Microbial Oxidation of *p*-Diethylbenzene*

Masato Tanabe,† Robert L. Dehn, and Mau H. Kuo

ABSTRACT: A *Pseudomonas* strain isolated from a fuel storage tank oxidizes *p*-diethylbenzene exclusively to the monocarboxylic acid, *p*-ethylphenylacetic acid. Deuterium labeling was used to elucidate the oxidation mechanism. Deuterium-labeled *p*-diethylbenzenes as substrates show an isotope effect of $k_H/k_D = 1.7$.

Retention of the deuterium atoms on the α carbon indicates the major pathway of oxidation involves direct terminal oxidation and not dehydrogenation followed by hydration.

The microbial oxidation of hydrocarbons to their terminal carboxylic acids has received much attention in recent years (McKenna and Kallio, 1965; Johnson, 1964). Generally, the overall pathway of oxidation of the hydrocarbon initially to the ω alcohol, followed by further oxidative conversion into the carboxylic acid (reaction 1), is well accepted (Foster, 1962), although ω-1 oxidation has been reported (Forney and Markovetz, 1970).

$$RCH_2CH_3 \xrightarrow{[O]} RCH_2CH_2OH \xrightarrow{[O]} RCH_2COOH \quad (1)$$

Many studies pertinent to the general mechanism of microbial hydrocarbon oxidation have appeared. Results obtained from experiments with a cell-free enzyme system have demonstrated a specific dependency on DPNH as a cofactor in the initial conversion of *n*-octane into 1-octanol. An Fe^{2+}-requiring protein rubredoxin and a hydroxylase have also been isolated from this cell-free system (Peterson and Coon, 1968).

The similarity of cofactor requirements and composition of the microbial oxidation system and the liver microsomal hydroxylating system has been pointed out (Coon and Lu, 1969).

Controversy still exists over the details of the initial hydrocarbon oxidation step and several mechanisms have been proposed (reaction 2). Reaction 2a involves initial dehydrogenation to the corresponding 1-alkene, followed by hydration across the double bond to the 1-alkanol (Senez and Azoulay, 1961). This hypothesis is further supported by the unambiguous isolation and identification of the 1-hexadecene from cultivation of alkene-free hexadecane with *Micrococcus cerifans*, *Mycobacterium phlei*, *Norcardra* sp., *Pseudomonas* sp., or *Rhodotorula* sp. in inorganic salt solutions (Wagner *et al.*, 1967). Internal monoalkenes have also been isolated from the culture broths of a *Nocardia salmonicolor* grown on hexadecane (Abbott and Casida, 1968).

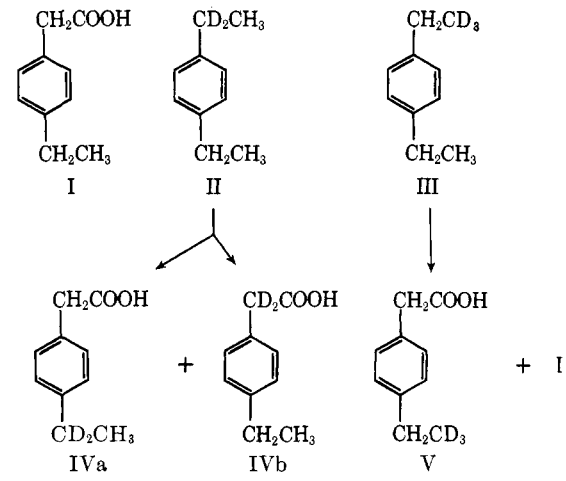
The other suggested pathway involves direct insertion of some oxygen species on the terminal methyl carbon (reaction 2b). This pathway has been demonstrated to be the major mode of oxidation by *Torulopsis gropeniesseri* by the use of tritium-labeled substrates (Jones and Howe, 1968). Incorporation of $^{18}O_2$ has been demonstrated in several studies (Stewart *et al.*, 1959; Foster, 1962).

$$RCH_2CH_3 \longrightarrow RCH{=}CH_2 \xrightarrow{H_2O} RCH_2CH_2OH \quad (2a)$$

$$RCH_2CH_3 \xrightarrow{[O]} RCH_2CH_2OH \quad (2b)$$

A strain of *Pseudomonas* that we have isolated from a local jet fuel storage tank oxidizes *p*-diethylbenzene to *p*-ethylphenylacetic acid (I) (Scheme I) in 20–30% yield. Analysis of the culture filtrate confirmed that only the monocarboxylic acid I was present, since neither the corresponding alcohol, dicarboxylic acid, nor ketonic material could be found. This monoterminal hydrocarbon oxidative system was therefore useful to study further the mechanistic aspects of microbial hydrocarbon oxidations. In the present study the use of deuterium-labeled substrates offered a means of identifying some of the features involved in the conversion of *p*-diethylbenzene into *p*-ethylphenylacetic acid.

SCHEME I



Deuterium-labeled substrates have been previously employed to obtain information on the mechanisms of microbial oxidations. The 11α hydroxylation of a steroid with *Rhizopus nigricans* occurs by stereospecific hydroxylation with displacement of an 11α-deuterium and retention of configuration (Corey *et al.*, 1958). No kinetic deuterium isotope effect was observed in this steroid conversion. Microbial oxidative study of hexadeuterioethane to deuterioacetic acid by *P. methanica*

* From the Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025. *Received October 2, 1970.* This work was supported in part by the Public Health Service under Research Grant AM-06629 from the National Institute of Arthritis and Metabolic Diseases.

† To whom to address correspondence.

TABLE I: Nuclear Magnetic Resonance[a] Analysis of *p*-Ethylphenylacetic Acids.

	Number of Protons Found at			
	τ 2.84 (Singlet)	τ 6.43 (Singlet)	τ 7.38 (Quartet)	τ 8.80 (Triplet)
Substrate	Aromatic H	Methylene H Adjacent to Carbonyl	Methylene H	Methyl H
p-Diethylbenzene	4.0	2.0	2.0	3.0
p-Diethylbenzene-α-d_2	4.0	1.0	0.96	3.0
p-Diethylbenzene-β-d_3	4.0	2.0	2.0	1.2

[a] Nuclear magnetic resonance spectra were taken in $CDCl_3$.

was useful in excluding one possible mechanistic pathway (Leadbetter and Foster, 1960).

Results

In order to establish whether mechanistic conclusions can be drawn about an olefin intermediate in the conversion of *p*-diethylbenzene into *p*-ethylphenylacetic acid, studies using the monomethylene dideuterated substrate II in the microbial oxidation were conducted. Other studies employing the monomethyl trideuterium-labeled substrate III permit the measurement of any biological kinetic isotope effects involved in the overall six-electron oxidation to the acids V and I. The intramolecular competitive oxidation in III (*i.e.*, CD_3 *vs.* CH_3) offers a convenient and simple method for determining the magnitude of the isotope effect by analysis of the deuterium content by nuclear magnetic resonance spectrometry of the acids V and I formed as metabolites. The nmr analysis of *p*-ethylphenylacetic and its deuterated analogs is of particular ease and convenient to interpret since the chemical shifts of all protons are well separated and readily identified (Table I).

The preparation of the deuterium-labeled substrates followed applications and minor variations of literature procedures for the introduction of deuterium substituents (Budzikiewicz *et al.*, 1964). Friedel–Crafts alkylation of ethylbenzene with acetyl-d_3 chloride yielded the intermediate *p*-ethylacetophenone-d_3. Since the deuteriums α to the carbonyl group are very labile and subject to rapid exchange in protic solvents, a synthetic method for the conversion of a ketone group into a methylene function in an aprotic medium was necessary. The use of lithium aluminum hydride in the presence of anhydrous aluminum chloride (Brown and White, 1957) offered the requisite conditions for the reduction and *p*-diethylbenzene-β-d_3 (III) of 90% isotopic purity was produced in the reduction. Application of the identical method of reduction to *p*-ethylacetophenone with lithium aluminum deuteride ($LiAlD_4$) in ether yielded *p*-diethylbenzene-α-d_2 (II) of 95% isotopic purity. The deuterium analysis for both isomers determined by nuclear magnetic resonance and combustion methods compared favorably.

The results of nuclear magnetic resonance analysis of the deuterated oxidation products from II and III as compared to nondeuterated I are summarized in Table I. The methylene deuterated substrate II yielded a mixture of acids which retained about 95% of the deuterium substitution.

The overall deuterium isotope effect was calculated from nuclear magnetic resonance analysis of *p*-ethylphenylacetic acid. It was assumed the only acids present in the *p*-ethylphenylacetic acid isolated were I and *p*-ethylphenylacetic-β-d_3 acid (V). It was further assumed from the amount of unoxidized *p*-diethylbenzene-β-d_3 recovered that reaction conditions were essentially steady state conditions and therefore the ratio of products is a measure of the rates of oxidation of the substrates. Nuclear magnetic resonance revealed 1.2 of the possible 3.0 protons of the methyl were reclaimed: the difference (1.8) is attributed to deuterium substitutions on the methyl of *p*-ethylphenylacetic acid (V). The isotope effect is therefore the ratio of 1.8 to 1.2, which after normalizing for 100% deuterium substitution in the starting *p*-diethylbenzene results in a deuterium isotope effect of $k_H/k_D = 1.7$.

Discussion

The isotope effect seen here is in good agreement with the magnitude of the effect recently observed in liver microsomal hydroxylations at a saturated carbon atom (Tanabe *et al.*, 1969; Mitoma *et al.*, 1967). Our observed effect is the composite of several oxidation steps, corresponding to several C–H bond breakings, and it is difficult to specify the magnitude of the effect at each oxidation level. However, the observation of this isotope effect clearly indicates at least one step, involving a C–H bond breaking, is rate determining.

In the conversion of alcohols into aldehydes catalyzed by alcohol dehydrogenases, deuterium isotope effects on the order of $k_H/k_D = 1.5$–1.7 have been observed (Mahler *et al.*, 1962). No information is available on the magnitude of the isotope effect involved in the biological oxidation of an aldehyde to an acid. Deuterium isotope effects of $k_H/k_D = 3$–5 have been observed in chemical transformations of aldehydes to acids with permanganate ion (Wiberg, 1955).

Near-quantitative retention of deuterium on the α carbon clearly indicates oxidation does not significantly involve an olefinic intermediate as postulated in reaction 2a. The significantly small loss of deuterium from the α-carbon atom cannot be assessed, since the results of other workers have indicated involvement of a dehydrogenated intermediate. The similarity of the general microbial oxidation system to the liver microsomal system has already been noted. Recent experiments have indicated that in the liver microsomal system, several enzymatic systems may be present and reacting simultaneously on a single substrate (Mitoma *et al.*, 1970). Although oxidation of *p*-diethylbenzene by our *Pseu-*

domonas strain takes place mainly by direct insertion of some oxygen species (reaction 2b), in other specific instances a different enzyme system can predominate and a dehydrogenated intermediate (reaction 2a) can become the major pathway.

Specification of the exact nature of the enzyme(s) involved in each specific instance awaits studies with cell-free systems and purification of the enzyme(s) involved.

Experimental Methods

Infrared spectra were recorded on the Perkin-Elmer Infracord Model 137. Nuclear magnetic resonance spectra were taken on a Varian A-60 spectrometer using 1% tetramethylsilane as an internal standard. Gas–liquid chromatographic analyses were performed on a Wilkens Aerograph A-90-P using a 5 ft × 0.25 in. 20% SF-96 column at 130°. Melting points were determined on a Fisher-Johns point block and are uncorrected. Organic solutions were dried over anhydrous Na_2SO_4. Deuterium analyses were performed by Dr. Josef Nemeth, Urbana, Ill.

Synthesis of Deuterated Compounds

p-Ethylacetophenone-α-d_3. A solution of 15 ml of dry carbon disulfide, 7.0 ml of ethylbenzene (0.056 mole), and 5.0 g of acetyl-d_3 chloride (0.065 mole) was cooled at 0° and 7.5 g of dry aluminum chloride (0.056 mole) was slowly added at such a rate to keep the temperature of the reaction mixture below +5°. The addition took 3 hr. The reaction mixture was stirred for 16 hr at room temperature and then slowly poured into 200 ml of ice water containing 5 ml of concentrated HCl. The aqueous solution was extracted with 50 ml of ether three times. The ether was combined, washed, dried, and flash distilled at atmospheric pressure. The residue was distilled at 12.5 mm to yield 5.8 g of *p*-ethylacetophenone-α-d_3, bp 145° (12.5 mm). Gas–liquid chromatographic analysis showed only one component which has a retention time identical with *p*-ethylacetophenone.

p-Diethylbenzene-β-d_3 (III). Eighty milliliters of dry ether and 0.84 g (0.02 mole) of lithium aluminum hydride in a flask which had been flamed under nitrogen was cooled to 0° and 8.0 g (0.06 mole) of aluminum chloride was added and stirred for 1.5 hr. Three grams of *p*-ethylacetophenone-α-d_3 (0.02 mole) in 20 ml of dry ether was slowly added. The reaction mixture was stirred for 16 hr at room temperature. Methyl formate (10 ml) was added to decompose any excess lithium aluminum hydride, followed by 20 ml of 20% sulfuric acid to decompose any excess aluminum chloride. The ether layer was separated and the aqueous layer extracted with 50 ml of ether three times. The ether was combined, washed, dried, and flash distilled. The residue was distilled at 20 mm to yield 2.0 g of *p*-diethylbenzene-β-d_3, bp 71.5° (20 mm).

Gas–liquid chromatographic analysis showed only one component with retention time identical with *p*-diethylbenzene; nuclear magnetic resonance (CCl_4) τ 3.18 (singlet), 4.0 protons; τ 7.50 (quartet), 3.9 protons; τ 8.80, 3.3 protons. The nuclear magnetic resonance analysis indicated the compound was 90 ± 2% deuterated. *Anal.* Calcd for $C_{10}H_{11}D_3$, 21.43. Found, 18.80. This is equivalent to a deuterium content of 87.7% of theory.

p-Diethylbenzene-α-d_2 (II). To 160 ml of dry ether in a flame-dried flask was added 1.65 g (0.038 mole) of lithium aluminum deuteride, followed by 16 g of aluminum chloride (0.12 mole), and the mixture was cooled to 0°. *p*-Ethylacetophenone, 6.0 g (0.04 mole) in 40 ml of dry ether, was slowly added. The reaction mixture was stirred at room temperature for 16 hr, and then poured into 250 ml of ice water. It was then acidified with 20% sulfuric acid until the colorless salts dissolved. The solution was extracted with 100 ml of ether twice and the ether extracts were combined, washed with brine, dried, and flash distilled. The residue was distilled at 20 mm to yield 3.7 g of *p*-diethylbenzene-α-d_2, bp 76.5–78.5° (20 mm); nuclear magnetic resonance (CCl_4) τ 3.18 (singlet), 4.0 protons; τ 8.80 (triplet), 6.0 protons; τ 7.50 (quartet), 2.0 protons. *Anal.* Calcd for $C_{10}H_{12}D_2$, 14.28. Found, 14.10. This is equivalent to a deuterium content of 98.8% that of theory.

Microbial Oxidation of p-Diethylbenzenes

Pseudomonas strain B-129 originally isolated from a nearby fuel storage tank was used for the experiment. One milliliter of active suspension of *Pseudomonas* B-129 was inoculated into the following medium: Bushnell–Hass medium (Bushnell and Hass, 1941), 100 ml, yeast extract (Difco), 0.01 g, and *p*-diethylbenzene or deuterated *p*-diethylbenzene, 0.5 g. Omission of yeast extract or use of washed bacterial cells frequently resulted in reduced yield of product. Growth was carried out in a series of 500-ml erlenmeyer flasks. Incubation took place on a gyratory shaker (New Brunswick Scientific Co.) at 200 oscillations/min at 37° for 7 days.

After incubation, the bacterial cells were removed by centrifugation in a refrigerated centrifuge (Servall Co.) at a speed of 12,000*g* for 15 min. The supernatant liquid was adjusted to pH 10 with 1 N NaOH and extracted with 50 ml of ether three times. The ether extracts were combined, washed with brine, and dried. Ether was removed by distillation at atmosphere pressure. The residue contained only *p*-diethylbenzene and residual ether as revealed by gas chromatographic and infrared analysis.

The aqueous phase was then adjusted to pH 3 with 2 N HCl and extracted with 50 ml of ether three times. The ether extract was washed and dried as described previously. Upon removal of ether by distillation, light yellow solid material (80 mg) remained. This material was sublimed at 90° and 0.2 mm to yield white crystalline *p*-ethylphenylacetic acid, mp 87–90° (Beilstein: 88–90°); λ_{max}^{Nujol} 2.9, 4.1 (broad), 5.85, and 12.08 μ.

Experiments with Deuterated p-Diethylbenzenes

Three flasks each containing 0.5 g of *p*-diethylbenzene-α-d_2 (or *p*-diethylbenzene-β-d_3) were incubated with the microorganism (B-129) at 37° for 7 days. Deuterated *p*-ethylphenylacetic acids were isolated and purified in the same manner previously described: *p*-diethylbenzene-α-d_2 (II) as substrate: *p*-ethylphenylacetic-α-d_2 acid (IV) (167 mg, mp 87–89°) (*Anal.* Calcd for $C_{10}H_{10}D_2O_2$, 16.66. Found, 15.50); *p*-diethylbenzene-α-d_2 (446 mg) (Calcd for $C_{10}H_{12}D_2$, 14.28. Found, 13.55); *p*-diethylbenzene-β-d_3 (III) as substrate: *p*-ethylphenylacetic-d_3 acid (V + I) (144 mg, 88–90°) (Calcd for $C_{10}H_9D_3O_2$, 25.00. Found, 15.90).

References

Abbott, J. A., and Casida, L. E., Jr. (1968), *J. Bacteriol. 96*, 925.

Brown, B. R., and White, A. M. S. (1957), *J. Chem. Soc.*, 3755.

Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1964), Structure Elucidation of Natural Products by Mass Spectrometry, Vol. I, San Francisco, Calif., Holden-Day, p 17.

Bushnell, L. D., and Haas, H. F. (1941), *J. Bacteriol. 41*, 653.
Coon, M. J., and Lu, A. J. (1969), *in* Microsomes and Drug Oxidations, Gillette, J. R., *et al.*, Ed., New York, N. Y., Academic.
Corey, E. J., Gregoriou, G. A., and Peterson, D. H. (1958), *J. Amer. Chem. Soc. 80*, 2338.
Forney, F. W., and Markovetz, A. J. (1970), *J. Bacteriol. 102*, 281.
Foster, J. W. (1962), *in* Oxygenases, Hayaishi, O., Ed., New York, N. Y., Academic, p 588.
Jones, D. F., and Howe, R. (1968), *J. Chem. Soc. C*, 2801.
Johnson, M. J. (1964), *Chem. Ind. (London)*, 1532.
Leadbetter, E. R., and Foster, J. W. (1960), *Arch. Mikrobiol. 35*, 92.
Mahler, H. R., Baker, R. H., Jr., and Shiner, V. J., Jr. (1962), *Biochemistry 1*, 47.
McKenna, J., and Kallio, R. E. (1965), *Annu. Rev. Microbiol. 19*, 183.
Mitoma, C., Dehn, R. L., and Tanabe, M. (1970), *Pharmacologist 12*, 255.
Mitoma C., Yasuda, D., Tagg J., and Tanabe, M. (1967), *Biochim. Biophys. Acta 136*, 566.
Peterson, J. A., and Coon, M. J. (1968), *J. Biol. Chem. 243*, 329.
Senez, J. C., and Azoulay, E. (1961), *Biochim. Biophys. Acta 47*, 307.
Stewart, J. E., Kallio, R. E., Stevenson, D. P., Jones, A. C., and Schissler, D. O. (1959), *J. Bacteriol. 78*, 441.
Tanabe, M., Yasuda, D., LeValley, S., and Mitoma, C. (1969), *Life Sci. 8*, 1123.
Wagner, F., Zahn, W., and Buhring, V. (1967), *Agnew. Chem., Int. Ed. Engl. 6*, 359.
Wiberg, K. B. (1955), *Chem. Rev. 55*, 713.

Phospholipid Metabolism during the Development of the Liver. The Incorporation of [1,2-^{14}C]Ethanolamine, [2-^{3}H]*myo*-Inositol and L-[U-^{14}C]Serine into Phospholipids by Liver Slices*

Paul A. Weinhold and Robert D. Sanders

ABSTRACT: The incorporation of [1,2-^{14}C]ethanolamine, L-[U-^{14}C]serine, and [2-^{3}H]*myo*-inositol into phospholipids has been determined with liver slices from fetal, young, and adult rats.

The rate of incorporation was determined with the concentration of precursor that gave a maximal amount of incorporation and gave a linear rate of incorporation for at least 1 hr. [1,2-^{14}C]Ethanolamine and [2-^{3}H]*myo*-inositol were incorporated intact into ethanolamine phosphoglyceride and inositol phosphoglyceride, respectively. The radioactivity from L-[U-^{14}C]serine was incorporated into all parts of the phospholipid molecule (glycerol, fatty acids, and bases). The amount of incorporation into each part was determined. The ability of liver slices to incorporate [1,2-^{14}C]ethanolamine into ethanolamine phosphoglycerides was low in −5-day fetal (20% of adult), increased to 60% of the adult before birth, and continued to increase to 80% of the adult by 1 day after birth. The rate of incorporation increased again between 5 and 6 days. The incorporation of [2-^{3}H]*myo*-inositol was also low in −5-day fetal and increased to 40% of the adult before birth. The developmental pattern was very similar to that observed for the incorporation of [1,2-^{14}C]ethanolamine. The incorporation of L-[U-^{14}C]serine was about 30% of the adult in −4-day fetal and increased continually until adult levels were reached at 4 days after birth. The incorporation of radioactivity from L-[U-^{14}C]serine into fatty acids, glycerol, and cholesterol was higher in the fetal liver than in adult liver. The kinetics of incorporation of [1,2-^{14}C]ethanolamine into phosphorylethanolamine and ethanolamine phosphoglycerides indicated that the developmental changes in the pathway occurred beyond the phosphorylation of ethanolamine.

The ultrastructure of hepatocytes and their organelles undergoes a regular sequence of changes during embryonic (Dadoune, 1963; Wood, 1967) and neonatal development (Stempak, 1967). The amount of smooth endoplasmic reticulum increases significantly during the period from 1 to 2 days before birth to 2–3 days after birth (Dallner *et al.*, 1966). Phospholipids, a major component of these membrane systems, increase in concentration in the liver immediately following birth (Weinhold and Villee, 1965). These observations suggest that the ability of hepatocytes to synthesize and modify the individual phospholipids changes during development. The investigation of these changes should contribute to an understanding of the mechanisms that control phospholipid synthesis as well as the formation and alteration of membranes.

We have previously reported the results from studies on the biosynthesis of choline phosphoglycerides by liver slices from fetal, newborn, and adult rats (Weinhold, 1969). In

* From the Department of Biological Chemistry and Department of Medicine (Nuclear), University of Michigan, and Veterans Administration Hospital, Ann Arbor, Michigan. *Received August 27, 1970.* This work was supported by a grant from the National Institute of Child Health and Human Development, U. S. Public Health Service.