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Bacterial Oxidation of 2-Tridecanone to 1-Undecanol[1]

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ABSTRACT

A study of the microbial utilization of long-chain methyl ketones was undertaken. In general, enrichment culture experiments revealed that soil microorganisms capable of utilizing these compounds as growth substrates are ubiquitous. Gram-negative, rod-shaped bacteria were the prominent organisms exhibiting this capability. In particular, a strain of *Pseudomonas* isolated from soil degraded 2-tridecanone into several products that were recovered from cell-free culture fluid. These products were identified by gas-liquid chromatography as 2-tridecanol, 1-undecanol, 1-decanol, and undecanoic acid. A large amount of the substrate was converted to 1-undecanol. This compound was characterized further by classical methods of organic analysis. Unequivocal identification of 1-undecanol has established that some unique mechanism that involves subterminal oxidation must exist to degrade 2-tridecanone. No such mechanism has been reported for the biological degradation of long-chain, aliphatic, methyl ketones. A pathway for utilization of 2-tridecanone was proposed that is consistent with, but not confirmed by, the data presented.

A direct biological origin for long-chain, aliphatic, methyl ketones is firmly established. These compounds have been identified from such diverse natural sources as plants and essential oils (2, 26), milk and dairy products (3, 24), and insects (9); their formation is well known in mammals (7), fungi (5, 6, 10), and bacteria (20). Moreover, methyl ketones do not accumulate to any great extent in the biosphere, and, consequently, their efficient utilization by microorganisms is established indirectly [but *see* Morrison and Bick (23)]. Yet, little is known about the degradation of methyl ketones other than acetone, and virtually nothing is available regarding the mechanism of this degradation for any biological system.

A small amount of information on the catabolism of long-chain methyl ketones exists; it has come only from studies of microbial systems, and most of it is based on indirect evidence. Studies in which growth and respiration experiments and gross chemical analyses were employed have shown that this group of compounds can support growth (13, 20), is oxidized in the presence or absence of growth (18, 20), and is reduced to secondary alcohols (6).

In 1963, the first direct isolation and identification of an intermediate formed by bacterial oxidation of a methyl ketone was reported by Lukins and Foster (20). These authors isolated and characterized acetol as a product of acetone degradation by a mycobacterium; presumptive evidence was presented for 1-hydroxy-2-butanone from 2-butanone. On the basis of these data, and by analogy with other data concerning oxidation of similarly structured hydrocarbons, Lukins and Foster envisaged a general relationship for bacterial utilization of compounds possessing saturated chains and terminal methyl groups, namely, methyl-group oxidation. Specifically, primary attack on saturated compounds with terminal methyl groups would be oxidation of one or both of these groups. It was inferred that this mode of primary attack might be the major, if not exclusive, mechanism employed by bacteria to degrade such compounds.

Studies outlined in this paper deal mainly with the catabolism of a long-chain methyl ketone, 2-tridecanone, by a pseudomonad. This report focuses on the identification of degradative prod-

[1] This report is part of a thesis submitted by the senior author to the Graduate College of the University of Iowa in partial fulfillment of the requirements for the M.S. degree.

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ucts of this ketone that could not arise from terminal methyl-group oxidation. The data presented allow speculation that other degradative mechanisms in addition to methyl-group oxidation exist for methyl ketones. A preliminary report of these data has appeared (F. W. Forney and R. E. Kallio, Bacteriol. Proc., p. 86, 1966).

MATERIALS AND METHODS

Organisms and cultural. A number of organisms were isolated from various soils by use of 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 2-undecanone, and 2-tridecanone as selective substrates in the basal-salts medium of Dworkin and Foster (4). Enrichment cultures were incubated at room temperature (25 to 30 C) without agitation.

One organism, which was isolated on 2-tridecanone, was partially characterized by one of us (F. W. F.). All cultural and biochemical characteristics were determined at 30 C except growth temperature requirements, which were determined at 28, 30, 37, and 42 C on nutrient agar slants, on meat infusion-agar slants, and in nutrient broth. Results were recorded after several transfers at each of these temperatures. Flagella were stained by the method of Bailey as modified by Fisher and Conn (29). Pigment production was examined on the media of King, Ward, and Raney (15). Sensitivity to antibacterial agents was determined on nutrient agar plates by use of Sensitivity Discs (Difco); the vibriostatic agent, 0/129 (2,4-diamino-6,7-diisopropylpteridine), was tested by sprinkling it on inoculated nutrient agar plates (28). All biochemical tests were performed by standard methods (29), except for the following: the Hugh and Leifson (14) test, the Kovacs (17) oxidase test as modified by Klinge (16), the cytochrome oxidase test of Gaby and Hadley (8), and the arginine metabolism test of Thornley (32).

Media for growth experiments consisted of 100 ml of basal-salts medium in 250-ml, cotton-stoppered Erlenmeyer flasks amended with 0.3% of the desired substrate (w/v solid or v/v liquid), unless otherwise indicated. Yeast extract (Difco) and peptone (Difco) at a concentration of 1.0% were dissolved in 100 ml of distilled water and adjusted to *p*H 7 to prepare these media. KNO_3 or $KClO_3$ at 0.5% concentration was used to supplement the media when specified. Large-scale growth media consisted of 1.0 liter of basal-salts medium amended with 0.3% 2-tridecanone in 2.8-liter Fernbach flasks.

Growth cultures were incubated at 30 C on a rotary shaker, except as noted. Growth was judged by direct observation of turbidity of experimental cultures compared with that of appropriate control cultures. For experiments in which cells grown on a particular substrate were used as inocula, growth culture fluid was centrifuged and the cells were washed two times with sterile basal medium. Washed cells were suspended in the same diluent, and this suspension was used to inoculate fresh growth media. For anaerobic experiments, growth cultures were rendered anaerobic in Brewer jars filled with a hydrogen-carbon dioxide mixture. Such cultures were incubated without agitation at 30 C, as were duplicate aerobic control cultures.

Analytical. To obtain products from ketone degradation, large-scale growth culture fluid was freed from cells, acidified to *p*H 1, and extracted with ether in a continuous-extraction apparatus for a minimum of 72 hr. Various classes of organic compounds in concentrated extract were detected by thin-layer chromatography on plates spread with Adsorbosil-1 (Applied Science Laboratories Inc., State College, Pa.), developed with hexane-diethyl ether-acetic acid (80:20:1), sprayed with 0.2% ethanolic 2′,7′-dichlorofluorescein, and examined with ultraviolet light. These classes of compounds were separated by column chromatography on silica gel H (Merck & Co., Inc., Rahway, N.J.) with the same solvent system as eluent. Further purification of individual column fractions was accomplished by preparative thin-layer chromatography. A major aliphatic alcohol component of ether extracts was purified by fractional distillation under reduced pressure. All purified fractions were analyzed by gas-liquid chromatography on polar, free fatty acid (21) columns [6 ft by $\frac{1}{8}$ inch (182.9 by 0.3 cm) or 7 ft by $\frac{1}{4}$ inch (213.4 by 0.6 cm) outside diameter, copper], and on a nonpolar, silicone gum rubber column [5 ft by $\frac{1}{8}$ inch (152.4 by 0.3 cm), stainless-steel, containing 5.0% SE-30 on 60/80 Chromosorb W]. The 5- and 6-ft (152.4- and 182.9-cm) columns were used in an Aerograph model 600-D, and the 7-ft (213.4-cm) column, in an F&M model 700 chromatograph, both equipped for flame ionization detection. Helium (25 ml/min) was employed as carrier gas. Methyl esters of fatty acids were prepared by the method of Radin, Hajra, and Akahori (25). Infrared-absorption spectra were obtained with a model 137B Perkin-Elmer spectrophotometer. Samples were analyzed without solvent in NaCl sandwich cells. Refractive indices were determined with an Abbe-Spencer refractometer. Pseudosaccharin ether derivatives were synthesized by the method of Meadoe and Reid (22), and *meta*-nitrophenylcarbamate derivatives, by the method of Hoeke (12). Melting points were obtained with a Fisher block and are corrected values taken from a thermometer calibration curve. Qualitative organic analyses were done by general methods outlined by Cheronis and Entriken (1). Carbon-hydrogen analyses were performed by the Chemistry Department of the University of Iowa.

RESULTS AND DISCUSSION

Soil survey. Success of an enrichment survey designed to find methyl ketone-utilizing organisms in soils is exemplified by the fact that 33 positive enrichment cultures were obtained from 8 different soil samples. Each soil yielded organisms capable of growth on 2-nonanone, 2-decanone, 2-undecanone, and 2-tridecanone. One soil yielded a positive 2-octanone culture, but none contained organisms that grew on 2-heptanone under the conditions employed. Subsequently, 35 pure cultures of microorganisms were isolated. Without exception, these organisms were gram-negative, rod-shaped bacteria. This survey il-

lustrates the ease with which ketone-utilizing organisms can be isolated from soil and furnishes direct evidence that their occurrence in nature is ubiquitous. The observation that all of the organisms were gram-negative rods suggests that this group of bacteria plays an important role in biodegradation of naturally occurring methyl ketones. This idea is strengthened by an observation of Lukins (Ph.D. Thesis, Univ. of Texas, Austin, 1962) that organisms isolated by soil enrichment on methyl ketones were also gram-negative, rod-shaped bacteria. It should not be implied that these substrates are unavailable to other groups of organisms, since methyl ketones serve as growth substrates for *Aspergillus versicolor* (13), are oxidizable by corynebacteria (18), and support the growth of mycobacteria (20). The ability to degrade methyl ketones is probably a general property of many soil organisms.

Organism. The culture of the organism used for most of this work consisted of gram-negative rods, slightly to moderately curved, with rounded ends, occurring singly or in pairs, and with a tendency for bipolar staining. Individual cells were actively motile in hanging-drop preparations; these were observed to have a polar flagellum in properly stained preparations. Cell size typically measured 0.7 to 1.1 μ by 2.8 to 4.2 μ with a monotrichous flagellum measuring 11.2 to 12.6 μ in length.

On nutrient agar, colonies appeared dull, granular, and irregular; they had umbonate, brown centers with thin, spreading, translucent, undulate margins bearing radial lines. These margins exhibited bluish iridescence by transmitted light. Colonies reached 2 mm in diameter in 24 hr, resembled "fried eggs," and appeared flesh-colored by reflected light. No diffusible pigment was detected.

On Stone's gelatin agar, colonies appeared much the same as those on nutrient agar. No gelatin hydrolysis was observed in 72 hr, and no diffusible pigment was produced. Neither pigments nor fluorescence was detected on King's media (15).

Optimal-growth temperature was between 28 and 30 C. The organism grew at 37 C but not at 42 C. Sensitivity to 5 μg of tetracycline and 15 μg of erythromycin was observed, in contrast to insensitivity to 2 units of penicillin and to the vibriostatic agent. The organism utilized citrate but not urea in 72 hr. It produced an alkaline reaction in 72 hr, but no reduction or coagulation after 1 week in litmus milk. Indole and hydrogen sulfide were not produced in S I M Medium (Difco). The methyl red test was negative and acetylmethyl carbinol was not produced after growth in M R-V P Medium (Difco). Nitrate was reduced to nitrite in nitrate broth. The organism was catalase-positive, oxidase-positive, and cytochrome oxidase-positive. Acid was produced oxidatively from glucose in Hugh and Leifson medium (14). A neutral reaction was observed under anaerobic conditions in the arginine medium of Thornley (32).

On the basis of these observations, the organism was assigned to the genus *Pseudomonas* (*Bergey's Manual*) and was designated strain 4G-9. It appears to be a nonfluorescent member of this genus, although it is atypical in certain respects from any of the species described. A more extensive characterization of this organism, based on a recent study of *Pseudomonas* taxonomy (30), will be forthcoming in a subsequent publication.

Utilization of various carbon sources by pseudomonad 4G-9. Cells grown on 2-tridecanone were checked for their ability to grow on various compounds as sole carbon and energy sources. Results of aerobic growth on several different classes of organic compounds are shown in Table 1. In addition to the substrates shown, acetate, glucose, and 2-tridecanol supported vigorous growth. In general, it is evident that the longer-chain compounds support more rapid and abundant growth than the shorter-chain compounds. Lukins and Foster (20) reported that short-chain ketones supported better growth of their methyl ketone-producing strains of *Mycobacterium* than did long-chain ketones. These results are an exception to our findings, but the data are not strictly comparable; different organisms as well as substrates were involved. Our data do support a suggestion by these authors that an organism isolated for its ability to grow on methyl ketones likewise may attack hydrocarbons. This should not be interpreted as support for their contention that methyl-group oxidation is a common mechanism for degrading hydrocarbons and methyl ketones. Organisms may be found that degrade these substrates by different mechanisms.

Metabolism of pseudomonad 4G-9 in relation to oxygen. A limited number of pseudomonads are capable of respiration with nitrate under anaerobic conditions (31). Pseudomonad strain 4G-9 can reduce nitrate to nitrite under aerobic conditions. For these reasons, various attempts were made to obtain anaerobic growth of the organism in complex media, containing yeast extract or peptone, and in defined media, containing 2-tridecanone, 2-tridecanol, or 1-undecanol, that were supplemented with nitrate or chlorate. Aerobic control cultures were not inhibited by these supplements. Results of the anaerobic experiments were uniformly negative. Pseudomonad 4G-9 is thus incapable of denitrification; its only mode of attack on any substrate

TABLE 1. *Growth of Pseudomonas 4G-9 on various substrates*

Substrate	Incubation	Relative growth[a]
	days	
2-Heptanone	12	−
2-Octanone	12	−
2-Nonanone	12	++++
2-Decanone	12	++++
2-Undecanone	12	++++
n-Octane	12	−
n-Nonane	12	++
n-Decane	12	+
n-Undecane	12	++
n-Dodecane	12	+++
n-Tridecane	12	++++
Nonanoate[b]	5	++
Decanoate	5	+++
Undecanoate	5	++++
Dodecanoate	5	++++
1-Nonanol	5	−
1-Decanol	5	++++
1-Undecanol	5	++++
1-Dodecanol	5	++++
1,2-Dodecanediol[b]	21	−
1,2-Tetradecanediol	21	++++

[a] Symbols: −, no growth; +, slight growth; ++, moderate growth; +++, abundant growth; ++++, maximal growth.

[b] Substrates used at 0.1% concentration (w/v).

TABLE 2. *Properties of 1-undecanol from oxidation of 2-tridecanone*

Determination	Value
Boiling point	
Reported (6 mm)	123–125[a]
Observed (6 mm)	124–126
Refractive index, $[n]_D^{23}$	
Reported	1.4392[a]
Observed	1.4392
Pseudosaccharin derivative (mp)	
Reported	58.5[b]
Observed	58.25
m-Nitrophenylcarbamate derivative (mp)	
Reported	56[c]
Authentic (synthesized)	74
Observed	74
Carbon	
Calculated	76.61
Observed	76.86
Hydrogen	
Calculated	13.93
Observed	13.58
Oxygen	
Calculated	9.46[d]
Observed	9.56[d]

[a] Heilbron (11).

[b] Meadoe and Reid (22).

[c] Hoeke (12).

[d] By difference.

must therefore be aerobic. It remains to be established whether molecular oxygen is involved in the primary oxidation of 2-tridecanone or any other methyl ketone.

Purification and characterization of 1-undecanol from 2-tridecanone oxidation. Starting with 29.6 g of ketone substrate (12 liters of growth medium), growth was allowed to proceed until no ketone could be detected by thin-layer chromatography. Extracts of this culture fluid were distilled in vacuo with a recovery of 2.3 g of purified alcohol. This figure does not represent complete recovery since alcohol was lost during distillation.

Purification of gram amounts of the alcohol made possible its further characterization. Table 2 summarizes some of the physical and chemical properties of this substance that identify it as 1-undecanol. Mixed melting points of known and experimental derivatives were unchanged. The obvious discrepancy between observed and reported melting points of *n*-undecyl *m*-nitrophenylcarbamate derivatives remains unexplained. Derivatives of authentic 1-undecanol from two different sources were prepared, and these, or mixtures of them with experimental derivative, gave the same observed melting point. Melting points of carbamate derivatives of C_{10} and C_{12} alcohols corresponded with values given in the literature.

In addition to the data shown in Table 2, results of qualitative organic analyses were positive for the hydroxyl function and for primary alcohol. Results in Fig. 1 show an infrared spectral analysis of experimentally derived 1-undecanol compared with analyses of the substrate and of authentic 1-undecanol. Spectra of the alcohols compare satisfactorily with each other. Also, each of these spectra corresponds accurately with published Sadtler standard spectra (27).

Identification of products recovered after 2-tridecanone oxidation. Preparative thin-layer chromatographic fractions were analyzed by gas-liquid chromatography. Identification of individual compounds in each fraction was based both on a comparison of retention time with that of authentic compound, and on an increase in relevant peak height and identity of retention time when standard compound was added and the mixture was rechromatographed. Analyses of of these fractions are presented in Table 3. Also included is the analysis of 1-undecanol obtained by distillation (labeled distillate 3); no other compounds were detected in this fraction. In each case, correspondence of retention time and a peak height increase occurred upon addition of appropriate known compounds one at a time to these

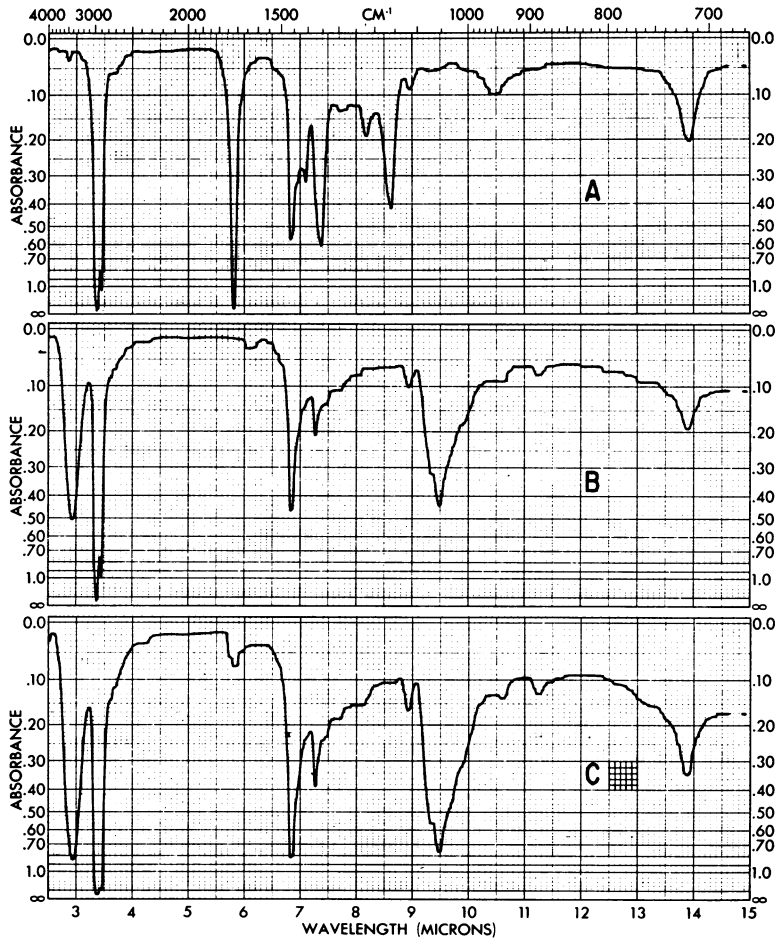

FIG. 1. *Infrared-absorption spectra of 2-tridecanone (A), authentic 1-undecanol (B), and experimentally derived 1-undecanol (C).*

fractions. Additional compounds, besides 1-undecanol, identified in this manner were 2-tridecanol, 1-decanol, and undecanoic acid.

Identification of products recovered after 2-tridecanol oxidation. Occurrence of 2-tridecanol with 1-undecanol, as seen in fraction 3 of Table 3, suggested some relationship between them: the former might be a precursor of the latter. This possibility was checked by analyzing cell-free culture fluid from 2-tridecanol-grown cells. Gas-liquid chromatographic results are shown in Table 4. Both 2-tridecanol and 1-undecanol were present. Cochromatography with these alcohols added individually confirmed the identifications and verified the fact that 1-undecanol can be produced from 2-tridecanol by the organism.

Proposed metabolism of 2-tridecanone. Identification of 1-undecanol produced during the metabolism of 2-tridecanone represents the first report of direct isolation and characterization of an intermediate formed during the metabolism of any methyl ketone other than acetone by bacteria. At present, no hypothesis concerning the metabolism of long-chain methyl ketones has been offered that is based on direct experimental data. We propose the pathway shown in Fig. 2 for utilization of 2-tridecanone. Subterminal oxidation of the substrate occurs to give a primary alcohol, 1-undecanol, as one product and some 2-carbon fragment as another product. Subsequent utilization of 1-undecanol by its oxidation to undecanoic acid is inferred from evidence that both the alcohol and the acid can support growth of the organism, and that undecanoic acid was isolated and identified from cultures grown on 2-tridecanone. More conclusive evidence for undecanoic acid as a direct intermediate in the pathway should be obtained by identifying this

TABLE 3. *Gas-liquid chromatographic identification of products recovered from culture fluid after oxidation of 2-tridecanone*[a]

Authentic compounds			Purified fractions		
Compound	Column		Fraction	Column	
	FFA[b]	SGR[c]		FFA[b]	SGR[c]
1. Undecanoic acid	4.2	6.5[d]	1	4.2	6.5[d]
2. 1-Undecanol	7.2	8.9	2 (a)	7.2	8.9
3. 2-Tridecanol	9.3	17.0	2 (b)	9.1	17.0
4. 1-Undecanol	11.6	8.9	3 (a)	11.4	8.9
5. 1-Decanol	7.0	5.4	3 (b)	6.9	5.4
6. 1-Undecanol	7.0	8.9	Distillate 3	7.0	8.9

[a] Results expressed as retention time, in minutes.
[b] FFA = free fatty acid. Conditions: Line 1, 7 ft, 180 C; lines 2 and 3, 7 ft, 120 C; lines 4 and 5, 7 ft, 110 C; line 6, 6 ft, 110 C.
[c] SGR = silicone gum rubber. Conditions: line 1, 145 C; lines 2–6, 140 C.
[d] Analyzed as methyl ester.

TABLE 4. *Gas-liquid chromatographic identification of alcohols recovered from culture fluid after oxidation of 2-tridecanol*[a]

Authentic compounds			Components in fraction		
Compound	Column		Component	Column	
	FFA[b]	SGR[c]		FFA[b]	SGR[c]
1-Undecanol	7.0	8.9	1	7.0	8.9
2-Tridecanol	9.8	17.0	2	9.6	17.0

[a] Results expressed as retention time, in minutes.
[b] Same as Table 3. Conditions: 6 ft, 110 C.
[c] Same as Table 3. Conditions: 140 C.

acid as an oxidation product of the organism grown on 1-undecanol. Preliminary data obtained by thin-layer chromatography indicate fatty acid(s) is present in such cultures, but no identification has been made. Utilization of undecanoate probably would occur by β-oxidation, since this classical pathway for fatty acid utilization operates in pseudomonads. The 2-carbon product of subterminal oxidation was not identified. It may be significant that acetate can support growth of the organism. Possibly, a 2-carbon compound could provide energy to drive the primary oxidation reaction.

The pathway that we have proposed for bacterial utilization of 2-tridecanone may turn out to be a general one. Subterminal oxidative cleavage of a ketone molecule may embody a more generalized principle governing the degradation of longer methyl ketones than the monoterminal oxidation principle (20), which may govern the degradation of a unique ketone, acetone. Symmetry of the acetone molecule offers little choice for oxidation. Longer ketones, on the other hand, in which it can be imagined that an aliphatic side chain has replaced a hydrogen atom of an acetone methyl group, present a structure to oxidative enzymes that contains multiple points where oxidation could occur. Some hint that other cleavage points are utilized is suggested by the presence of trace amounts of 1-decanol in 2-tridecanone growth cultures.

Mechanisms for methyl ketone oxidation. A reaction mechanism mediating the primary attack on methyl ketones by bacteria has yet to be elucidated, although several have been proposed. In 1941, Goepfert (10) offered evidence for a C_1-C_2 split of acetone, presumably after initial oxidation. Even more convincing evidence for the direct oxidation of acetone was secured later by Levine and Krampitz (19), who concluded that acetone was oxidized to acetol, then to acetaldehyde and a C_1 product, by a soil diphtheroid. More recently, Lukins and Foster (20) advanced their methyl-group oxidation mechanism. All of these mechanisms deal with acetone oxidation; none is directly concerned with the oxidation of long-chain methyl ketones.

We have obtained no experimental evidence that would allow us to propose a reliable mechanism by which 2-tridecanone is attacked and split, but we have raised the possibility that some reaction involving subterminal oxidation is occurring. Further work with our system is required to establish a biochemical basis for occurrence of the intermediates that have been identified. An extension of similar studies to other systems also is necessary to elucidate more fully how microorganisms catabolize unsymmetrical methyl ketones.

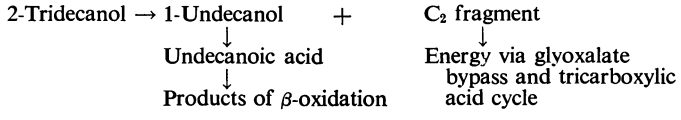

FIG. 2. *Proposed pathway for utilization of 2-tridecanone.*

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