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Microbial Cooxidations Involving Hydrocarbons

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INTRODUCTION

The late Jackson W. Foster defined *cooxidation*, a technique originally described by Leadbetter and Foster (45, 46), as follows. "Nongrowth hydrocarbons are oxidized when present as co-substrates in a medium in which one or more different hydrocarbons are furnished for growth. Employing this principle with *Pseudomonas methanica* growing at the expense of methane, a series of homologous oxidation products was obtained from co-substrate gases: from ethane, ethanol, acetaldehyde, and acetic acid were produced; from propane, *n*-propanol, propionic acid, and acetone; from *n*-butane, *n*-butanol, *n*-butyric acid, and 2-butanone" (19).

Foster realized the implications and utility of the cooxidation technique both to the microbial physiologist and to those involved in industrial fermentations. He suggested "that the inability to grow at the expense of a particular hydrocarbon is not a consequence only of an organism's inability to attack the substrate; obviously, failure to grow may be due then to its inability to assimilate the oxidation products" (20). Concerning the potential use of cooxidation in industrial fermentations, he stated, "Commercial appeal of the oxidative conversion of a large variety of non-growth hydrocarbons as co-substrates for organisms growing at the expense of cheap substrates, such as methane or natural gas, is obvious" (20).

Studies by Lukins and Foster (47) demonstrated the microbial oxidation of nongrowth substrates by non-proliferating cells of *Mycobacterium smegmatis* whereby washed suspensions of propane-grown *M. smegmatis* cells readily oxidized ethane, ethylene, and propylene but did not grow at the expense of any of these substrates. In fact, ethane was oxidized at a more rapid rate than propane, which had served as the growth substrate. They also reported that *M. smegmatis*, after growth on *n*-alkanes, oxidized several ketones that were not utilized by this species as sources of carbon and energy. In Foster's writing he did not term (or evidently consider) the oxidation of substrates that did not support growth cooxidation, probably because there was no cosubstrate present during the oxidative process.

The accumulation of metabolic products by non-proliferating cell oxidation or bioconversion of substrates is a time-honored technique. Replacement culture was described by mold physiologists over 50 years ago in their studies on metabolic pathways and in their attempts to accumulate useful intermediate products of sugar metabolism. The replacement technique as originally proposed by Butkevich was brought into general use and designated "Pilzdeck" by the noted mold physiologist K. Bernhauer (5-8). Bernhauer's reports confirm that mycelial mats placed on the surface of a glucose medium oxidize the glucose to organic acids and that these mycelial mats retain oxidative activity through several successive replacements on a sugar medium.

Since the original publications by Foster, another term, *cometabolism*, has been introduced and is used by many workers (for a review, see Horvath [27]). The term cometabolism has been applied appropriately in describing the conversion of pesticides but has also been extended to include cooxidation, as well as the utilization of substrates by non-proliferating cellular suspensions. One might question whether the term cooxidation, as defined by Foster, would include the oxidation of substrates by non-proliferating cells. According to *Webster's Third New International Dictionary* (1966) the following definitions can be considered: *co-* —with: together: joint: shared; *metabolism*—the sum of processes concerned in the building of protoplasm and its

destruction incidental to life; *oxidation*—the art or process of oxidizing. The term cooxidation as described by Foster is appropriate in context with etymology, and when used in describing a biological process it should properly not be considered synonymous with cometabolism. Indeed, usage of either the term cometabolism or the term cooxidation to describe conversions of nongrowth substrates by resting (non-proliferating) cellular suspensions in the absence of a metabolizable cosubstrate would be inappropriate. The enzymatic conversion of a substrate by a nonproliferating cellular suspension because an enzyme of broad specificity and capable of the conversion is in proximity to the substrate might best be described as bioconversion. There is no "co-" (with, together) concerned with such an event.

Recently, Hulbert and Krawiec wrote an elegant critique on the use of the term cometabolism and concluded that "use of the term [cometabolism] may lead to serious misconceptions about the immediate capacity of micro-organisms to rid the environment of noxious materials....we propose that use of the word co-metabolism be abandoned" (30).

This discussion is concerned with two general areas of cooxidation relative to hydrocarbons: (i) the oxidation of hydrocarbon cosubstrates that cannot support growth of a species concomitant with growth of the species on a utilizable substrate and (ii) the oxidation of a cosubstrate other than a hydrocarbon during growth of the organism on a hydrocarbon substrate.

Potential for Cooxidation in Nature

The widespread occurrence of molecules, both man-made and as products of living systems, that are recalcitrant (a recalcitrant molecule will be defined as one that is resistant to biological degradation but that does disappear from the environment at a slow rate; this persistence is due to the inability of microorganisms to manage such molecules readily [for a discussion of molecular recalcitrance, see Alexander (1)]) or, in many cases, virtually non-biodegradable is well documented (1, 2). The term recalcitrant molecule(s) was first applied by E. J. McKenna and R. E. Kallio in 1963 in a symposium on "Microbial Aspects of Waste Disposal" (Bacteriol Proc., p. 17, 1963). These workers found that the biodegradability of an alkylbenzene sulfonate was markedly affected by the character of the alkyl side chain, and they described the degree of resistance to microbial attack as the level of recalcitrance. Many synthetic polymers, e.g., polyvinyl chloride, nylon, polydichlorostyrene, and polyurethane, are apparently impervious to microbial attack. These compounds persist in nature, and there is little evidence that they are removed to a considerable extent from the environment by either biological or abiotic mechanisms. Many pesticides [e.g., polychlorinated biphenyls, aldrin, mirex, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, and other chemicals] persist in the environment, although many of these are slowly modified by biological and/or abiotic agents. Generally, recalcitrant compounds are those that are not utilized by any single microbial species as sources of carbon and/or energy but are biodegraded by a series of microbes of differing substrate specificity. Physical forces in the environment also play a role in the reactions involved in mineralization of these compounds. The initial attack via cooxidation on a recalcitrant molecule in an environmental niche is a coincidental attack on that compound that is probably of little consequence to the microorganism involved in the oxidative reaction. Neither energy nor carbon for biosynthesis results from this oxidation, and it is probable that the reaction occurs at some expense in energy for the microbes involved.

It is interesting to consider that recalcitrant molecules which yield products of cooxidation that are structurally similar to naturally occurring metabolites disappear more quickly from the environment than those that do not. Thus, dalapon, lindane, and cyclohexane, which are rarely utilized by single microbial species, are much less persistent in the environment than such compounds as 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane and polychlorinated biphenyl. Dehalogenation of dalapon would yield propionate, and dehalogenation of lindane would yield a molecule structurally similar to inositol.

The potential role of cooxidative processes in the biodegradation of recalcitrant molecules has received considerable attention. This is particularly evident in studies related to pesticide degradation and will not be discussed in this article (for a review, see Horvath [27]). That cooxidation is involved in the biodegradation of some hydrocarbons was suggested by studies presented by Beam and Perry (3, 4). There are few reports claiming cycloparaffin biodegradation in axenic culture or that unsubstituted cycloparaffins can serve as sole sources of carbon and energy for microbial growth (except for a brief report, by Imelik [35], that a strain of *P. aeruginosa* can utilize cyclohexane). Results reported simultaneously from two laboratories suggest that the initial oxidative breach of the pristine cycloparaffin molecule occurs via cooxidation (with hydrocarbon as cosubstrate) and that the oxygenated product is utilized by another microbial species (3, 4, 17). That organisms are present in nature that can utilize cycloparaffins was con-

firmed recently by the isolation and description of an axenic culture that can grow with cyclohexane as the sole substrate (69).

The incorporation of oxygen into diverse substrates is often a prerequisite to the mineralization of those molecules. Oxygenases play an important role in the environment, as these enzymes of broad substrate specificity (13) serve as an indispensable initiator of biodegradative attack. In a sense, they might be considered as a scavenger for recalcitrant compounds of low solubility, as incorporation of oxygen into the molecules renders them more amenable to biodegradation. The role that cooxidation involving molecular oxygenases would play in natural environments is then quite evident. These enzymes can be induced in microorganisms by a wide variety of gratuitous inducers, and their activities can yield partially oxidized compounds that might then be subject to mineralization by a host of bacteria in the environment.

Oxygenases That Attack Hydrocarbons

The initial oxidative attack on the reduced hydrocarbon molecule is an aerobic one and involves molecular oxygen (19, 20), although ZoBell and Prokop have stated, "Laboratory and field observations amply attest the microbial decomposition of various kinds of mineral oils under strictly anaerobic conditions accompanied by the growth of sulfate reducers. That conditions are anaerobic is indicated by the growth of sulfate reducers. The decomposition of the mineral oils is indicated by changes in their properties and quantities" (80). The problems encountered in refining these natural anaerobic conditions to laboratory quantification render experimental demonstration of such metabolic capacities difficult to confirm.

The initial clear-cut demonstration that molecular oxygen is involved in hydrocarbon utilization was offered in a classic report by Hansen and Kallio in 1957 (24). They demonstrated that *P. stutzeri*, using nitrate as a terminal electron acceptor, evolves N_2 anaerobically while oxidizing oxygen-containing compounds that are considered intermediates in the biodegradation of hydrocarbons; i.e., *P. stutzeri* cell suspensions generate N_2 anaerobically with dodecanol, dodecanal, or dodecanoic acid as the substrate. However, no evolution of N_2 is evident with *n*-dodecane or 1-dodecene as the substrate under anaerobic conditions. All substrates are readily oxidized aerobically. These experiments provide evidence that molecular oxygen is required for the insertion of oxygen into the hydrocarbon molecule but not for the subsequent utilization of the oxygenated intermediates. There are other reports (26, 44) of experiments with $^{18}O_2$ that support the contention that molecular oxygen is required for hydrocarbon oxidation.

There is ample evidence that, in microbes, the oxygenase for hydrocarbon substrates is an inducible enzyme, and van der Linden and Thijsse in a comprehensive review stated, "The adaptive nature of the hydrocarbon-oxidizing enzymes seems well established by numerous investigators and appears to be a general phenomenon. Yet, a few investigators have reported on experiments which might go to show that there is more to this problem" (74). The inducible nature of the hydrocarbon-oxidizing system was also discussed in a review by Humphrey (31). Ladd (43) reported that a *Corynebacterium* sp., after growth on nutrient broth, oxidized both alkanes and 1-alkenes without a lag phase but did not oxidize cyclics, e.g., cyclohexane, tetrahydronaphthalene, benzene, or toluene. Similar results suggesting the possible constitutive nature of some oxygenases were reported in other species (24, 25).

Induction of Oxygenases by Nonsubstrates

There have been a number of reports in recent years suggesting that the hydrocarbon-oxidizing system can be induced in bacteria by substrates other than hydrocarbons. Some of these compounds that can act as inducers of the enzyme require a molecular oxygenase for biodegradation, and others probably would not. Perry and Scheld (54) reported that a significantly greater percentage of the microorganisms isolated from soil by enrichment with *o*-phthalate could oxidize propane without a lag phase after growth on *o*-phthalate but had a lag phase of 40 to 50 min after growth on glucose before oxidation of propane would occur. These results suggest that the propane-oxidizing enzyme(s) might be induced by *o*-phthalate, since the enzymes involved in utilization of intermediates of propane oxidation, e.g., 1-propanol, 2-propanol, etc., are constitutive in this organism. Robinson (59) studied a pseudomonad that oxidized *n*-octane without lag in respirometry experiments when grown on octane but not after growth on glucose. However, this pseudomonad oxidized 1-octene, which was not utilized as growth substrate, after growth on octanoic acid, suggesting that the homologous fatty acid could induce the alkene-oxidizing system. Nyns et al. (51) reported similar results with the yeast *Candida lipolytica*, which has an adaptive oxidizing system for *n*-hexadecane, and this system can be induced by *n*-hexadecane, cetyl alcohol, palmitaldehyde, or palmitic acid. Two significant articles have ap-

peared (73, 75) which expand on the experimental results reported earlier. van der Linden and Huybregtse (73) found that growth of *P. aeruginosa* on 1,6-hexanediol resulted in the induction of an epoxidizing and alkane-oxidizing system. Other diols, from 1,3-propanediol to 1,8-octanediol, had little or no inducing effect. They suggested that the singular success in induction of the oxygenase by the 1,6-diol might be due to a particular product of biodegradation of this compound that can act as the oxygenase inducer. van Eyk and Bartels (75) made an extensive survey of compounds that served as repressors or inducers of the adaptive oxygenase system in *P. aeruginosa*. Several metabolic intermediates, e.g., α-ketoglutarate, fumarate, malate, and succinate, effectively repressed synthesis of the inducible oxygenase when added to a complete medium with *n*-hexane as the substrate. Peptone–yeast extract was also an effective repressor of the paraffin-oxidizing system. They found, however, that several nonhydrocarbon compounds not utilized for growth by *P. aeruginosa* would nevertheless effectively induce the paraffin-oxidizing system. Among these compounds were: 1,2-dimethoxymethane, diethoxymethane, cyclopropane, dicyclopropylmethane, and spiro-[2,4]-heptane. Others have reported (11, 12) that oxygenases for specific substrates can be induced by compounds other than the known substrate and, in certain cases, by intermediates that occur in the biodegradative pathway subsequent to the enzyme involved. Kachholz and Rehm (38) made the interesting observation that of 14 different types of bacilli and 100 isolates from soil samples, none could assimilate C_{14} to C_{18} alkanes as sole carbon sources. However, five types of bacilli could oxidize alkanes in the presence of other carbon sources.

Stability of the Oxygenase System in Microbes

Whether the capacity to produce the oxygenase is lost after prolonged growth on nonhydrocarbon substrates is open to question. Davis et al. (14) reported that, of two strains of *Mycobacterium paraffinicum* isolated on ethane, one lost the ability to oxidize ethane after subculture on ethanol, and both lost the capacity after growth on acetate. Similar results were reported with kerosene-utilizing microorganisms isolated from soil that irreversibly lost the ability to utilize hydrocarbons after subculture on nonhydrocarbon substrates (33). They suggested that gram-negative organisms lose this oxidative capacity more readily than do gram-positive organisms. Blevins and Perry (10) tested several species of *Mycobacterium* for loss of the inducible hydrocarbon-oxidizing system after continued subculture on acetate and reported that all strains tested retained propane-oxidizing capacity after continued subculturing and outgrowth over a period of months.

Pitfalls of Laboratory Cooxidation Studies

Studies on cooxidation with certain hydrocarbons as cosubstrates should be interpreted with caution. This is particularly applicable in respirometry experiments since the observation that some hydrocarbon substrates can stimulate respiration in intact microbial cells (49). McKenna and Kallio report that "there appears to be a problem with certain hydrocarbons (and perhaps other substances) which may, in some way, increase the endogenous respiration of the test organisms without being oxidized. If a manometric experiment is cleancut and the experimental oxygen consumption is well in excess of a low auto-respiratory rate then the conventional technique of subtracting the latter from the former may be valid. On the other hand, if oxygen is consumed by an organism in the presence of hydrocarbon (which does not support growth) at a level only slightly, but definitely above the endogenous, the question of true oxidation versus stimulation of auto-respiration may be properly raised and would require clarification" (49).

Klein (40) isolated a bacterium from the aquatic environment, tentatively identified as an *Arthrobacter*, that had limited ability to grow with glucose as substrate, but addition of 0.5% *n*-hexadecane to the medium resulted in vigorous growth. The isolate would not utilize the paraffinic hydrocarbon as a sole source of carbon and energy, nor was there evidence that the alkane was oxidized to the homologous alcohol under these conditions. The results suggest that hydrocarbons might exert some physiological effect on the bacterium without being oxidized or cooxidized and that this effect could erroneously be ascribed to either of these processes.

COOXIDATION—PARAFFINIC HYDROCARBONS

The original study on the phenomenon later termed cooxidation (20) was the subject of a classical paper in 1959 by Leadbetter and Foster, who reported that *P. methanica*, an obligate methylotroph, would yield oxidation products from other gaseous alkanes if these alkanes were present during growth on methane (45). A later paper (46) provided detailed identification of the products formed via cooxidation of cosubstrates with methane as substrate. The products isolated and identified from the growth medium with [1,2-^{14}C]ethane, [2-^{14}C]propane, and unla-

beled butane as cosubstrates are presented in Table 1.

These experiments clearly demonstrated that microorganisms, while growing on a utilizable substrate, can concomitantly oxidize another compound present in the medium even though that compound cannot serve as a source of carbon and energy. Whittenbury et al. (78) found that cooxidation is prevalent in methane utilizers, as several microbial species of obligate methylotrophs would cooxidize ethane to ethanol or acetaldehyde. They also found that one of these species, *Methylosinus trichosporium*, reduced acetylene to ethylene during growth on methane. Kachholz and Rehm (38a) reported that five species of the genus *Bacillus* (strains of *B. macerans, B. lentus, B. subtilis, B. coagulans,* and *B. stearothermophilus*) would degrade *n*-alkanes by cooxidation. The substrates for growth were glucose, peptone, and yeast extract. The major products of cooxidation were ketones, diols, ketols, and diketones. At 30°C, *B. stearothermophilus* formed 20 times the amount of oxidation product formed in the same medium at 50°C.

Hubley et al. (29) reported that the obligate methylotrophs *Methylomonas albus* and *M. trichosporium*, after growth on methane, would oxidize carbon monoxide to carbon dioxide. Although this is an example of oxidation in the absence of the substrate, it indicates that the oxygenase systems in methane-utilizing organisms can act on carbon monoxide. Ferenci et al. (18), in an extensive report, presented evidence that methane and carbon monoxide oxidation in *P. methanica* is catalyzed by a single enzyme system. They suggest that the carbon monoxide or ethane monooxygenase activities are secondary activity of the methane monooxygenase system and that the reaction occurs as outlined in Fig. 1. Their results suggest that alcohol oxidation can supply reducing power to the monooxygenase system in vivo. The only significant difference between carbon monoxide, ethane, and methane oxidations by intact cells is that a cooxidizable substrate is required with *P. methanica* for carbon monoxide oxidation and that it also stimulates ethane oxidation. Since the monooxygenase would be active in propane or butane oxidation, the metabolic sequence as postulated would be operative in the cooxidation system originally described by Foster (19).

TABLE 1. *Cooxidation of gaseous alkanes by P. methanica growing with methane as substrate*

Cosubstrate	Product	% of total radioactivity recovered
[1,2-^{14}C]ethane[a]	Acetic acid	69.0
	Ethanol	1.0
	Acetaldehyde	17.3
	CO_2	5.5
	Cell material	3.8
	Extracellular	3.6
[2-^{14}C]propane[b]	Propionic acid	9.5
	1-Propanol	1.0
	Acetone	18.0
	CO_2	31.6
	Cell material	8.5
	Extracellular	31.7
Butane	Butyric acid	ND[c]
	1-Butanol	ND
	2-Butanone	ND

[a] Initial composition of the gas phase: ethane, 5%; methane, 45%; air, 50%.

[b] Initial composition of the gas phase: propane, 30%; methane, 40%; air, 30%.

[c] Not determined.

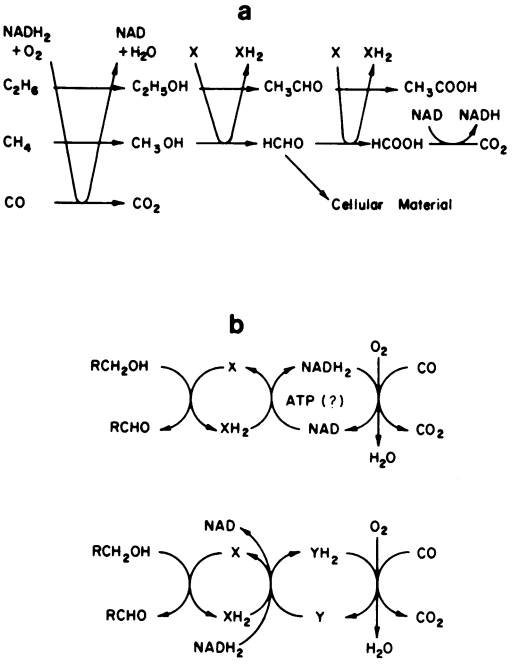

FIG. 1. *(a) Outline of the oxidative metabolism of P. methanica. X, Unknown physiological electron acceptor for reactions catalyzed by the alcohol dehydrogenase. (b) Two possible routes (i and ii) for the channeling of reductant from alcohol oxidation to the CO monooxygenase. X, As in (a); Y, postulated carrier or set of electron carriers between reduced nicotinamide adenine dinucleotide and the CO monooxygenase. Abbreviations: NAD, nicotinamide adenine dinucleotide; $NADH_2$, reduced NAD; ATP, adenosine 5'-triphosphate. Reprinted from Ferenci et al. (18) by permission of Cambridge University Press, New York.*

An interesting example of cooxidation was reported by de Bont and Mulder in 1974. They isolated a methane-oxidizing bacterium (designated strain 41) from garden soil on a nitrogen-free medium (16). Nitrogenase activity could be assayed by acetylene reduction when the bacterium was grown on methanol, but no ethylene was determined after growth on methane. Analysis for nitrogen fixation with $^{15}N_2$ yielded clear evidence for nitrogen fixation with both methanol and methane as substrates. The results indicate that during growth of strain 41 on methane there is the induction of a paraffin-oxidizing system which cooxidizes the ethylene produced from acetylene. The inducible oxygenase was not present in cells growing on methanol, and there was an accumulation of the ethylene produced from acetylene.

The conversion of a growth substrate to homologous oxygenated products could also be considered a form of cooxidation. There have been a number of observations (for a summary, see Bird and Molton [9]) of the accumulation of oxygenated metabolic products during growth of bacteria on alkane substrates, and many of these have had carbon numbers equivalent to those of the substrates. The production of oxygenated metabolites that can be utilized by microbes that cannot utilize the hydrocarbon substrate has led to problems in enrichment for and culturing of some hydrocarbon-oxidizing bacteria. This is particularly evident for microbes that utilize methane, and because of these difficulties in obtaining axenic cultures some studies have been reported with mixed cultures (76). Recently, Wilkinson et al. (79) reported on the microbial interactions that can occur in mixed bacterial populations during growth on methane. While growing on methane, the active oxygenase system of a *Pseudomonas* sp. present cooxidized considerable amounts of methane to methanol, and accumulation of methanol can cause an autoinhibition of growth. Growth of an accompanying species, *Hyphomicrobium* sp., on the methanol alleviated the toxicity and allowed both to grow. The *Hyphomicrobium* sp. apparently lacks a molecular oxygenase, and consequently the two species are interdependent for survival, at least under laboratory conditions with methane as substrate. The transient accumulation of fatty alcohols of chain lengths equivalent to those of the substrates was observed by Suzuki and Ogawa (70) with the glutamate-producing *Arthrobacter paraffineus*. When *n*-alkanes from C_{12} to C_{14} were substrates, the homologous alcohols accumulated significantly in the initial 6 to 8 h of the fermentation, and these were gradually converted to the fatty acids of equivalent chain lengths.

The diterminal oxidation of *n*-alkane substrates without chain shortening, as originally described by Kester and Foster (39), should also be considered cooxidation. An example of fatty acids produced from *n*-alkanes by *Mycobacterium rhodochrous* strain 7E1C (originally described as *Corynebacterium* 7E1C) is: *n*-decane → decanoic acid, 10-hydroxydecanoic acid, or 1,10-decanedioic acid. These fatty acids cannot serve as sources of carbon and energy for this strain. Indications are that the added monocarboxylic acid can be oxidized to the corresponding dicarboxylic acid provided that an *n*-alkane is present as the growth substrate. Iizuka et al. (32) reported similar results with a strain of *Candida rugosa*, isolated from aircraft fuel, that would oxidize *n*-decane to the dicarboxylic acid. Krauel et al. (41) reported on *Candida guilliermondii* strain H17, which produced nonanoic acid during growth on *n*-nonane but yielded shorter-chain dicarboxylic acids during growth on *n*-alkanes from C_{10} to C_{17}; e.g., growth on *n*-decane resulted in the production of adipic acid.

An interesting example of dicarboxylic acid synthesis by yeast was reported by Shiio and Uchio (62) with strain 310 of *Candida cloacae*. This organism, while growing on a medium containing both yeast extract and *n*-alkane, oxidized the alkane to the dicarboxylic acids containing the same number of or fewer carbon atoms. The dicarboxylic acids from even-chain-length *n*-alkanes (C_{10} to C_{18}) were all of even carbon number, and those from odd-chain-length *n*-alkanes (C_9 to C_{17}) were of odd chain length. These workers (71) derived a mutant from strain 310 by *n*-methyl-*n*-nitro-N^1-nitrosoguanidine treatment (designated strain M-1) which would not assimilate dicarboxylic acids. Resting cells grown on a medium containing acetate and *n*-alkane produced significant quantities of dicarboxylic acid homologous to the alkane cosubstrate. The highest yield of dicarboxylic acid produced was with *n*-hexadecane as substrate (29.3 g of hexadecanedioic acid per liter was produced). Since strain M-1 did not grow well on *n*-alkanes as compared with the parent (strain 310), the addition of other carbon sources which supported the growth of strain M-1 was necessary for the conversion of *n*-alkane to dicarboxylic acid by growing cells. When acetic acid was added as a growth substrate, the yeast produced 21.8 g of hexadecanedioic acid per liter from *n*-hexadecane. Later, Uchio and Shiio (72) derived a mutant of *C. cloacae* strain M-1 which was completely unable to assimilate *n*-alkanes and accumulated the homologous dicarboxylic acid when an *n*-alkane was present as cosubstrate with a substrate that would support growth of the mutant. This mutant, designated strain MR-

12, cooxidized significant quantities of *n*-alkane to the homologous dicarboxylic acid during growth on acetate (Table 2). These results suggest that the paraffin-oxidizing system is intact in these organisms but that the ability to utilize the oxygenated product is not.

There is another example of cooxidation of a substrate to a homologous oxygenated compound without degradation: the oxidation of *n*-alkanes to the homologous fatty acids or alcohols and the formation of esters from these products. Stewart et al. (68) were the first to recover a product of aliphatic alkane oxidation with a carbon number identical to the alkane serving as substrate. They isolated a gram-negative coccus (*Micrococcus cerificans* H.O.1) by enrichment with *n*-hexadecane as the carbon source that converted a part of the substrate to extracellular cetyl palmitate. These workers postulated that the ester was formed from cetyl alcohol and palmitic acid produced from the substrate alkane. Later, Stewart and Kallio (67) reported that the products in Table 3 were obtained from *M. cerificans*. The waxes produced from paraffins (within the ranges tested) have alcohol moieties with the same carbon skeletons as those of the paraffins from which they are derived. The fatty acid in the waxes was *n*-hexadecanoic acid except with *n*-octadecane as substrate, where one-half of the ester produced had *n*-octadecanoic acid as the acid moiety. Stevenson et al. (66) demonstrated that strain H.O.1 would produce esters from *n*-heptadecane and identified heptadecylpentadecanoate, heptadecylpalmitate, and heptadecylmargarate in the growth supernatant after growth on this paraffin. Raymond and Davis (55) isolated a *Nocardia* sp. from soil that formed cetyl palmitate during growth on *n*-hexadecane and octadecylstearate when grown on *n*-octadecane. This species deposited more of the wax inside the cells than in the supernatant. The production of esters by fungi was reported by Iizuka et al. (34) with *Hormodendrum hordei* and a *Cladosporium* sp. The esters formed from *n*-pentadecane and *n*-hexadecane were believed to be unsaturated fatty acid esters but were not identified further. Later, Pelz and Rehm (53) isolated a number of molds from soil (*Mucorales* and *Moniliales*) and reported that these isolates (and several laboratory strains) produced ketonic substances from *n*-dodecane and *n*-tetradecane and esters from *n*-tetradecane and *n*-pentadecane. McCarthy (48) reported that straight-chain paraffins can be metabolized to the homologous fatty acid in mammalian systems.

COOXIDATION—CYCLOPARAFFINS

The oxidation of cycloparaffins to homologous cycloketones was first reported by Ooyama and Foster (52). These workers isolated a strain from soil with 2-methylbutane as substrate (designated strain JOB-5, later determined to be a strain of *Mycobacterium vaccae*) that produced neutral hydrazones from cycloalkanes that were not utilized by this strain as growth substrates. In the experiments carried out by Ooyama and Foster, resting-cell suspensions were used, and

TABLE 3. *Esters produced by M. cerificans during growth on n-alkane substrates*

Substrate	Ester
n-Octadecane	CH_3—$(CH_2)_{16}$—$COOCH_2$—$(CH_2)_{16}$—CH_3, CH_3—$(CH_2)_{14}$—$COOCH_2$—$(CH_2)_{16}$—CH_3 (1:1 mixture)
n-Hexadecane	CH_3—$(CH_2)_{14}$—$COOCH_2$—$(CH_2)_{14}$—CH_3
n-Tetradecane	CH_3—$(CH_2)_{14}$—$COOCH_2$—$(CH_2)_{12}$—CH_3
n-Dodecane	CH_3—$(CH_2)_{14}$—COO-X (unknown)

TABLE 2. *Production of dicarboxylic acids from n-alkanes by C. cloacae via cooxidation during growth on acetate*

Cosubstrate	*n*-Alkane		Dicarboxylic acid produced	
	Added (vol/vol)	Consumed (%)	Name	Amt (mg/ml)
n-Dodecane	5	83	1,12-Dodecanedioic acid	13.9
n-Tridecane	5	82	1,13-Tridecanedioic acid	16.1
n-Hexadecane	5	95	1,16-Hexadecanedioic acid	24.9
n-Dodecane +	5	69	1,12-Dodecanedioic +	17.2
n-Hexadecane	5	57	1,16-Hexadecanedioic acid	9.9
n-Tridecane +	5	72	1,13-Tridecanedioic +	15.4
n-Hexadecane	5	57	1,16-Hexadecanedioic acid	10.7

they stated, "The formation of products from such substrates obviously is an example of 'nongrowth oxidation'" (52). These cycloalkanes, when present as cosubstrates in a medium with propane as the growth substrate, can also be transformed via the cooxidative process (3, 4). The major part of their study, which will be considered here, is the production of cycloalkanones which occurred by either nongrowth oxidation with resting cells or by cooxidation with growing cells. The ketonic derivatives obtained from oxidation of the cycloparaffins are illustrated in Fig. 2.

Since it has been reported by many workers (3, 17, 23, 43, 53) that unsubstituted cycloparaffins are not readily degraded by axenic cultures (for positive reports, see references 35 and 69), cooxidation has been suggested as a means in nature for mineralizing these ubiquitous constituents of crude oil. The ready utilization of cycloalkanones by bacteria commonly found in soil has been reported (4, 61).

Haider et al. (23) isolated microorganisms by enrichment from field, grassland, and forest soil that could utilize phenol and benzene as carbon sources. These organisms, of the genera *Pseudomonas* and *Nocardia*, produce ^{14}C-labeled

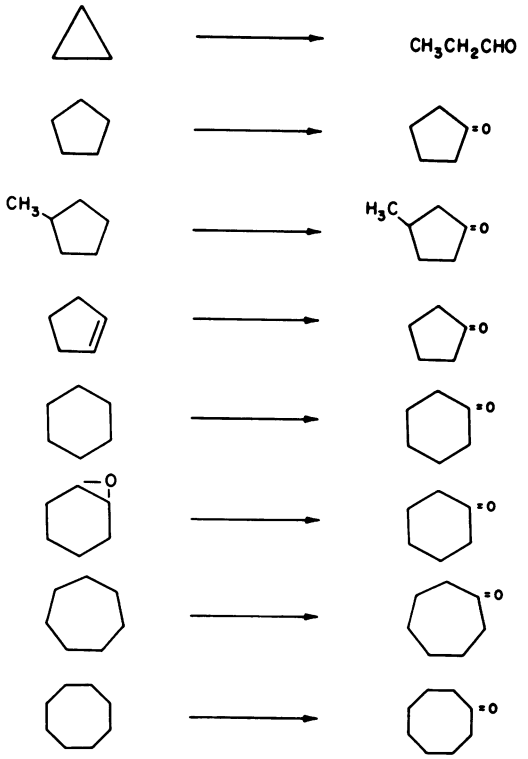

FIG. 2. *Oxidation of cycloparaffins by M. vaccae strain JOB-5.*

chlorinated phenols from benzene but did not produce $^{14}CO_2$ from ^{14}C-cycloalkanes. However, when ^{14}C-cycloalkanes were added to the soils from which the organisms were isolated, $^{14}CO_2$ was released. This suggests that the mixed microbial population of soil contains organisms that can mineralize the cycloalkane. Beam and Perry (3, 4) demonstrated that $^{14}CO_2$ was released from intact soil when [^{14}C]cyclohexane was added but could not isolate organisms by enrichment that could grow with this cycloalkane as substrate. Extensive tests with hydrocarbon-utilizing organisms suggested that the cycloalkane molecule could not be utilized as a growth substrate. They found that *M. vaccae*, while growing with propane as substrate, would cooxidize cyclohexane to cyclohexanone. They isolated a strain from soil with cyclohexanone as substrate, designated strain CY-6 (which could not grow on cyclohexane or propane), and demonstrated that the ^{14}C label from [^{14}C]cyclohexane was incorporated into the cellular protein of CY-6 if *M. vaccae*, strain CY-6, propane, and [^{14}C]cyclohexane were combined. This suggests that *M. vaccae* cooxidizes cyclohexane to a substrate (cyclohexanone) that is utilized by another strain: an example of commensalism. Similar results were reported separately by de Klerk and van der Linden (17) with two species of *Pseudomonas*. One of the species, while growing on *n*-hexane, cooxidized cyclohexane to cyclohexanol, which was utilized by the second pseudomonad as a source of carbon and energy. These studies with cyclohexane might well be a model for the role that cooxidation can play in the biodegradation of relatively recalcitrant hydrocarbon molecules in nature.

COOXIDATION—AROMATICS

Davis and Raymond (15) were the first to publish extensively on the cooxidation of alkyl-substituted cyclic hydrocarbons. The principal strain used was *Nocardia* strain 107-332, which can utilize gaseous and liquid alkanes, e.g., ethane, *n*-butane, *n*-decane, *n*-hexadecane, or *n*-octadecane, as growth substrates. Paraffinic hydrocarbons are utilized by this organism to completion, and extracellular products other than capsular slime do not accumulate in the growth medium in the absence of cyclic hydrocarbons. Several cooxidation reactions described for alkyl-substituted cyclics with *n*-hexadecane as cosubstrate are presented in Fig. 3.

The product from *n*-butylcyclohexane cooxidation (cyclohexylacetic) was obtained with another *Nocardia* (strain M.O.). Neither ethylbenzene, *n*-butylbenzene, *p*-isopropyltoluene, nor *n*-butylcyclohexane can serve as substrate for the

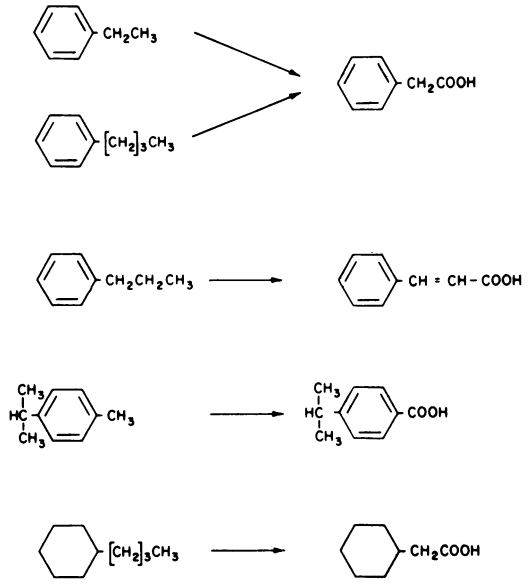

FIG. 3. *Cooxidative reactions by Nocardia strain 107-332 on alkyl-substituted cyclic hydrocarbons.*

two strains of *Nocardia* tested. *Nocardia* 107-332 can utilize *n*-nonylbenzene or *n*-dodecylbenzene as a carbon and energy source. When *n*-dodecylbenzene was utilized as substrate, the product of oxidation was phenylacetic acid, whereas oxidation of the odd-carbon-chain substrate, *n*-nonylbenzene, resulted in the dissimilation of the cyclic ring and also accumulation of low levels of phenylacrylic and phenylpropionic acids.

Gibson et al. (22) devised a novel cooxidative method for accumulating intermediates of the oxidative attack on halogenated aromatic compounds. A strain of *P. putida* that can grow with toluene as substrate was exposed to chlorinated derivatives of toluene, and cooxidative products were isolated. From *p*-chlorotoluene they isolated (+)-*cis*-4-chloro-2,3-dihydroxyl-1-methylcyclohexa-4,6-diene and 4-chloro-2,3-dihydroxy-1-methylbenzene.

Raymond et al. (56) investigated the cooxidation of several methyl-substituted mono- and dicyclic aromatic hydrocarbons by *Nocardia* cultures. These organisms were isolated from soil by enrichment with *n*-paraffins as the carbon sources. The organisms isolated were described as *Nocardia corallina* A-6, *N. corallina* A-11, *N. salmonicolor* A-100, *N. albicans* A-116, *N. minima* A-138, and *Nocardia* sp. V-33. All grew readily on *n*-hexadecane, and a compilation of their results with aromatic cosubstrates is shown in Fig. 4. The following were not cooxidized in the nocardial system: *m*-xylene, α-methylnaphthalene, 1,8-dimethylnaphthalene, 1,5-dimethylnaphthalene, and 1,4-dimethylnaphthalene. Only those substituted naphthalenes with the methyl group in the β position were oxidized, and nuclear magnetic resonance studies indicated that all oxidations occurred with the methyl group in this position.

Later, Raymond et al. (57) reported on a novel procedure for accumulating greater amounts of product during cooxidation. The addition of ion exchange resins to liquid media or agar plates resulted in a considerable increase in product. Ion exchange resins of two types were added to cooxidation systems: (i) a weakly basic polystyrene–polyamine type, IR 45, and (ii) a macroreticular type, IRRA-93. The strains used in this study were *N. corallina* A-6 and V-49 and *N. salmonicolor* A-100. Some representative data on the effect of IR-45 resin concentration on *p*-toluic acid and dihydroxy-*p*-toluic acid production in shake flask culture are presented in Table 4. The resin did not affect the mineral balance of the medium provided that the resin was properly conditioned before use. Raymond et al. state, "anion-exchange resins in hydrocarbon fermentations probably serves a number of functions which contribute to the increased yields. These functions are: (i) pH control, (ii) con-

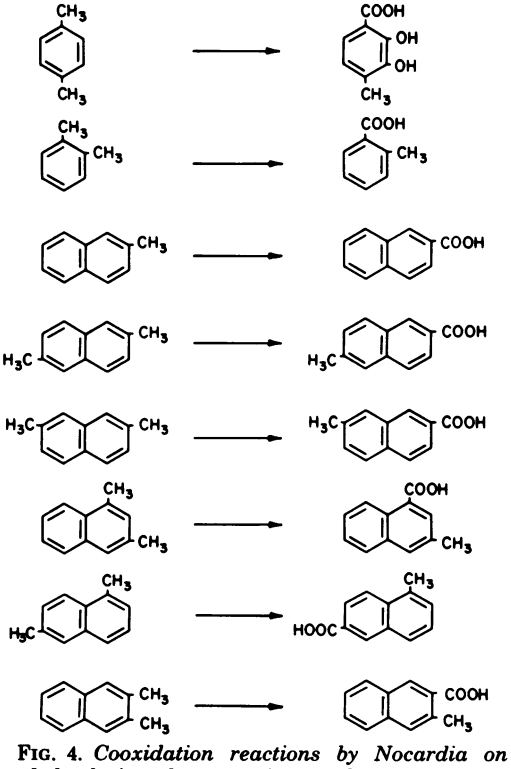

FIG. 4. *Cooxidation reactions by Nocardia on methyl-substituted aromatic cosubstrates during growth on n-hexadecane.*

TABLE 4. *The effect of IR-45 resin concentration on accumulation of dihydroxy-p-toluic acid (DHPT) in shake culture by N. corallina strain A-6 and N. salmonicolor strain A-100*

Resin concn (ml/liter)	Nocardial strain			
	A-6		A-100	
	Total acids (g/liter)	% DHPT	Total acids (g/liter)	% DHPT
0	0.1	0	0.1	0
150	4.5	18	3.0	20
300	5.5	22	5.3	28

trolled feeding of the hydrocarbon substrate, (iii) removal of product to prevent further oxidation, and (iv) removal of products which tend to repress the desired reaction" (57).

Studies by Jamison et al. (36) with *N. corallina* V-49, a strain isolated on *n*-hexadecane, suggest that this strain can cooxidize several aromatic compounds with *n*-hexadecane as utilizable substrate and that ring cleavage can occur under these conditions. The degradation of *p*-xylene proceeded as shown in Fig. 5. This strain of *N. corallina* cooxidized several cyclic aromatic hydrocarbons when *n*-hexadecane served as the growth substrate, as illustrated in Table 5.

The production of *α,α'-cis,cis*-dimethylmuconic acid and 2,3-dihydroxy-*p*-toluic acid from *p*-xylene by *N. corallina* V-49 and *N. salmonicolor* A-100 under stirred fermentor conditions has been reported (28). The oxidative pathways for the cooxidative fermentation of *m*-xylene by nocardial strains with *n*-paraffin as the growth substrate proceeded as shown in Fig. 6. The organisms were pregrown in the fermentor with benzene or *n*-alkane as substrate. After growth of the *Nocardia* to a suitable density, *p*-xylene was fed into the fermentor along with *n*-paraffins. It was essential that the addition of *p*-xylene be made at low levels under carefully controlled conditions, as *p*-xylene was toxic to the strains. The relative amounts of *n*-alkane and *p*-xylene were also critical. In a typical fermentation, about 16 g of *α,α'-cis,cis*-dimethylmuconic acid per liter was produced from *p*-xylene by *N. corallina*; *N. salmonicolor* yielded a mixed acid product consisting of 5.5 g of *p*-toluic acid and 2.5 g of dihydroxy-*p*-toluic acid per liter.

Jamison et al. (37) made an extensive survey of hydrocarbon cooxidation by *N. corallina* strain V-49. The fermentations were carried out on agar-resin plates (57) or in 40-liter stirred fermentors (28). When the basic growth substrates (*n*-hexadecane and Cerelose) were tested as a control, generally less than 2 mg of product

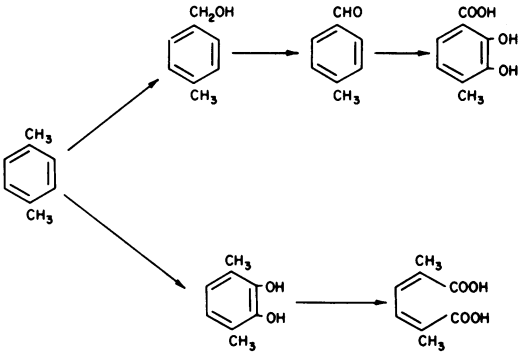

FIG. 5. *Ring cleavage of p-xylene by N. corallina V-49 under cooxidation conditions.*

TABLE 5. *Cooxidation of aromatic substrates by N. corallina V-49*

Cosubstrate	Major product	
	Name	Yield (g/liter)
Benzene	Muconic acid	0.1
Toluene	Methylmuconic acid	0.2
p-Chlorotoluene	*p*-Chlorotoluic acid	1.5
1,2,4-Trimethylbenzene	3,4-Dimethylbenzoic acid	
	2,3-Dihydroxy-4,6-dimethylbenzoic acid	0.7
	Trimethylmuconic acid	
m-Xylene	*m*-Toluic acid	0.7
2-Methylnaphthalene	2-Naphthoic acid	0.1
2,6-Dimethylnaphthalene	6-Methyl-2-naphthoic acid	2.7
2,7-Dimethylnaphthalene	7-Methyl-2-naphthoic acid	6.2

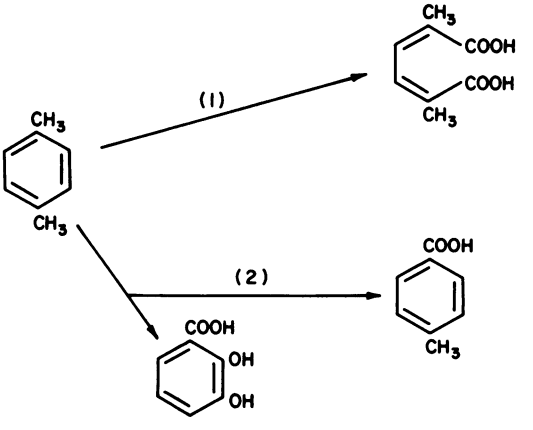

FIG. 6. *Cooxidation products from stirred fermentors. (1) N. corallina V-49; (2) N. salmonicolor A-100.*

(per plate) was recovered. The cosubstrates added were cycloparaffins, monocyclic aromat-

ics, dicyclic aromatics, and polycyclic aromatics. The seven cycloparaffinic hydrocarbons tested were cyclohexane, dicyclohexyl, methylcyclopentane, 1,3-dimethyladamantane, cyclooctane, cyclooctene, and Decalin. All except methylcyclopentane and Decalin yielded small amounts of product but none of the products were characterized. Several methyl- and ethyl-substituted aromatics were tested, and those that yielded small amounts of product (uncharacterized) were as follows: 1,3,5-trimethylbenzene; 1,2,4,5-tetramethylbenzene; 1,2,3,4,5-pentamethylbenzene; and 1,2,3,4,5,6-hexamethylbenzene. Ethylbenzene and *o*-diethylbenzene yielded significant amounts of product, but the structures were not determined. Those substituted aromatic cosubstrates that yielded identifiable amounts of product and the reaction sequences are presented in Fig. 7.

Several benzene derivatives with larger groups substituted on the ring were subjected to cooxidation with *n*-hexadecane as cosubstrate. The strain was *N. corallina* V-49. Those that gave significant yields of oxidized product (which was not characterized) were cumene, *p*-cymene, and phenylcyclohexane. However, biphenyl was cooxidized with ring fission as shown in Fig. 8.

The dicyclic aromatic hydrocarbons that yielded small amounts (none characterized) or no product were: naphthalene, indan, 1,4-dimethylnaphthalene, 1,8-dimethylnaphthalene, and 1,5-dimethylnaphthalene. Significant amounts of product were formed by cooxidation of: 1,3-dimethylnaphthalene, 1,6-dimethylnaphthalene, and 2,3-dimethylnaphthalene, but unfortunately none of the oxidation products were characterized. The dicyclics that did yield identifiable cooxidation products and the structures are shown in Fig. 9.

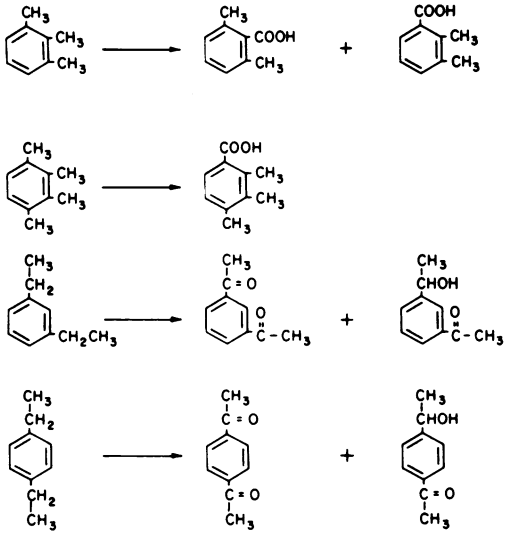

FIG. 7. *Cooxidation of aromatic hydrocarbons by N. corallina V-49.*

FIG. 8. *Ring fission in biphenyl by cooxidation. The strain was N. corallina V-49.*

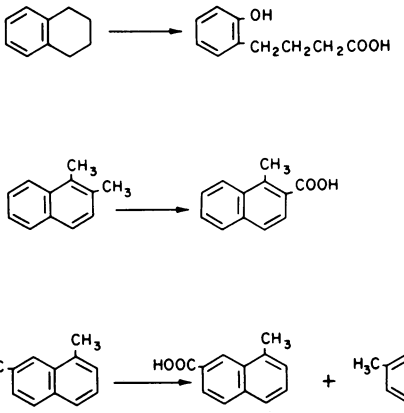

FIG. 9. *Cooxidation of dicyclic aromatic hydrocarbons by N. corallina V-49.*

Polycyclic aromatic hydrocarbons were also tested in the *N. corallina* cooxidation system. Anthracene, octahydroanthracene, and phenanthrene yielded small amounts of uncharacterized product. Significant amounts of oxidation product were evident from 2-methylanthracene, 9,10-dimethylanthracene, 3-methylphenanthrene, retene, and acenaphthylene, but none of the products were characterized.

In another study with *N. corallina* strain V-49 (58) pseudocumene was reported to be cooxidized and several products were recovered (Fig. 10). Along with these products were trace amounts of a product of ring fission believed to be trimethylmuconic acid. These workers also isolated a soil species that brought about ring fission of naphthalene cooxidatively when Cerelose or *n*-hexadecane was present as the growth substrate, as shown in Fig. 11.

Sagardía et al. (60) isolated a strain from oil-contaminated soil, *P. aeruginosa* PRG-1, that selectively degraded thianaphthene in a 5% oil-basal medium. Thianaphthene was also degraded when yeast extract was present as cosubstrate. The strain cannot grow with thianaphthene as a sole source of carbon and energy. These results suggest that strain PRG-1 while growing on yeast extract or oil can cooxidize thianaphthene, a process that might be used to rid oil of undesirable components.

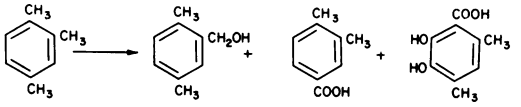

FIG. 10. *Cooxidation of pseudocumene by N. corallina V-49.*

FIG. 11. *Ring fission of naphthalene under cooxidative conditions. The species was an undescribed soil isolate.*

Cooxidation of pesticides by microorganisms during growth on hydrocarbon substrates has been reported. Analogs of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane were oxidized by a *Pseudomonas* sp. while growing on diphenylethane (21), and biodegradation of malathion in soil was augmented by the incorporation of *n*-heptadecane as cosubstrate (50). The potential for oxidation of *n*-alkanes in animal systems without alterations in chain length has also been reported (42, 49, 77).

The cooxidation of 2,6-dimethylnaphthalene was studied by Skryabin et al. (64). They found that this compound could be cooxidized to 2,6-naphthalenedicarboxylic acid by strains of *Pseudomonas* and *Nocardia*, with carbohydrate or hydrocarbon as the growth substrate. Skryabin et al. (65) isolated over 100 cultures of mycobacteria and tested them for the capacity to cooxidize aromatic hydrocarbons during growth on *n*-hexadecane or glucose and found that the major products were phenols and aromatic acids. They suggested that there are two oxidative types of mycobacteria: (i) those that cooxidize the aromatic nucleus and (ii) those that attack the alkyl substituents. The mycobacteria can be divided into six groups on the basis of their cooxidative products. Skryabin and Golovleva (63) also tested a wide array of bacteria, yeasts, and molds for their cooxidative capacities. They used *n*-alkanes, isoalkanes, glucose, corn steep liquor, yeast extract, amino acids, etc., as carbon and energy sources. Paraffinic, cycloparaffinic, aromatic, and heterocyclic compounds were tested as cosubstrates with these organisms, and they obtained as products the corresponding organic acids, phenols, alcohols, ethers, and ketones.

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