Oxidation of Alkyl-substituted Cyclic Hydrocarbons by a Nocardia during Growth on *n*-Alkanes

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ABSTRACT

DAVIS, J. B. (Socony Mobil Oil Company, Inc., Dallas, Texas), AND R. L. RAYMOND. Oxidation of alkyl-substituted cyclic hydrocarbons by a Nocardia during growth on *n*-alkanes. Appl. Microbiol. **9**:383–388. 1961.—Nocardia 107–332, a soil isolate, oxidizes short-chain alkyl-substituted cyclic hydrocarbons to cyclic acids while growing on *n*-alkanes. Cyclic acids are produced also from relatively long-chain alkyl-substituted cyclics such as *n*-nonylbenzene or *n*-dodecylbenzene which alone support growth in a mineral-salts medium. ω-Oxidation of the alkyl substituents is followed by β-oxidation. It is of particular interest that cyclic acids such as cyclohexaneacetic and phenylacetic with C_2 residual carboxylic acid substituents are resistant to further oxidation by the nocardia but cyclic acids with C_1 or C_3 substituents are readily oxidized and utilized for growth.

The specificity of microbial oxidations is demonstrated by the conversion of *p*-isopropyltoluene (*p*-cymene) to *p*-isopropylbenzoic acid in *n*-alkane, growth-supported nocardia cultures.

Oxidation of cyclic hydrocarbons by microbes has received considerable attention (Tausson, 1929; Murphy, 1953; Walker and Wiltshire, 1953; Treccani, Walker, and Wiltshire, 1954; Strawinski and Stone, 1955; Webley, Duff, and Farmer, 1956; Kitagawa, 1956; Rogoff and Wender, 1957; and others). Tausson and Veselov (1934), using crude cultures, suggested the possibility of bacterial reduction of sulfates through the oxidation of phenanthrene and naphthalene. But in our opinion these and subsequent reports have not clearly implicated sulfate-reducing bacteria or other anaerobic microbes in the oxidation of hydrocarbons.

Strawinski and Stone (1955), using a pseudomonas closely resembling *Pseudomonas aeruginosa*, obtained up to about 29% conversion of 1% naphthalene to salicylic acid in vigorously aerated cultures. Webley et al. (1956), with resting cells of a nutrient-grown nocardia species (strain P_2 obtained from V. Treccani, University of Milan), reported high yields of phenylacetic acid from phenyldecane, phenyldodecane, and phenyloctadecane. Shorter chain alkylbenzenes, ethyl, *n*-propyl, and *n*-butyl were not oxidized. 1-(α-Naphthyl) hendecane yielded β-(α-naphthyl) propionic acid, and 3-phenyleicosane yielded phenylethylacetic acid. These data strongly suggested β-oxidation preceded by ω-oxidation of the alkyl substituents of benzene. Kitagawa (1956), using acetone-dried cells of *P. aeruginosa* adapted to toluene, obtained oxidation of toluene to catechol and identified benzyl alcohol, benzaldehyde, and benzoic acid as oxidative intermediates. These examples represent a variety of approaches to the study of the microbial oxidation of cyclic hydrocarbons.

While studying the utilization of white mineral oil by a nocardia species, we noted the accumulation of organic acids in the culture medium; infrared spectral examination indicated that these were cyclic acids. The observation was of particular interest since this strain of nocardia had failed to utilize a variety of cyclic hydrocarbons. It suggested that oxidation of cyclic hydrocarbons by the nocardia occurred during growth on alkanes or alkyl substituents in the hydrocarbon mixture.

This paper describes experiments which confirm the premise that certain cyclic hydrocarbons with short alkyl substituents which are not utilized for growth by this nocardial strain, and which are relatively if not totally resistant to oxidation by resting cells, can be converted to oxidized products in substantial yields in a two-component hydrocarbon system. Normal alkanes provide the growth substrate, whereas cyclic hydrocarbons with short alkyl substituents provide the specific product substrate. If an alkyl substituent is of sufficient length to support growth, the addition of an alkane hydrocarbon to the culture medium is unnecessary for product formation; cyclic oxidized products accumulate in the culture in either the presence or absence of added alkane.

MATERIALS AND METHODS

Microbe. The principal microbe used in these studies was a nocardia employed previously by Raymond and Davis (1960). We maintain it in our culture collection of soil isolates under the tentative designation *Nocardia* 107–332 since it does not conform precisely to any description of *Nocardia* species, Breed, Murray, and Smith

(1957). It conforms closely with *Nocardia salmonicolor* except that it utilizes phenol. Growth on liquid alkanes such as *n*-decane, *n*-hexadecane, or *n*-octadecane is accelerated by vigorous agitation of the aqueous culture medium consisting of, for example, urea, 0.1%; KH_2PO_4 0.2%; Na_2HPO_4, 0.3%; $MgSO_4 \cdot 7H_2O$, 0.08%; and about 1% hydrocarbon. *n*-Alkane hydrocarbons are utilized to completion and extracellular products other than capsular slime do not accumulate in the culture medium in the absence of cyclic hydrocarbons.

Cultural conditions. The impeller agitated culture systems used in this work were described previously (Raymond and Davis, 1960) and ordinarily contained 2 liters of culture media. Small culture systems consisted of 500-ml round bottles fitted with screw-caps to prevent the escape of volatile hydrocarbons. These usually contained 100 ml of culture media and were agitated on a gyratory shaker at 30 C.

The cyclic hydrocarbons tested included methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *n*-nonyl-, and *n*-dodecylbenzene; methyl- and *n*-butylcyclohexane; *p*-isopropyltoluene (*p*-cymene); and isopropylbenzene (cumene). The specific conditions for the individual experiments are described under Results. In general, the following conditions were employed. For the impeller-agitated systems, growth of the nocardia proceeded with *n*-octadecane or *n*-hexadecane for 24 hr during which time the systems were sparged with air. The cyclic hydrocarbon tested was then added with additional *n*-alkane and growth was allowed to proceed for another 72 hr without sparging to prevent excessive loss of the volatile cyclic hydrocarbons. The 500-ml bottle cultures were sealed after the addition of 0.6% *n*-alkane (C_{10}, C_{16}, or C_{18}) and 0.2% of the respective cyclic hydrocarbon.

Cyclic acids of the type isolated as products were tested in both growth systems (500-ml bottles) and resting-cell respirometer experiments. These acids[1] included cyclohexanecarboxylic, cyclohexaneacetic, cyclohexanepropionic, cyclohexanebutyric, benzoic, *m*-hydroxybenzoic, phenylacetic, phenylacrylic, and phenylpropionic.

Analytical methods. Following growth in the hydrocarbon systems, the cells were removed by centrifugation at 16,500 rev/min. The cell-free culture liquor was ordinarily steam distilled under alkaline, then acid conditions. The acidified residual was extracted with ethyl or petroleum ether.

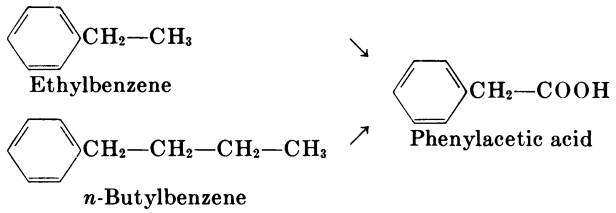
Products were analyzed by infrared spectrometry, melting point determination, neutralization equivalent, and gas chromatography. Instruments included the Perkin-Elmer model 221 infrared spectrometer[2] and the F & M model 300-B linear programmed temperature gas chromatograph.[3]

Methyl esters of the aromatic acids, prepared using boron trifluoride as catalyst (Metcalfe, 1960), were fractionated better on the silicone-gum rubber column employed than were the acids and thus were preferable in comparing fractograms of known and unknown compounds. Methyl esters were prepared by dissolving milligram amounts of the aromatic acids in about 0.5 ml of methanol, bubbling in boron trifluoride[4] for about 2 min, then adding a small amount of water and extracting the esters with *n*-heptane.

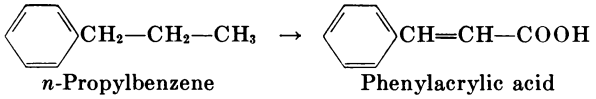
RESULTS

Cyclic hydrocarbons with short alkyl substituents. Growth of *Nocardia* 107–332 was negative on the following cyclic hydrocarbons: methylbenzene (toluene), ethylbenzene, *n*-propylbenzene, *n*-butylbenzene, methyl cyclohexane, *n*-butylcyclohexane, isopropylbenzene (cumene), and *p*-isopropyltoluene (*p*-cymene). Growth was tested in both liquid cultures and on streaked mineral-salts agar plates, incubated in the presence of the respective hydrocarbon vapor and air. Under similar conditions, growth of the nocardia on ethane, *n*-butane, *n*-hexadecane, or other *n*-alkanes is profuse. With the exception of *n*-butylbenzene and *n*-butylcyclohexane, oxidation of the above cyclic hydrocarbons also was negative with either ethane- or nutrient-grown nocardia cells in respirometer (resting-cell) experiments.

Ethylbenzene, *n*-propylbenzene, *n*-butylbenzene, *n*-butylcyclohexane, and *p*-isopropyltoluene were oxidized and yielded cyclic acid products when added along with *n*-hexadecane or *n*-octadecane to mineral-



salts media inoculated with nocardia. Ethylbenzene and *n*-butylbenzene each yielded phenylacetic acid. *n*-Propylbenzene yielded phenylacrylic (cinnamic acid).



These *n*-alkylbenzenes were tested in 500-ml culture bottles. *n*-Hexadecane, 0.6%, was the alkane growth substrate with 0.2% cyclic hydrocarbon in mineral-salts medium. Incubation time was 96 hr at 30 C. The cyclic acids, extracted with ethyl ether, were identified by infrared spectrometry and gas chromatography. For

[1] Purchased from Distillation Products Industries (Eastman) Rochester, N. Y., with the exception of cyclohexaneacetic acid, which was prepared from *n*-butylcyclohexane by the oxidation of Nocardia 107–332.

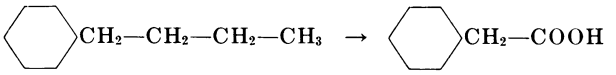
[2] Perkin-Elmer Corporation, Norwalk, Conn.

[3] F & M Scientific Corporation, New Castle, Del.

[4] Purchased from The Matheson Company, Inc., East Rutherford, N. J.

example, Fig. 1 shows the infrared spectra of phenylacetic acid derived from ethyl and *n*-butylbenzene, respectively.

n-Butylcyclohexane was converted to cyclohexaneacetic acid by *Nocardia* 107–332 and also by another strain of nocardia (*Nocardia* M.O.) originally isolated from soil using a crude oil enrichment. This cyclic hydrocarbon was tested in the impeller-agitated culture systems employing *n*-octadecane as the growth substrate. To 2 liters of mineral-salts medium, 1.6 g of *n*-octadecane were added and the system agitated and



n-Butylcyclohexane Cyclohexaneacetic acid

sparged with air for 24 hr at 30 C. By this time growth had begun and 1.6 g of *n*-butylcyclohexane were added to the *Nocardia* 107–332 system; 0.8 g to the *Nocardia* M.O. system. Forced aeration was stopped to conserve the added volatile cyclic hydrocarbon. The systems were agitated another 5 days after which the cells were harvested, the concentrated alkalized culture liquor acidified, and steam distilled. *Nocardia* 107–332 yielded 0.863 g of dried cells and 0.384 g (26% yield) of cyclohexaneacetic acid was recovered from the culture distillate; identified by melting point, neutralization equivalent, infrared spectroscopy, and gas chromatography. The *Nocardia* M.O. system yielded 0.892 g of dried cells and 0.293 g (41% yield) of cyclohexaneacetic acid. The yields cited are based upon complete utilization of the *n*-butylcyclohexane and are minimum. Partial loss of the volatile cyclic hydrocarbon undoubtedly occurred. Residual substrate consisting primarily of *n*-octadecane was 0.328 and 0.258 g in the *Nocardia* 107–332 and *Nocardia* M.O. systems, respectively.

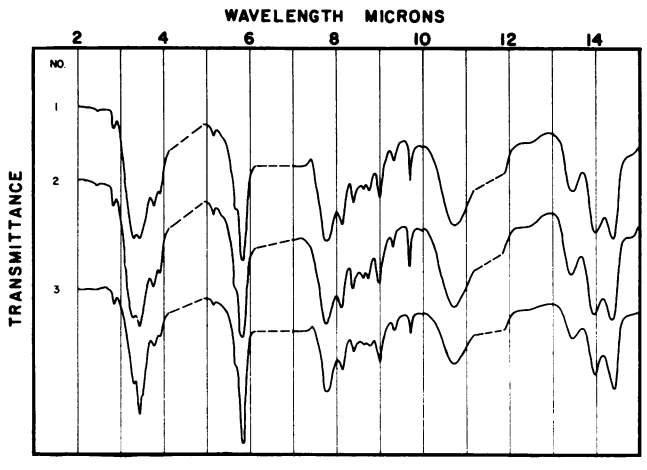
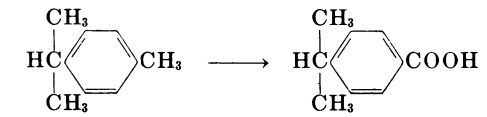


FIG. 1. *Infrared spectra of phenylacetic acid (1) and products (2) and (3) derived from the oxidation of ethylbenzene and n-butylbenzene, respectively, by Nocardia 107–332 while growing on n-hexadecane.*

p-Isopropyltoluene (*p*-cymene) was converted to *p*-isopropylbenzoic acid by *Nocardia* 107–332 growing

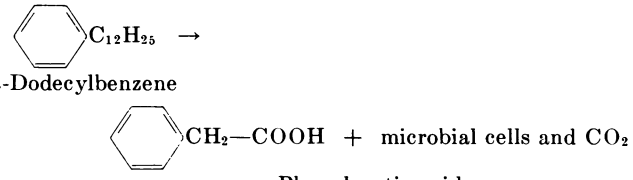


p-Isopropyltoluene (*p*-cymene) *p*-Isopropylbenzoic (cuminic) acid

on *n*-hexadecane. To an actively growing 2-liter culture in an impeller-agitated system, 8 g of *n*-hexadecane and 5 g of *p*-isopropyltoluene were added. Incubation proceeded for 5 days while vapor of the cyclic hydrocarbon was slowly added in an air stream to the system to replace lost volatile hydrocarbon and provide aeration. The cell-free alkalized culture liquor was reduced to 200 ml by evaporation. A copious crystalline precipitate (0.5 g) formed when the concentrated solution was made acid (pH 2 to 3) with HCl. The melting point of the precipitate was 117 C and the amide prepared with thionyl chloride melted at 144 C. Titration with standard base indicated that the compound had only one carboxyl group and it was tentatively identified as cuminic (*p*-isopropylbenzoic) acid. This was confirmed by gas chromatography comparing a fractogram of the methyl ester with that of the known compound.

Cyclic hydrocarbons with relatively long alkyl substituents. Both *n*-nonylbenzene and *n*-dodecylbenzene supported the growth of *Nocardia* 107–332. In an impeller-agitated system, 2.2 g of *n*-dodecylbenzene in 2 liters of inoculated mineral-salts medium yielded 1.29 g of dried nocardial cells and 0.98 g of phenylacetic acid, recovered by ethyl ether extraction and identified by infrared spectroscopy. The phenylacetic acid recovered was about 80% of the theoretical based on carbon.

The amount of carbon in the alkyl substituent, excluding that calculated as available for phenylacetic



n-Dodecylbenzene

Phenylacetic acid

acid formation, amounted to 1.08 g of the 2.2 g dodecylbenzene utilized. This was ample for the production of 1.29 g of nocardial cells consisting of 58% carbon.

Phenylacetic acid was identified also in *n*-dodecylbenzene cultures of two other nocardia, *Nocardia* M.O. and *Nocardia* (Bough), and a mycobacterium, *Mycobacterium* 107–227, soil isolates recovered from ethane enrichments.

A sparing effect was observed relative to phenylacetic acid formation by adding either *n*-decane or *n*-octadecane to small scale *n*-dodecylbenzene cultures of *Nocardia* (Bough) and *Nocardia* M.O. (Table 1).

n-Nonylbenzene was utilized by *Nocardia* 107–332 with the accumulation of small amounts of phenylacrylic and phenylpropionic acids. About 50 mg of *n*-nonylbenzene were added to a small culture system containing 50 ml of inoculated mineral-salts medium. The system was agitated on a gyratory shaker at 30 C. After 48 hr an additional 450 mg of the cyclic hydrocarbon were added to the growing culture. The cells, harvested after a total incubation of 6 days, had a dry weight of 320 mg. Thirty-one milligrams of ether-extractable material were recovered from the culture liquor and this material was identified by infrared spectroscopy as consisting of cyclic acids. Further investigation employing *n*-nonylbenzene cultures showed that the principal cyclic acid accumulating was phenylacrylic with about 8% of the total apparently in the form of phenylpropionic acid, as determined by gas chromatography of the methyl esters.

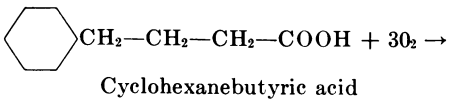
If the odd-numbered carbon chain substituents of *n*-nonylbenzene were utilized exclusively for growth, about 40% of the compound would be converted to microbial cells and carbon dioxide; approximately 60% would be oxidized to cyclic acids. However, the data show that only about 5% of the utilized *n*-nonylbenzene could be accounted for as cyclic acids; 95% including the cyclic portion of the molecule was apparently oxidized to cells and CO_2. This aspect will be discussed later in detail. The observation led to an examination of the oxidation of various cyclic acids by *Nocardia* 107–332.

Oxidation and utilization of cyclic acids. A series of cyclic acids including naphthenic and aromatic acids were subjected to oxidation by *Nocardia* 107-332 cultures under both resting-cell (pregrown) and growth conditions (Table 2). The two compounds that resisted oxidation and consequently utilization for growth were cyclohexaneacetic and phenylacetic acids. Not only do these results conform with an accumulation of these products in cultures as previously noted, but there is indicated a peculiar resistance of the C_2 carboxylic acid-substituted cyclics to oxidation.

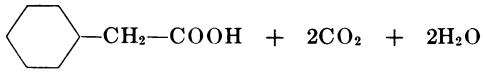
TABLE 1. *Sparing effect of n-alkanes on phenylacetic acid formation*

Microbe	Substrate added	Cells, dry wt	Phenylacetic acid formed
		mg	*mg*
Nocardia (Bough)	*n*-Dodecylbenzene, 44 mg	12	18
	n-Decane, 44 mg + *n*-dodecylbenzene, 44 mg	16	7
Nocardia M.O.	*n*-Dodecylbenzene, 44 mg	19	21
	n-Octadecane, 44 mg + *n* - dodecylbenzene, 44 mg	16	8

Oxidation of cyclohexanebutyric acid was studied in particular since no growth was observed with this compound, although it was oxidized. The results of experiments showed that for each micromole of cyclohexanebutyric acid oxidized, three micromoles of oxygen were consumed (Fig. 2) and two micromoles of carbon dioxide produced. These data suggest the following molar balance:



Cyclohexanebutyric acid



Cyclohexaneacetic acid

The oxidative product, cyclohexaneacetic acid, is re-

TABLE 2. *Utilization and/or oxidation of cyclic acids by Nocardia 107–332*

Cyclic acid	Growth* (duplicates)	Microliters O_2 uptake/hr†
Cyclohexanecarboxylic	+	237
Cyclohexaneacetic	−	10
Cyclohexanepropionic	+	460
Cyclohexanebutyric	−	198
Benzoic	+	463
m-Hydroxybenzoic	+	461
Phenylacetic	−	6
Phenylpropionic	+	393
Phenylacrylic	+	378

* + = Growth on sodium salt of cyclic acid (0.2%) in mineral salts medium. − = No growth after 1 week of incubation at 30 C.

† Used 5 μmoles of respective sodium salt, incubated with 5 mg (dry weight) of microbial cells in 0.05 M phosphate solution, pH 7; values corrected for endogenous respiration.

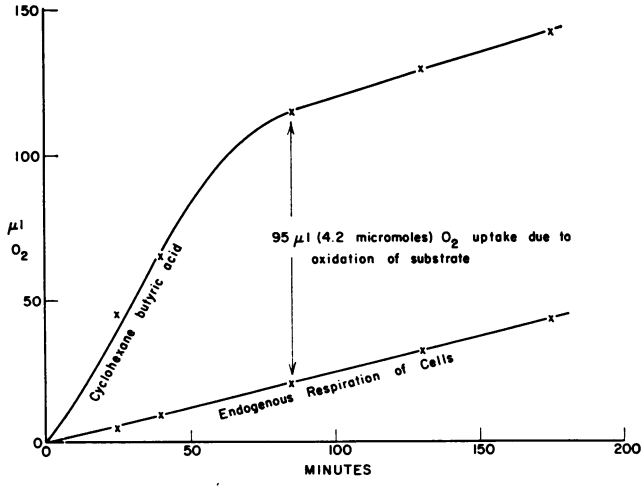


FIG. 2. *Oxidation of 1.4 μmoles of cyclohexanebutyric acid by Nocardia 107–332: molar ratio of oxygen uptake to substrate employed is 3:1.*

sistant to further oxidation and lack of growth of the nocardia on cyclohexanebutyric acid presumably is due to inability of the nocardia to grow at the expense of the two carbons oxidatively removed from the molecule.

Discussion

Webley et al. (1956), using resting-cell suspensions of nocardia and *n*-alkyl-substituted cyclic hydrocarbons, observed results strongly indicating β-oxidation of the alkyl substituents which conformed with classic biochemical oxidation of aliphatic chains. In addition, they assumed that ω-oxidation was the logical preliminary to β-oxidation of the alkyl substituent. The data reported here clearly substantiate this view. ω-Oxidation is evidenced by the formation of phenylacetic acid from ethylbenzene and phenylacrylic acid from *n*-propylbenzene. β-Oxidation is indicated by the residual cyclic acids derived from the *n*-alkyl-substituted cyclic hydrocarbons. Those with even-numbered carbon chains, such as *n*-butylcyclohexane, *n*-butylbenzene, and *n*-dodecylbenzene, yielded cyclic acids with a C_2 carboxylic acid substituent. Those cyclic hydrocarbons with an odd-number of carbons in the *n*-alkyl substituent, *n*-propylbenzene and *n*-nonylbenzene, yielded much smaller relative amounts of residual cyclic acids with a C_3 carboxylic acid substituent. The oxidations were carried out by *Nocardia* 107-332, actively growing on *n*-alkane hydrocarbons, except in the case of *n*-dodecylbenzene and *n*-nonylbenzene which alone supported growth. Because of the distinctive odor of phenylacetic acid, its production in cultures could be followed readily. It was detected very early in cultures containing either ethylbenzene plus *n*-hexadecane, *n*-butylbenzene plus *n*-hexadecane, or *n*-dodecylbenzene.

n-Nonylbenzene cultures yielded principally phenylacrylic acid, but the small amount of cyclic acids which accumulated in the cultures indicated that they were being utilized further for growth. This was confirmed by tests of cyclic acids (Table 2). Those with an odd-number of carbons in the fat acid substituent were readily utilized; and, in addition, the cyclic portion of acids, such as benzoic, *m*-hydroxybenzoic, phenylpropionic, phenylacrylic, and cyclohexanepropionic, was utilized by *Nocardia* 107-332. In contrast, acids, such as phenylacetic, cyclohexaneacetic, and cyclohexanebutyric, with an even-number of carbons in the fat acid substituent, are resistant to utilization for growth; although the latter is oxidized to cyclohexaneacetic acid.

The reason for the resistance of the cyclic portion of acids with an even-number of carbons in the substituent is not clear. But the refractory nature of these compounds to utilization by the nocardia apparently lies in the residual C_2 carboxylic acid substituent of either phenylacetic or cyclohexaneacetic acids. This may be due to a peculiar steric hindrance of this configuration to enzyme activation. In contrast, the ultimate β-oxidized intermediate in *n*-propylbenzene or *n*-nonylbenzene oxidations, benzoic acid (C_1 carboxylic acid acid substituent not actually detected in cultures), is readily oxidized and utilized for growth by the nocardia.

A soil isolated pseudomonas, closely resembling *P. aeruginosa*, produced phenylacetic acid in *n*-dodecylbenzene cultures and phenylacrylic (cinnamic) acid in *n*-nonylbenzene cultures as does *Nocardia* 107-332 (*see* Fig. 3). Tested on the cyclic acids listed in Table 2, growth by the pseudomonas was observed only in the case of benzoic. That nocardial strains or species vary in their ability to utilize C_2- and C_3-substituted aromatic acids was noted by Webley et al. (1956). *Nocardia opaca* strain T_{16} oxidized both phenylacetic and phenylpropionic acids, the latter yielding phenylacrylic and benzoic acids as oxidative intermediates; *Nocardia* sp. strain P_2 did not attack phenylacetate.

Certain cyclic hydrocarbons (which alone were resistant to oxidation by *Nocardia* 107–332), such as methylcyclohexane, methylbenzene, and isopropylbenzene, still were resistant to oxidation in growing *n*-alkane cultures of the nocardia. Admittedly, we have not exhausted the variety of cultural conditions that can be conceived to test the oxidation of these compounds by *Nocardia* 107–332.

An interesting example of the specific microbial oxidation of a cyclic hydrocarbon is the conversion of *p*-isopropyltoluene (*p*-cymene) to *p*-isopropylbenzoic acid. This conversion was accomplished only when the cyclic hydrocarbon was added to cultures growing on *n*-alkanes. The isopropyl substituent is more susceptible to chemical oxidation, but the microbial oxidative

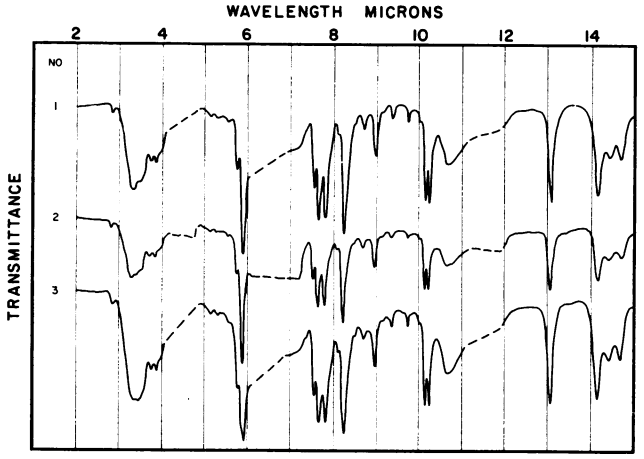


FIG. 3. *Infrared spectra of phenylacrylic (trans-cinnamic) acid (1) and products (2) and (3) of n-nonylbenzene oxidation by Nocardia 107-332 and Pseudomonas aeruginosa (soil isolate), respectively.*

system peculiarly chose to oxidize the methyl substituent.

Organic acids and other products in microbial cultures or fermentations ordinarily result when the microbe involved produces a particular metabolic intermediate or product that accumulates under the conditions of substrate concentration, aeration or pH. Thus in most cases the growth substrate also serves as the product substrate. However, exceptions to this involving a multiple substrate system are well known. As one of many examples, *Rhizopus nigricans* oxidizes progesterone to 11-α-hydroxyprogesterone while growing on other organic components in the medium (Murray and Peterson, 1952). Still another well-known process employs pregrown microbial cells wherein principal conversion of substrate to product is performed following growth of the cells.

For exemplary purposes the oxidation of ethylbenzene to phenylacetic acid and *p*-isopropyltoluene to *p*-isopropylbenzoic acid by *Nocardia* 107–332 are mentioned as representing the tenor of this paper. These cyclic hydrocarbons do not support the growth of the nocardia; nor does oxidation of these hydrocarbons occur readily with pregrown cells. However, the cyclic hydrocarbons are converted to oxidized products in cultures of the nocardia growing on *n*-alkanes. The refractory nature of the short-chain alkyl-substituted benzenes to oxidation in the absence of *n*-alkanes in a growth situation is not presently understood.

One obvious feature of interest is the effect of concentration of a short-chain alkyl-substituted cyclic hydrocarbon such as ethylbenzene compared with *n*-dodecylbenzene. One-half per cent *n*-dodecylbenzene is readily utilized for growth by the nocardia in a mineral-salts medium, but, with 0.2% ethylbenzene, 0.3% of the alkane, *n*-hexadecane, is not utilized for growth until after a lag of several days. Increasing the concentration of ethylbenzene to 0.5% completely inhibits growth. This implies a toxic effect of ethylbenzene which in practice can be circumvented by maintaining the cyclic hydrocarbon at a low concentration relative to the *n*-alkane in the medium.

By employing a two-component hydrocarbon system such as has been described, oxidative products are obtained without consumption of the cyclic portion of certain hydrocarbons. Theoretically, a specific cyclic hydrocarbon thus may be converted to a specific oxidized product in very high yield. In oxidations of cyclic hydrocarbons involving metabolic decomposition for growth, specificity of the oxidation process would be less as well as the yields of cyclic acids.

ACKNOWLEDGMENTS

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