the parental strain 335 from which it was derived were provided by B. Johnson. Liquid cultures were grown on a rotary shaker at 30° in HMB medium (Stanier *et al.*, 1966) with 5 mM fructose or sodium succinate as carbon and energy source and 2 mM sodium benzoate as inducer or substrate. For the conversion of benzoic acid to I, B9 cultures were grown to cell density of 5×10^8/ml, harvested by centrifugation, resuspended to 1.5×10^9 cells/ml in 0.05 M Na_2H–KH_2–phosphate buffer (pH 6.8) containing 1 mM benzoic acid and shaken at 30°. Conversion of benzoate to I was measured by ultraviolet spectroscopy, and additional aliquots of benzoic acid were added at approximately hourly intervals, to maintain a concentration of 1–2 mM. After complete conversion of benzoic acid, cells were removed by centrifugation.

Preparation of Compound I. Compound I was isolated by concentrating the cell-free supernatant fluid 50-fold by flash evaporation under reduced pressure at 45–50°. The concentrated solution was acidified at 0° to pH 2.0 with HCl, and extracted repeatedly with ethyl acetate at 0° (partition of I between H_2O and ethyl acetate, 7:1). The ethyl acetate extracts were pooled and concentrated to 2–10 ml by flash evaporation at 25°–30°. Compound I was then back-extracted into an equal volume of H_2O at 0°.

To prepare I as the free acid, the aqueous solution was added to a 60 to 100-fold excess of diethyl ether. The ether was dried with $MgSO_4$ and evaporated at room temperature until a crust of white crystals formed. The ether solution was then placed at −76° to permit further crystallization to occur. To prepare the sodium salt of I, the aqueous solution following back-extraction from ethyl acetate was neutralized with NaOH and crystallized from EtOH–H_2O. The yield was approximately 50% based on the number of moles of benzoic acid added.

Analytical Methods. Ultraviolet spectra were determined with a Cary Model 14 recording spectrophotometer. Infrared spectra were obtained with a Perkin-Elmer 137 spectrophotometer. Nuclear magnetic resonance spectra were determined with a Varian Model T-60 spectrometer, with tetramethylsilane used as internal or external standard.

Mass spectra and mass measurements were obtained with a Consolidated Electrodynamics Corp. Model 21-110B double-focusing high-resolution mass spectrometer.[1] Solid samples were introduced directly using a probe at a source temperature of 98°. Gaseous samples were introduced from an accessory reservoir. Measurements of optical rotation were made using a Bendix Model ETL-NDL, type 143A instrument.

Materials. $[^{18}O]O_2$ (93.35% enriched) was from Miles Laboratories, Elkart, Ind. 2-Chloro-, 3-chloro-, 4-chloro-, 2-fluoro-, and 3-fluorobenzoic acids, *trans*-cyclohexane-1,2-diol, and a mixture of *cis*- and *trans*-cyclohexane-1,2-diol were from the Aldrich Chemical Co., Milwaukee, Wis. 2-Methyl-, 3-methyl-, 4-methyl-, 2-amino-, 2-nitro-, 3-nitro-, and 4-nitrobenzoic acids were from MC & B, Norwood, Ohio. 4-Fluorobenzoic acid was from Pierce Chemicals, Rockford, Ill. 4-Aminobenzoic acid was from Eastman Chemical, Rochester, N. Y. 3-Methyl- and 4-methylcatechol were from K & K Laboratories, Plainview, N. J.

Results

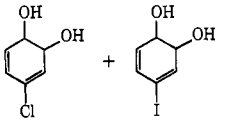
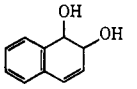
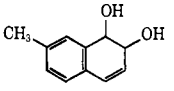
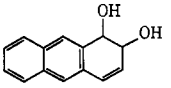
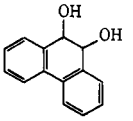
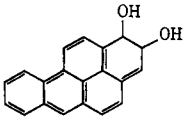
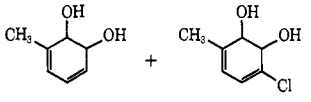
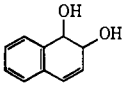
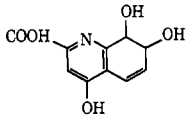
The Mutant Organism. A. eutrophus grows at the expense of benzoic acid, which it metabolizes *via* catechol and the β-ketoadipate pathway (Figure 1). Strain B9 was isolated from *A. eutrophus* strain 335 by B. Johnson, who screened mutagenized cells for the inability to grow with benzoic acid as sole carbon and energy source. In addition to the lesion which leads to the accumulation of I from benzoic acid, B9 also has a separate mutation which causes it to accumulate *cis,cis*-muconic acid from catechol (Figure 1) (B. Johnson and R. Y. Stanier, in preparation). Compound I and *cis,cis*-muconic acid have very similar ultraviolet spectra (Figure 2). That the product accumulated from benzoic acid was not muconic acid was discovered when A. M. R. attempted to use B9 to prepare fluoromuconic acid from fluorobenzoic acid as part of a separate investigation.

Identification of Compound I. Compound I was identified as 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid from its analysis, spectral properties, and identification of its decomposition products under acidic conditions. Compound I had a melting point of 85° (dec). *Anal.* Calcd for $C_7H_8O_4$: C, 53.85; H, 5.16. Found: C, 53.99; H, 5.16.[2] The ultraviolet spectrum of the Na^+ salt (pH 7.0, H_2O) showed λ_{max} 262 nm (ϵ 3350). Two closely related compounds (+)-1-methyl-4,6-cyclohexadiene-2,3-diol and 3,5-cyclohexadiene-1,2-diol show $\lambda_{max}^{H_2O}$ 265

[1] Purchased with funds provided by National Science Foundation Grant GP5323. Mass spectra and mass measurements were determined by F. Balistreri of the Department of Chemistry, University of California, Berkeley.

[2] Performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.

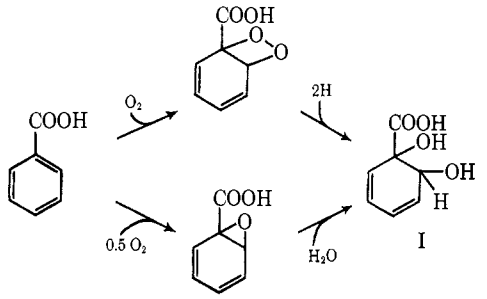
TABLE I: Dihydroxycyclohexadiene Products from the Oxidation of Aromatic Compounds.

Compound	Biological Source	Suggested Intermediate in Formation	Evidence for Mechanism	Reference
	Mammalian	Oxide	Trans isomer	Sato *et al.* (1963)
	Mammalian	Oxide	Trans isomer	Smith *et al.* (1950) Azouz *et al.* (1953)
	Mammalian Mammalian	Oxide Oxide	^{18}O incorporation, trans isomer Isolation of oxide	Holtzman *et al.* (1967) Jerina *et al.* (1968)
	Mammalian			Grimes and Young (1956)
	Mammalian	Oxide	Trans isomer	Booth and Boyland (1949)
	Mammalian	Oxide	Trans isomer	Boyland and Wolf (1950)
	Mammalian			Weigert and Mottram (1946)
	Pseudomonas	Peroxide	^{18}O incorporation, cis isomer	Gibson *et al.* (1970a)
	Pseudomonas	Peroxides	Cis isomers	Gibson *et al.* (1968, 1970b)
	Baccillus Pseudomonad	Oxide Oxide	Trans isomer Trans isomer	Walker and Wiltshire (1953) Griffiths and Evans (1965)
	Pseudomonas	Oxide	^{18}O incorporation	Taniuchi and Hayaishi (1963) and Hayaishi (1966)

nm (ϵ 5220) (Gibson *et al.*, 1970b) and 262 nm (ϵ 3715) (Nakajima *et al.*, 1959), respectively. The nuclear magnetic resonance (in δ) spectrum of I (free acid, Me_2SO-d_6) showed signals at 4.66 (1 H, doublet, J = 2.5 Hz) and 5.6-6.2 (4 H, complex multiplet). The chemical shift of the carboxyl portion is 11.5 ppm in this solvent. The infrared spectrum (in μ) of the Na^+ salt (Nujol) showed major absorption maxima at 2.8, 3.0 (broad), 6.05, 6.25 (broad, 6.85 (broad), 7.30 (broad), and 9.1. The pK_a was 2.9. The $[\alpha]_D^{25}$ = −123.8°, Na^+ salt (1.78%, w/v) in H_2O.

Compound I decomposes readily under acidic conditions When an unneutralized aqueous solution of I was warmed to 45°, crystals precipitated, bubbles formed, and the odor of phenol became evident. The crystalline compound was identified as salicylic acid from its melting point (159°), infrared spectrum, and ultraviolet spectra at basic, neutral, and acidic pH. Phenol was extracted from the aqueous solution and its identity was confirmed from its ultraviolet spectra at basic, neutral, and acidic pH. The gas was assumed to be CO_2 evolved in the formation of phenol. Two structural isomers

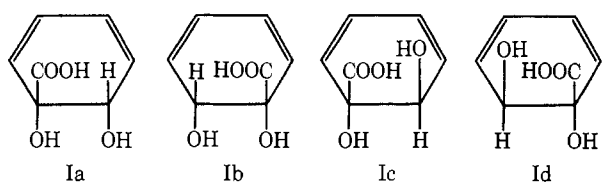
SCHEME I



of I, along with their thermal decomposition products, are shown in Figure 3.

Incorporation of ^{18}O into Compound I. Both peroxide and epoxide mechanisms have been proposed for the biological oxidation of aromatic compounds to diols (Table I). The formation of I by each of these mechanisms is shown in Scheme I. In order to distinguish between these mechanisms B9 cells were incubated with benzoic acid in the presence of a mixture of $[^{16}O]O_2$ and $[^{18}O]O_2$. Cells were prepared as described (Materials and Methods). The suspension (175 ml) was placed in a modified 500-ml suction flask provided with a stopcock at its top and a serum cap on its vacuum outlet (Rothberg and Hayaishi, 1957). The flask was alternately flushed with N_2 and evacuated five times, and then filled in sequence with $[^{18}O]O_2$ (93.35% enriched), N_2, and air to reach approximately a calculated composition of 9.8% $[^{18}O]O_2$, 8.5% $[^{16}O]O_2$, and 81.7% N_2 (corresponding to molecular oxygen compositions of 8.5% $[^{18}O]O$–$[^{18}O]O$, 8.6% $[^{16}O]O$–$[^{16}O]O$, 1.2%$[^{16}O]O$–$[^{18}O]O$). The flask was then incubated at 30° and aerated with the aid of a magnetic stirrer. Additional portions of benzoic acid were added by injection through the serum cap. Compound I formed was purified as before, and crystallized three times. The mass spectra (Figure 4) show the parent molecular ion of the diol at *m/e* 156 for the compound which incorporates $[^{16}O]O$–$[^{16}O]O$, and at *m/e* 160 for the compound which incorporates $[^{18}O]O$–$[^{18}O]O$. The absence of a major peak at *m/e* 158 (Figure 4) indicates that two oxygen atoms are incorporated into I, and that both are derived from the same oxygen molecule. The minor peak at *m/e* 158 is expected from the $[^{18}O]O$–$[^{16}O]O$ molecules present. These conclusions are confirmed by accurate mass measurements (Table II).

Optical Isomer of 3,5-Cyclohexadiene-1,2-diol-1-carboxylic Acid Formed from Benzoic Acid by A. eutrophus B9. Four different structures consistent with the foregoing evidence may be drawn



Two of these (Ia and Ib) are *cis-vic-* and two *trans-vic*-diols (Ic and Id). In order to distinguish between these two structural classes, experiments of two types were performed. Owing to the great instability of I to low pH and high temperature these were necessarily somewhat indirect.

RATE OF REACTION WITH PERIODATE. Using *trans-* and mixed *cis-* and *trans*-cyclohexane-1,2-diol as reference compounds, I was observed to react faster with periodate than either reference, but at a rate much greater than the *trans*-1,2-diol (Visconti *et al.*, 1955).

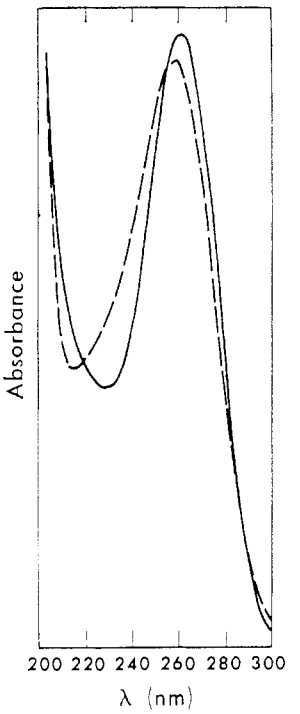


FIGURE 2: Ultraviolet spectra of I (solid line) and of *cis,cis*-muconic acid (dashed line), both at pH 7.0. For I, ϵ_{max} 3350; for *cis,cis*-muconic acid, ϵ_{max} 17,200.

COLOR REACTION WITH POTASSIUM TRIACETYLOSMATE. A positive color reaction with this reagent was observed with I and with mixed *cis-* and *trans*-cyclohexane-1,2-diol, but not with *trans*-cyclohexane-1,2-diol. The deep orange color was not discharged by addition of potassium acetate (Criegee *et al.*, 1942). A similar but lighter yellow color was observed with salicylic acid, a known decomposition product of I. Phenol, another decomposition product, also formed a pale yellow adduct, but this color was discharged by addition of potassium acetate.

These results constitute presumptive evidence in favor of a cis configuration, *i.e.*, structure Ia or Ib.

Substituted 3,5-Cyclohexadiene-1,2-diol-1-carboxylic Acids Accumulated by B9. Several analogs of I could be prepared by

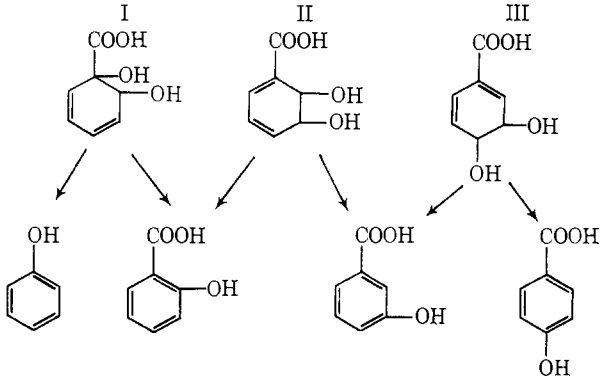


FIGURE 3: Thermal degradation products of I and two of its isomers. Results for II and III are from Young *et al.* (1969a,b).

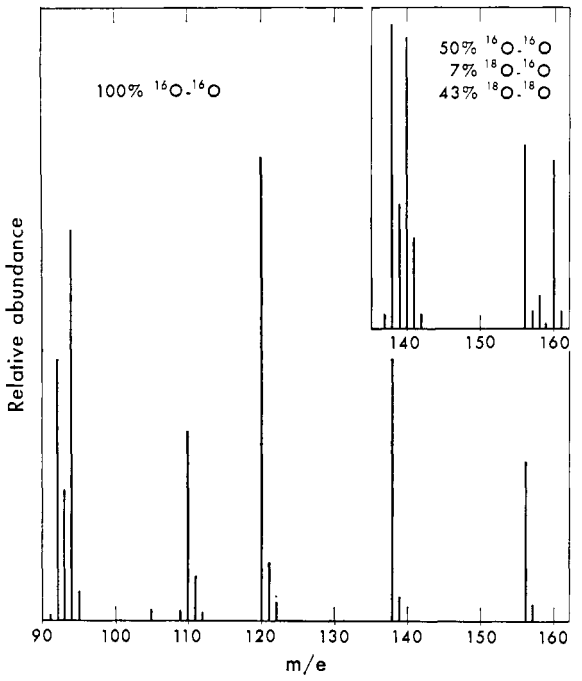


FIGURE 4: Mass spectra of I formed in air and (inset) an $[^{18}O]O_2$-enriched atmosphere.

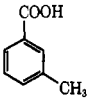
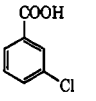
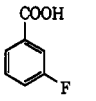
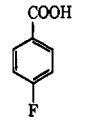
incubating benzoic acid induced cells of strain B9 with various substituted benzoic acids. The 2, 3, and 4 isomers of CH_3-, Cl-, F-, NH_2-, and NO_2-benzoate were incubated with separate cell suspensions under conditions identical with those sued to prepare I from benzoic acid. Four of these 15 suspensions accumulated a product detectable by ultraviolet spectroscopy (Table III).

TABLE II: Accurate Mass-to-Charge Ratios in the Mass Spectrum of I Formed in a Normal and in an$[^{18}O]O_2$-Enriched Atmosphere.

Formula	*m/e* Calcd	*m/e* Found	Rel Abundance in Parent Ion Region[a] (%)
Normal atmosphere (air)			
$C_7H_8{}^{16}O_4$	156.0423	156.0415	91
$C_7H_8{}^{16}O_3{}^{18}O$	158.0485	158	<1
$C_7H_8{}^{16}O_2{}^{18}O_2$	160.0507	160	<1
$[^{18}O]O_2$-enriched atmosphere[b]			
$C_7H_8{}^{16}O_4$	156.0423	156.0425	44
$C_7H_8{}^{16}O_3{}^{18}O$	158.0485	158.0475	8
$C_7H_8{}^{16}O_2{}^{18}O_2$	160.0507	160.0514	38

[a] The parent ion region was considered to be *m/e* 154 to 162. Minor species in this region were considered in calculating the relative abundance, but the values are not shown. The calculations were performed on rough scans done under the same conditions as the accurate mass measurements. [b] The isotopic composition of the oxygen portion of the atmosphere at the end of the experiment was 50% $[^{16}O]O$-$[^{16}O]O$, 7% $[^{18}O]O$-$[^{16}O]O$, 43% $[^{18}O]O$-$[^{18}O]O$ as measured by mass spectroscopy.

TABLE III: Products from the Oxidation of Substituted Benzoic Acid by B9 Cell Suspensions.[a]

Substrate	Product	λ_{max} of Product (nm)	Approx Rate of Formation (I = 100)
	IV	267	85
	V	270	15
	VI	261	40
	VII	263	40

[a] Benzoic acid induced cells of strain B9 were prepared as before and were incubated in 0.05 M Na_2H–KH_2PO_4 buffer (pH 6.8) with substrate (1 mM). Incubation was terminated when the ultraviolet absorption attributable to the substrate had disappeared.

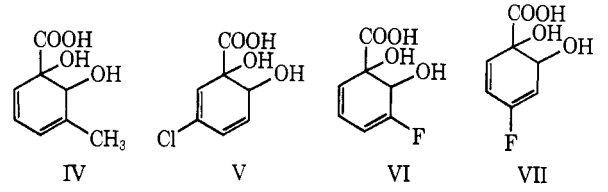
Compounds IV–VIII which accumulated were identified with the aid of 3,5-cyclohexadiene-1,2-diol-1-carboxylic (NAD) dehydrogenase (decarboxylating), a previously undescribed enzyme which catalyzes the NAD-dependent conversion of I into catechol and which is present in many genera of bacteria during their growth on benzoic acid (A. M. Reiner, in preparation). Upon incubation of samples of the four cell-free suspension fluids with a preparation of this enzyme purified 100-fold from an extract of *A. eutrophus* strain 335, IV–

TABLE IV: Spectral Characteristics of Substituted Catechols.[a]

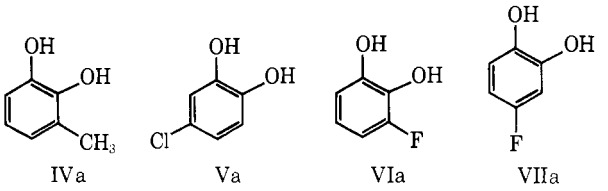
Compound	λ_{max} (nm), pH 7.0	λ_{max} (nm), pH 10.3
3-Methylcatechol	273	
4-Methylcatechol	280	
IVa	273	
3-Chlorocatechol[b]	275	270, 317
4-Chlorocatechol[b]	283	314
Va	283	314
VIa	275	
VIIa	280	

[a] Compounds IVa–VIIa were extracted from the reaction mixtures with ether. Spectra were determined in aqueous solution after removing the ether with a stream of N_2. Data for authentic methylcatechols were determined using commercial compounds. [b] Data from Taniuchi and Hayaishi (1963).

VII were each converted to the corresponding catechols (IVa–VIIa, respectively). The isomeric forms of IVa and of Va were determined from ultraviolet absorption data (Table IV). Compound VIIa is the only expected product from 4-fluorobenzoic acid, and VIa is the only other fluorocatechol isomer. From these results, structures IV–VII were assigned. The spectral data do not exclude VII as a concomitant product of 3-fluorobenzoic acid oxidation.



IV V VI VII

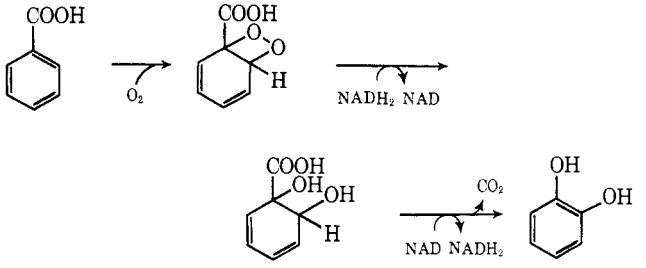


IVa Va VIa VIIa

Discussion

The hydroxylation of benzoic acid by bacteria to form catechol was known to occur at C-1 and -2 (Sleeper, 1951). Compound I had been proposed as one of a series of intermediates in the formation of catechol *via* an epoxide from benzoic acid (Taniuchi *et al.*, 1964). Here we have shown that I is formed from benzoic acid by a mutant of *A. eutrophus*, and that it can be isolated as a stable product. Compound I was not known previously either as a synthetic product or as a biological metabolite, and is the first cyclohexadienediol described with a substituent group on a hydroxyl carbon.

The $[^{18}O]O_2$ results presented here suggest strongly that I is formed from a peroxide. Evidence that I is a *cis*-diol is also consistent with this interpretation. We conclude that the first enzyme in what was previously termed the benzoate oxidase system is a dioxygenase (Hayaishi, 1966), and suggest the following general scheme for the oxidative conversion of benzoic acid to catechol by bacteria. Proof that I is an inter-



mediate in the conversion of benzoic acid into catechol in *A. eutrophus* and other bacteria, and additional evidence for this scheme will be presented separately (manuscript in preparation).

The specificity of the first two enzymes of this suggested sequence is not great, since compounds analogous to I are formed at appreciable rates by induced cells of strain B9 from methyl-, chloro-, and fluorobenzoates monosubstituted in the 3 or 4 positions (Table III). This finding is in agreement with observations on the homologous enzyme system in other bacteria (Ichihara *et al.*, 1962)

Peroxides have been implicated as possible intermediates in the bacterial oxidation of benzene *via* cyclohexadienediol (Gibson *et al.*, 1968), in the oxidation of toluene *via* methylcyclohexadienediol (Gibson *et al.*, 1970b), and in the oxidations of anthranilic acid and 2-fluorobenzoic acid to catechol, where no stable intermediates were demonstrated (Kobayashi *et al.*, 1964; Milne *et al.*, 1968). In various mammalian systems, formation of dihydroxycyclohexadiene compounds apparently occurs *via* an oxide intermediate (Table I). One can speculate that mammalian systems operate exclusively by the oxide mechanism while bacteria may use both mechanisms, but this is a tentative conclusion which awaits examination of additional cases.

Acknowledgments

We thank R. Y. Stanier for advice and for affording the hospitality of his laboratory and B. Johnson for the gift of strains. We thank G. L. Kenyon and D. T. Browne for determining nuclear magnetic resonance spectra and for valuable advice and suggestions on interpretations of these and other data.

Added in Proof

In a recent note Jerina *et al.* (1971) identified *cis*-dehydrodihydroxynaphthalene as the bacterial oxidation product of naphthalene, and cast doubt on previous reports which had implicated *trans*-dihydrodiol products of bacterial aromatic oxidations.

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Biosynthesis of Wax Esters in Fish. Metabolism of Dietary Alcohols*

D. M. Sand, Jean L. Hehl, and H. Schlenk†

ABSTRACT: Dietary fatty alcohols are incorporated by the gourami (*Trichogaster cosby*) as alcohols and acids into the roe wax esters. The course of this incorporation was studied by feeding 1-^{3}H- and 1-^{3}H,U-^{14}C-labeled palmityl and oleyl alcohols and by analyzing the lipids 24 hr after ingestion. Levels of incorporation into roe wax esters were 15–60% of ^{14}C but only 0.5–4% of ^{3}H that had been offered. The ratios ^{3}H/^{14}C in the alcohols of those wax esters showed that the dietary alcohols had undergone extensive oxidation to the corresponding fatty acids and were then reduced again for esterification. Some direct esterification of dietary alcohols is indicated by a small amount of tritium in position 1 of the alcohols recovered but it did not exceed 16% of the ^{14}C-labeled chains that were found as alcohols in the wax esters. Direct esterification may occur to a relatively greater extent in body wax esters which are present only in trace amounts. There an enrichment of ^{3}H compared to ^{14}C was observed which remains unexplained. Some of the tritium derived from labeled dietary alcohols is used for reduction of fatty acids since it is found in position 1 of alcohols other than the ones fed. Amounts of tritium similar to those in roe wax esters have entered the glycerol moiety of body triglycerides and phosphatidylcholines, likely by reduction of a triose phosphate. Very little tritium has been used for synthesis of lipid chains.

The roe lipids of the opaline gourami (*Trichogaster cosby*), a tropical freshwater fish, consist mainly of wax esters, whereas the body lipids contain mainly triglycerides (Sand and Schlenk, 1969). We have previously shown that dietary fatty alcohols and acids are efficiently incorporated and interconverted in this fish (Sand *et al.*, 1969). However, those experiments had been carried out with ^{14}C-labeled compounds and they did not indicate the pathways of oxidation and reduction that may be involved when dietary alcohols are incorporated into wax esters. Part of the alcohols was oxidized to acids which were found in wax esters and other lipids but part of them was found as alcohols in the roe wax esters. This latter portion may have been esterified directly or may first have been oxidized to acid and then reduced again to alcohol for esterification. Possibly both of these pathways were concurrently active in the formation of wax esters in opaline gourami.

In order to evaluate these possibilities, palmityl and oleyl alcohols labeled with ^{3}H in position 1 were fed to gouramis either as such or together with U-^{14}C-labeled alcohols. After 24 hr the fish were sacrificed and their lipids analyzed. In roe, the level of ^{3}H in the alcohol bound as wax esters showed that nearly all dietary alcohol had been oxidized and then part of it reduced again to alcohol for esterification. However, some direct esterification of dietary alcohols had taken place since part of the tritium in wax esters was in position 1 of the alcohol that had been fed.

The body lipids of gouramis contain a very small amount of wax esters, and direct esterification may play a greater role in their formation as indicated by the higher specific activity of tritium. Significant amounts of tritium were found in the glycerol moiety of triglycerides and phosphatidylcholines. Very little tritium was in alkyl chains such as 16:0 and 18:1 which the fish can synthesize *de novo*.

Materials and Procedures

Compounds. [1-$^{3}H_2$]Palmityl and oleyl alcohols were prepared from the acid methyl esters (purchased from The Hormel Institute Lipids Preparation Laboratory) by reduction with [^{3}H]$LiAlH_4$ (New England Nuclear Corp., Boston, Mass.). The alcohols were purified by column chromatography on SiO_2 and recrystallized twice from ethanol without

* From The Hormel Institute, University of Minnesota, Austin, Minnesota 55912. *Received March 9, 1971*. The investigation was supported in part by Public Health Service Grant AM 13424 from the National Institutes of Health; Public Health Service Research Grant HE 08214 from the Program Projects Branch, Extramural Programs, National Heart Institute; and by The Hormel Foundation.

† To whom to address correspondence.