Intermediates from the Microbial Oxidation of Aliphatic Hydrocarbons

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ABSTRACT

Oxidation of aliphatic saturated and unsaturated hydrocarbons by bacteria, yeasts, and fungi leads to the production of a variety of intermediates, e.g., mono- and dicarboxylic acids, primary alcohols, isomeric alcohols and their corresponding ketones, diols, epoxides, and hydroxy acids. Further degradation of isomeric ketones in two species of *Pseudomonas* occurs by a flavoprotein monooxygenase leading to the formation of ester intermediates. In the case of 2-tridecanone→undecyl acetate, the esterase active on the acetate ester also has been characterized. Oxidation of pristane gives rise to several branched chain intermediates of varying carbon length. Products from other branched chain hydrocarbons are also presented. Oxidation of hydrocarbons that are not growth substrates but are nonetheless oxidized by microorganisms is reviewed. The examples presented reiterate the fact that microorganisms are a metabolically diverse group capable of altering an incredibly wide array of organic compounds of which aliphatic and alicyclic hydrocarbons as a class represent but one example.

The title indicates that the formation of novel compounds of industrial significance is to be covered. My presentation will be inadequate on both points since most of the intermediate oxidation products of aliphatic hydrocarbons are not particularly "unique," and further, I have no particular insight into what compounds are or are not of industrial significance. Over the past 15 years, microbial oxidation of hydrocarbons has been reviewed repeatedly, but in the past several years little has been added to our overall knowledge as far as new intermediates arising from the transformation of aliphatic hydrocarbons is concerned. Since this short presentation can present only a limited review of the field, I have made no attempt to cover any aspect exhaustively.

The following section is summarized in Figure 1. Stewart, Kallio and co-workers (1) provided the first strong evidence that methyl group oxidation was occurring when they isolated the C_{32} ester, cetyl palmitate, from cultures of *Micrococcus cerificans* (now *Acinetobacter* sp.) growing on the C_{16} alkane, hexadecane. This indicated that methyl group oxidation formed a primary alcohol as the first stable intermediate followed by oxidation to the monocarboxylic acid that was subsequently esterified. Kallio's (1) laboratory also found that molecular oxygen was required for this methyl group oxidation.

Coon and co-workers (2,3), working with *Pseudomonas oleovorans*, showed that three protein components participated in the hydroxylation of the methyl group: (a) rubredoxin, (b) NADH-rubredoxin reductase, and (c) the hydroxylase. Cardini and Jurtshuk (4) found that oxidation of octane by a *Corynebacterium* sp. required NADH and molecular oxygen, but in contrast to *P. oleovorans*, the corynebacterium hydroxylase system was composed of two proteins, one of which exhibited the spectral characteristics of cytochrome P-450, and the other of a flavoprotein.

Kester and Foster (5) provided evidence that a *Corynebacterium* sp. could diterminally oxidize alkanes with the formation of ω-hydroxy acids and dicarboxylic acids. Tulloch et al. (6), and Jones and Howe (7), using two different species of the yeast *Torulopsis* found that alkanes were converted to the ω and ω-1 hydroxy acids. Coon's group also reported that the alkane hydroxylase from *P. oleovorans* would also ω-oxidize a series of fatty acids (8).

Products arising from methyl group oxidation of olefinic hydrocarbons have also been reported. Thijsse and van der Linden (9) found that a pseudomonad grown on heptane produced 6-heptenoic acid when given 1-heptene. Figure 2 summarizes findings from our laboratory of intermediates produced from growth on 1-hexadecene by the yeast *Cadida lipolytica* (10-12). 15-Hexadecen-1-ol and 15-hexadecenoic acid arise from methyl group oxidation. It was also interesting to note that the ω-unsaturated C_{16} acid was elongated to the ω-unsaturated C_{18} acid. Further, the C_{18} dienoic acid, unsaturated in the ω- and the Δ^9-position, was also produced. The ω-unsaturated C_{16} secondary alcohol was detected; secondary alcohols will be mentioned later.

Candida lipolytica also possesses the ability of attacking the terminal double bond of 1-alkenes. Bruyn (13) isolated 1,2-hexadecanediol from cultures growing on 1-hexadecene, and subsequently atmospheric oxygen was implicated in diol formation (14). Figure 3 summarizes intermediates identified in our laboratory from 1-hexadecene oxidation (10-12). Some are arranged in a sequence to indicate our concept of olefin degradation. The C_{16} epoxide, 1,2-diol, and α-hydroxy acid were seen, as were the C_{15} and the C_{17} monocarboxylic acids. Also, the Δ^9 C_{17} monoenoic acid was observed. Primary and secondary saturated C_{16} alcohols were also produced, presumably by reaction at the double bond.

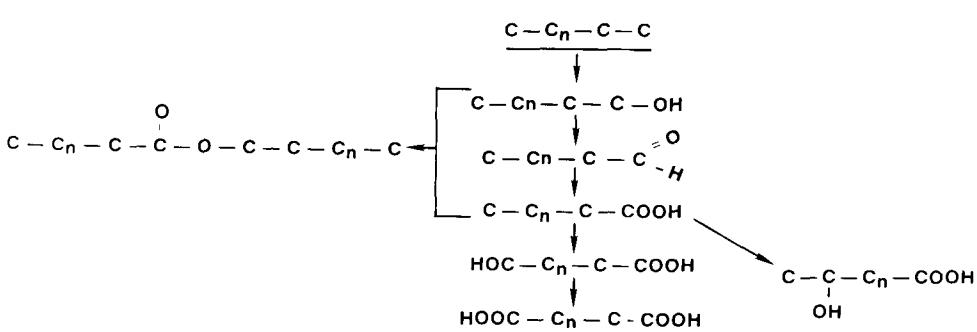

FIG. 1. A scheme summarizing reactions involved in methyl group oxidation by microorganisms.

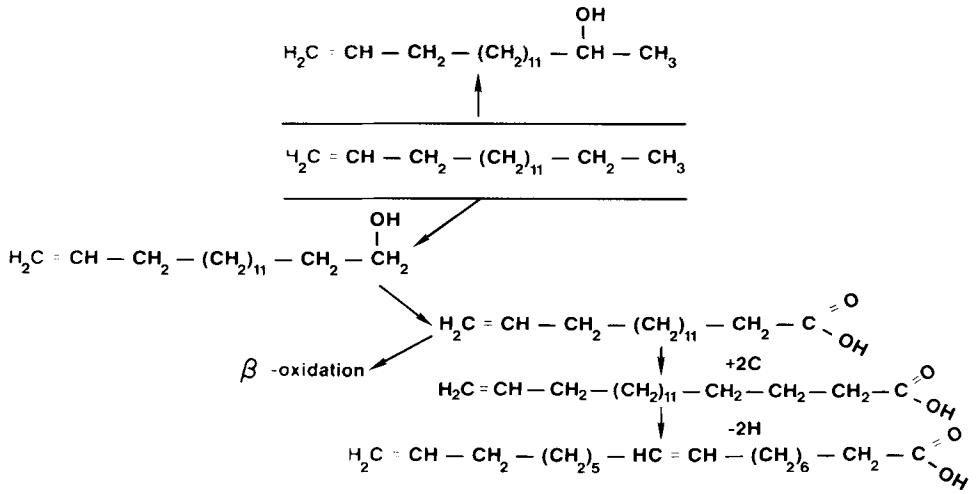

FIG. 2. Products from methyl group oxidation of a 1-alkene by *Candida lipolytica*.

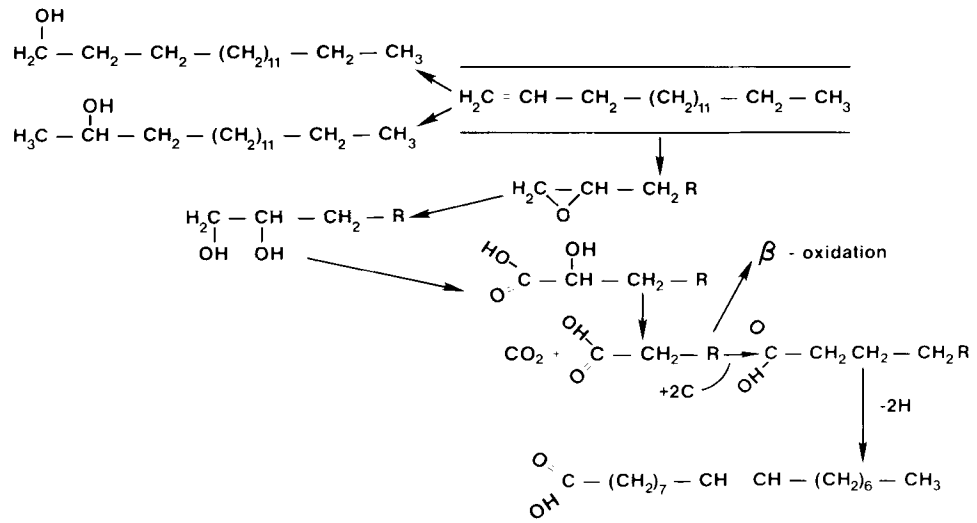

FIG. 3. Intermediates from oxidation of the double bond of a 1-alkene by *Candida lipolytica*.

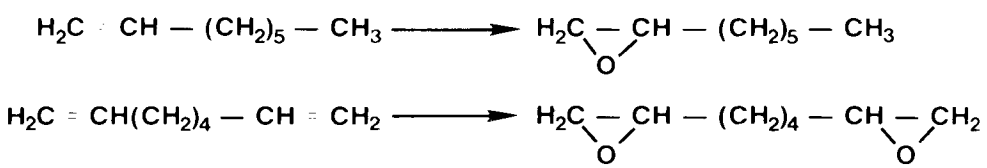

FIG. 4. Products from 1-octene and 1,7-octadiene.

May and Abbot (15) obtained the ω-hydroxylation enzyme system of *P. oleovorans* from Coon and tested it on 1-octene and 1,7-octadiene (Fig. 4). The latter was converted to both 1,2,7,8-diepoxyoctane and the mono-epoxy derivative, 7,8-epoxy-1-octene. 1-Octene was oxidized to 1,2-epoxyoctane and 7-octene-1-ol (not shown in Fig. 4). Thus the same hydroxylase system, at least in *P. oleovorans*, can attack the methyl group or the double bond.

Abbott and Casida (16) reported on the formation of internally unsaturated alkenes from alkanes by a resting cell suspension of a *Nocardia* sp. grown on glucose. When octadecane was the substrate, a mono-alkene mixture was formed with 9-octadecene being predominant, followed by decidedly decreasing amounts of the 8- , 7-, 6-, and 5-isomers. The alkenes did not appear to lead to any oxygenated products.

Ketones and secondary alcohols have also been implicated as intermediates in aliphatic hydrocarbon oxidation by microorganisms (Fig. 5). Lukins and Foster (17) showed that the methyl ketones, acetone, 2-butanone, 2-pentanone, and 2-hexanone were produced from the respective *n*-alkanes by *Micobacterium smegmatis*. Fredricks (18) identified 2-, 3-, 4-, and 5-decanone together with the corresponding secondary alcohols, from the oxidation of decane by *Pseudomonas aeruginosa*. An *Arthrobacter* sp. was shown by Klein and Henning (19) to co-oxidatively transform the C_{16} alkane, hexadecane, to the 2-, 3-, and 4-ketones and the corresponding secondary alcohols. The alkane would not serve as sole carbon and energy source for growth, and the ketones formed apparently were not metabolized further. In our laboratory we found that a *Penicillium* sp. growing on n-tetradecane or 1-tetradecene produced the 2-, 3-, and 4-ketones and alcohols (20). When the olefin was the substrate, these intermediates were ω-unsaturated. We also were able to obtain a cell-free extract from *Penicillium* sp. grown on tridecane that converted the

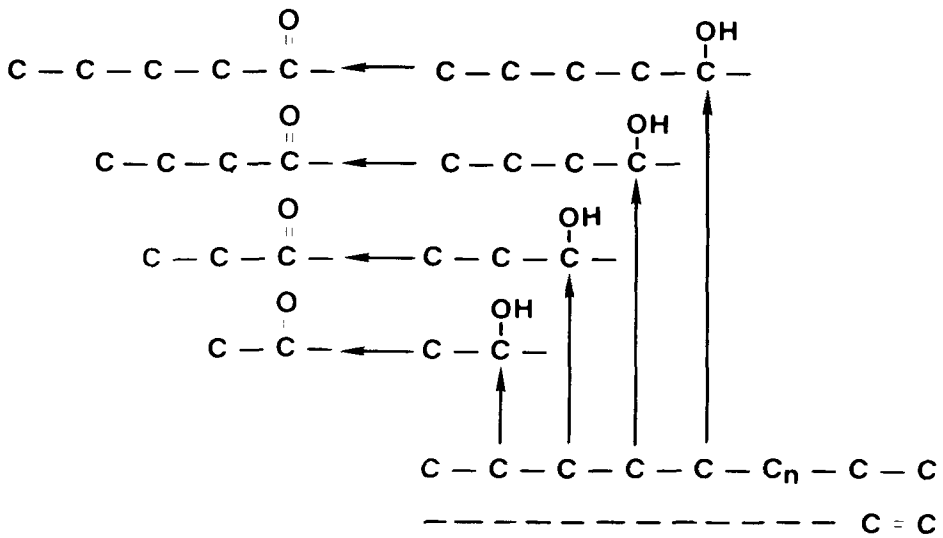

FIG. 5. A scheme summarizing subterminal oxidation of aliphatic hydrocarbons by microorganisms.

TABLE I

Specificity of 2-Tridecanone Oxygenase for Methyl Ketones

Substrate	Specific activity (μmoles O_2/min/mg)	Specific activity with catalase present	Ester detected
2-tetradecanone	0.47	0.46	dodecyl acetate
2-tridecanone	0.49	0.49	undecyl acetate
2-dodecanone	0.48	0.48	decyl acetate
2-undecanone	0.10	0.10	nonyl acetate
2-decanone	0.35	0.32	octyl acetate
2-nonanone	0.19	0.18	heptyl acetate
2-octanone	0.11	0.11	hexyl acetate
2-heptanone	0.16	0.14	pentyl acetate
2-butanone	0.07	0.08	---
acetone	0	---	---
acetophenone	0	---	---

TABLE II

Specificity of 2-Tridecanone Oxygenase for Isomers of Tridecanone

Substrate	Specific activity (μmoles O_2/min/mg)	Specific activity with catalase present	Ester detected
2-tridecanone	0.49	0.49	undecyl acetate
3-tridecanone	0.26	0.26	decyl propionate
4-tridecanone	0.27	0.27	nonyl butyrate
5-tridecanone	0.63	0.63	octyl pentanoate
6-tridecanone	0.60	0.60	heptyl hexanoate pentyl octanoate
7-tridecanone	0.62	0.62	hexyl heptanoate

hydrocarbon to the isomeric alcohols and ketones in the presence of NADPH (21).

The further oxidation of ketones gives rise to an interesting intermediate. Several years ago by enrichment culturing we obtained *Pseudomonas cepacia* which grew on the methyl ketone, 2-tridecanone, as sole carbon source. The main intermediate produced was a primary alsohol two carbons shorter than the ketone substrate (22); Subsequently, an intermediate was isolated that was produced in low yield; it was shown to be the acetate ester, undecyl acetate (23). If this were the true intermediate in 2-tridecanone oxidation, then its cleavage would yield the primary alcohol shorter by two carbons. We theorized that the functioning of an active esterase did not permit the accumulation of appreciable quantities of the ester. Addition of the esterase inhibitor, tetraethyl pyrophosphate (TEPP) allowed the accumulation of undecyl acetate (24).

This pathway is analogous to what Rahim and Sih (25) found in the fungal conversion of progesterone to testosterone. In this process progesterone, with its methyl ketone side chain, was converted to testosterone acetate by a NADPH-specific oxygenase, and the ester was hydrolyzed by an esterase to yield testosterone and acetate.

Our pseudomonad esterase was purified and was found to be an isoenzyme with a molecular weight of 34,000 daltons (26). The esterase was induced by growth on the ketone and on the acetate ester but not by other growth substrates (27). The activity of the esterase using a variety of other acetate esters was tested. A decrease in activity was apparent when the alkyl side chain was shortened. The presence of a terminal double bond in the undecyl acetate caused a slight reduction in enzyme activity.

Studies with cell-free extracts in an $^{18}O_2$-enriched atmosphere showed that molecular oxygen was incor-

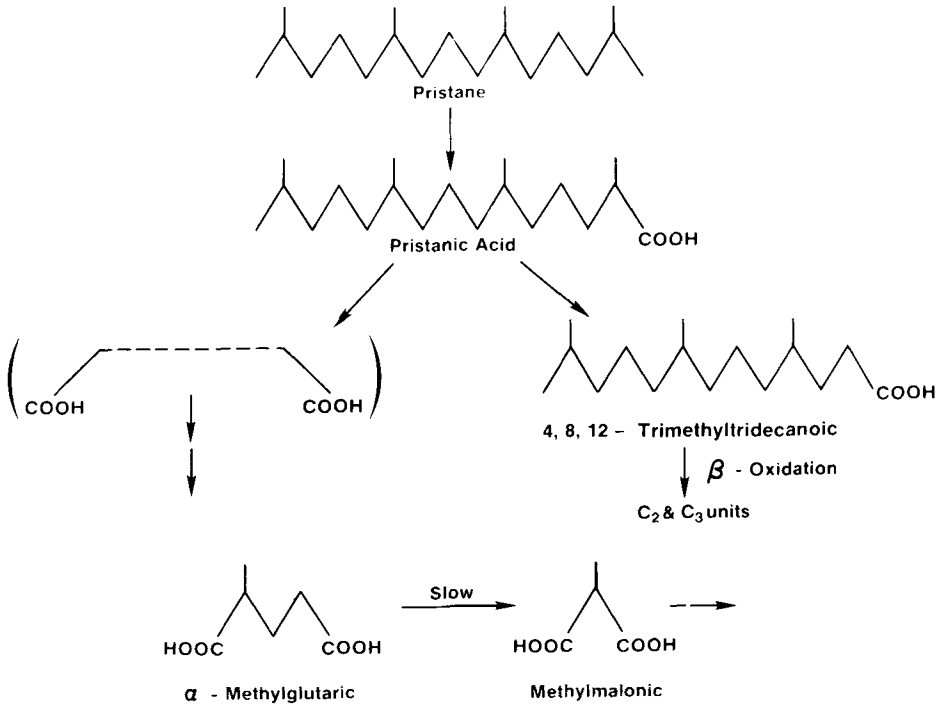

FIG. 6. Pristane degradation by a *Corynebacterium* (30).

porated into the ketone to form undecyl acetate (24):

$$CH_3(CH_2)_9CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 \xrightarrow[NADPH]{^{18}O_2} CH_3(CH_2)_9CH_2\text{-}^{18}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 \xrightarrow{H_2O}$$

$$CH_3(CH_2)_9CH_2{}^{18}OH + HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3$$

Recently the 2-tridecanone oxygenase has been purified and characterized (28). The enzyme has a molecular weight of 110,000 and is composed of two subunits of 55,000. NADPH is required, and the enzyme is a flavoprotein containing FAD. The specificity of 2-tridecanone oxygenase for other methyl ketones is presented in Table I. Activity was assayed by O_2 uptake using an oxygen electrode. A common property among external flavoprotein monooxygenases is emerging with respect to the role of substrates and analogs as effectors. Effectors combine with the oxidized enzyme and dramatically increase the rate of oxidation of reduced pyridine nucleotides. With substrate analogs, this increased rate of reduced pyridine nucleotide oxidation results in the formation of hydrogen peroxide instead of oxygenated product and water as expected with true substrates. In an assay measuring oxygen uptake, the fermentation of hydrogen peroxide would still appear as activity. Therefore, large amounts of catalase were added to reaction vessels with the assumption that there would be a significant decrease in oxygen uptake if hydrogen peroxide were being produced from methyl ketones acting as substrate analogs. The results show that most of the methyl ketones exhibited approximately the same activity in either the absence or presence of catalase. Further evidence that these methyl ketones were true substrates was provided by detection of the respective acetate esters by gas liquid chromatography. With the exception of the unexplained low activity of 2-undecanone, there was a general decrease in activity with decreasing methyl ketone size. The specificity of the oxygenase for isomers of 2-tridecanone is summarized in Table II. Esters detected from oxidation of 3-, 4-, and 5-tridecanone were formed by insertion of an oxygen atom on the side of the carbonyl with the longest alkyl group. However, with 6-tridecanone the detection of two esters indicated oxygen insertion on either side of the carbonyl group. 7-Tridecanone is a symmetrical molecule, and insertion on either side would result in the same ester. It was interesting to note that there was greater activity with 5-, 6-, and 7-tridecanone than with 2-tridecanone, the substrate used for growth of the organism. *P. aeruginosa* also carries out these reactions, and in contrast to *P. cepacia*, evidence indicates that *P. aeruginosa* has the ability to oxidize alkanes via ketone and ester intermediates (29).

McKenna and Kallio (30) reported on the metabolism of the isoprenoid alkane, pristane, by a *Corynebacterium* able to utilize this compound for growth (Fig. 6). In general, pristane was considered to be "biologically inert." Identification of 4,8,12-trimethyltridecanoic acid and α-methylglutaric acid indicates that both β-oxidation and ω-oxidation of fatty acid intermediates operates in the microorganisms. Methyl substitution of n-alkane molecules has a profound influence on the availability of a given hydrocarbon as a growth substrate, but it appears that if the position of the methyl group blocks β-oxidation, then the methyl-substituted hydrocarbon will not support growth. This does not mean that methyl-substituted alkanes cannot undergo an initial oxidation via co-oxidation.

As a digression from aliphatic hydrocarbons, I would like to consider intermediates from the oxidation of the alicyclic hydrocarbon, cyclohexane. Despite earlier reports that cyclohexane could serve as sole carbon source for microorganisms, numerous attempts to isolate such an organism failed. Beam and Perry (31) found that several cultures, after growth on propane, could oxidize cyclohexane to cyclohexanone via co-oxidation (Fig. 7B). de Klerk and van der Linden (32) described a system containing two strains of pseudomonads that could metabolize cyclohexane to completion (Fig. 7A). The first organism co-oxidatively converted cyclohexane to cyclohexanol while growing on n-heptane, and the second organism mediated the complete oxidation of cyclohexanol. Stirling, et al. (33) recently reported that a single strain of *Nocardia* could grow on cyclohexane. The hydrocarbon was oxidized to cyclohexanol, and the rest of the pathway is the same as that reported for cyclohexanol degradation by *Acineto-*

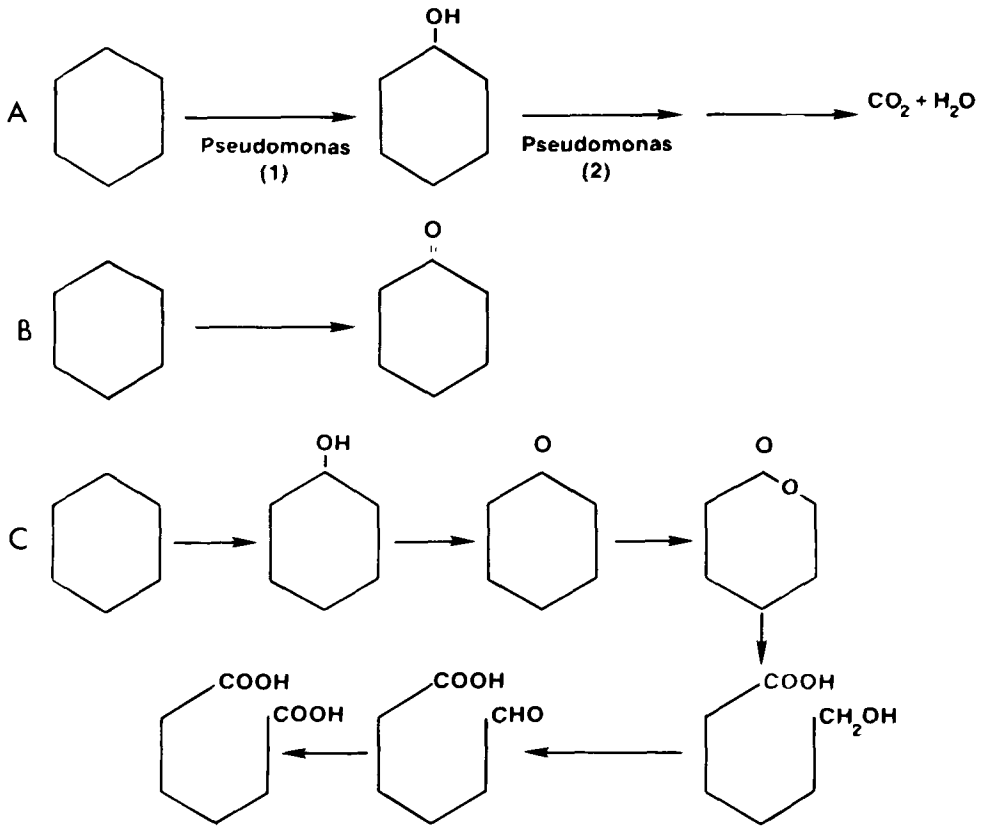

FIG. 7. Cyclohexane transformation: A. complete oxidation by two strains of *Pseudomonas* spp.; B. oxidation to hexanone; C. complete oxidation by a *Nocardia* via adipate.

bacter in work by Donoghue and Trudgill (34), i.e., alcohol to the ketone, followed by an oxygenase reaction resulting in the formation of a lactone which is cleaved to 6-hydroxyhexanoate and on to adipate (Fig. 7C). The oxygenase has been purified, and it is interesting to note that it is a flavoprotein with a subunit molecular weight similar to the methyl ketone oxygenase mentioned previously.

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