JOURNAL OF BACTERIOLOGY, May 1974, p. 394-399
Vol. 118, No. 2

Printed in U.S.A.

Microbial Degradation and Assimilation of *n*-Alkyl-Substituted Cycloparaffins[1]

H. W. BEAM AND J. J. PERRY

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27607

Received for publication 26 December 1973

Studies were conducted on the oxidation and assimilation of *n*-alkyl-substituted cycloalkane substrates by several hydrocarbon-utilizing microorganisms. These microorganisms utilized heptadecylcyclohexane and dodecylcyclohexane as the sole source of carbon and energy. Neither methylcyclohexane nor ethylcyclohexane was utilized as a growth substrate by any organisms tested. Gas-liquid chromatographic analyses of fatty acids present in cells after growth on dodecylcyclohexane confirm direct incorporation of both alpha- and beta-oxidation products. Growth patterns of these organisms on *n*-alkyl-substituted cyclohexane fatty acids of varying chain lengths suggest a greater probability of ring cleavage when the side chain contains an odd number of carbons.

The literature offers few reports (11, 14, 24) of microorganisms that can utilize cyclohexane as the sole source of carbon and energy. Although microbial oxidation of alkyl-substituted cycloalkanes has been observed, the commercially more important alkylbenzenes have received most of the attention of workers in this field (2, 6, 7, 19, 29).

Johnson et al. (13) noted the resistance of short-chain *n*-alkyl-substituted cycloalkanes (e.g., methyl- and ethylcyclohexane) to microbial oxidation. Recently, however, two reports (1, 25) suggest hydroxylation of ethylcyclohexane by *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. Longer *n*-alkyl substituents of cyclic compounds appear to be degraded by the classical beta-oxidation sequence (29).

This study was undertaken to gain more information on microbial degradation and concomitant assimilation of *n*-alkyl-substituted cycloalkanes.

MATERIALS AND METHODS

Microorganisms. The organisms used in this study were: *Mycobacterium vaccae* strain JOB5 (26); *M. rhodochrous* strains OFS (8) and 7E1C (9); *Nocardia asteroides* strain A-116; and *M. convolutum* strain R-22 (3). Stock cultures were maintained on mineral salts medium (15) with propane (50:50 [vol/vol] with air) as substrate.

Substrates. All hydrocarbons and other chemicals used were obtained from commercial sources and had a minimum purity of 99%. The sodium salts of acidic substrates and the hydrocarbons were sterilized by filtration.

[1] Paper no. 4153 of the Journal Series of the North Carolina State University Agricultural Experiment Station.

Substrate specificity. Substrates were tested at a concentration of 0.2% (vol/vol or wt/vol) in 125-ml Erlenmeyer flasks containing 30 ml of L-salts medium. Cultures were maintained in shake culture at 26 C for 7 days before growth was assessed.

Analysis of metabolites. Cultures of *M. convolutum* strain R-22, *M. vaccae* strain JOB5, and *M. rhodochrous* strain OFS were established in 2-liter Erlenmeyer flasks containing 500 ml of L-salts medium with 0.2% of either dodecylcyclohexane or heptadecylcyclohexane as the sole carbon and energy source. After 6 days of growth in shake culture at 26 C, the cultures were centrifuged and the resulting supernatants were rendered free of cells by passage through a membrane filter (0.45 μm; Millipore Corp.). Retrieval of accumulated fatty acids was accomplished by extraction of the aqueous supernatant twice with equal volumes of diethyl ether. Methyl esters of the fatty acids were prepared with BF_3 in methanol (8) and subjected to gas-liquid chromatographic analysis. The column used was copper tubing (6.35 by 1.83 mm) packed with 15% diethyleneglycol-succinate on 60/70 mesh Gas Chrom P (Applied Science Laboratories, State College, Pa). Column temperature was maintained at 140 C. The chromatographic system was an F & M model 810 equipped with a hydrogen flame ionization detector. Relative retention times of unknown peaks were compared to a standard curve prepared with known ω-cyclohexyl-substituted fatty acid methyl esters. Quantitation of methyl esters was by the method of Horning et al. (10).

Cellular fatty acid analysis. Fatty acid composition of strains JOB5, OFS, and R-22 was examined after growth on dodecylcyclohexane. Fatty acid methyl esters were prepared as previously described (8). Residual hydrocarbon substrate was removed from the esters by thin-layer chromatography (TLC). The esterified material was spotted on Silica Gel G TLC plates with a gel thickness of 0.5 mm. A solvent

system of hexane-diethyl ether-acetic acid (90:10:1) was employed. After drying, the ester band was scraped from the plate, eluted with benzene, and subjected to gas-liquid chromatographic analysis.

Phospholipid analysis. Strain R-22 cells (0.75 g) grown on dodecylcyclohexane were subjected to total lipid extraction with $CHCl_3$-CH_3OH (2:1 vol/vol). The lipid extraction procedure was essentially that used by Makula and Finnerty (18). The extract was concentrated under vacuum to a volume of 200 μliters and spotted on a Silica Gel G TLC plate with a gel thickness of 0.5 mm. The plate was placed in a system of $CHCl_3$-CH_3OH-7 N NH_4OH (65:35:5), and the solvent was allowed to proceed halfway up the plate. This separated the phospholipid fraction from the neutral lipids. After drying under N_2 for 30 min, the plates were placed in a system of hexane-diethyl ether-acetic acid (90:10:1), and solvent was allowed to run the entire length of the plate. This procedure rid the phospholipid fraction of residual hydrocarbon substrate and free fatty acids (22). After this one-dimension, two-phase chromatographic procedure was completed, the phospholipid fraction was eluted from the silica gel with benzene and transesterified with BF_3 in methanol. The reliability of this separation technique was checked by subjecting the lipids to column chromatography (17). The fatty acid esters were identified by gas-liquid chromatography (GLC). The relative amount of each fatty acid in the lipid was determined as previously described (8, 9, 27).

Several unknown peaks were encountered during analysis of both the cellular fatty acids and the phospholipid fraction. A James plot (12) was constructed to ascertain whether these new peaks might constitute a family of compounds. Confirmation of molecular structure was accomplished with a Varian model 1200 gas chromatograph-Finnigan model 1015 electron impact and chemical ionization mass spectrophotometer system.

Enrichment methods. Enrichment for organisms capable of growth on various substrates was performed as follows. (i) Soil was placed in a crystallization dish (12.5 by 6.5 cm) to a depth of 1.5 to 2 cm, and the substrates were mixed into the soil at concentrations from 0.1 to 2.0% (wt/vol). The soil was supplemented with L-salts medium, and tap water was added periodically to prevent drying. The dishes were incubated at 27 C for as long as 2 months. (ii) Approximately 100 mg of soil was placed in a 50-ml flask and placed either on a shaker for 7 to 14 days or incubated in stationary culture for 15 to 30 days. (iii) Dilutions of soil were spread on the surface of L-salts agar and incubated in desiccators containing substrate in the vapor phase, or the substrate was added directly to a 1.5-cm well cut into the center of the agar surface. This provided a gradient of substrate across the agar surface.

RESULTS

The ability of several organisms to utilize substituted cycloparaffins is depicted in Table 1. All organisms tested grew on dodecyl- and heptadecylcyclohexane as the sole source of carbon and energy. However, neither methyl- nor ethylcyclohexane supported the growth on any organism tested. Varying inoculum size and substrate concentration was not effective in promoting the growth of any organism tested. Attempts to isolate organisms from over 200 soil samples by enrichment culture that utilized cycloparaffins with short n-alkyl substituents were unsuccessful. Conversely, organisms capable of utilizing dodecyl- or heptadecylcyclohexane could be routinely found in soils from many geographical locations.

All hydrocarbon-utilizing microorganisms tested grew at the expense of the sodium salt of cyclohexyl n-alkyl fatty acids containing one or three carbons in the side chain. Acids containing an even number of carbons in the side group (e.g., two or four) were not utilized. Numerous organisms were isolated from soils enriched with the sodium salt of either cyclohexylmethanoic or 1-cyclohexylpropionic acid. Less success was realized, however, when cyclohexylacetic (Na) or 1-cyclohexylbutyric acid (Na) were employed as enrichment substrate.

The predominant fatty acids found in the supernatant fraction of cultures of strain R-22, JOB5, or OFS after growth on dodecylcyclohexane contained an even number of carbons in the side chain. Cyclohexylacetic acid comprised 79% of the profile for strain R-22 (Table 2). Total fatty acids with odd-carbon-number side chains (one or three carbons) amounted to 20.6% of the total acids present. After growth of R-22 on heptadecylcyclohexane, the predominant fatty acid found in the supernatant fraction was 1-cyclohexypropionic acid (Table 2). Cyclohexyl acids with even-carbon-number side chains were 35% of the total profile.

The relative amount of cyclohexylacetic acid produced during dodecyl- and heptadecylcyclohexane degradation by strain R-22 was determined by quantitative extraction of the supernatants with diethyl ether. The 12-carbon n-alkyl-substituted cycloparaffin yielded 7.4 mg of cyclohexylacetic acid, and 21 μg was obtained from the 17-carbon n-alkyl-substituted heptadecylcyclohexane. Equivalent total cell yield was attained on these substrates.

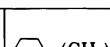
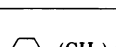
A James plot (12) revealed that several unknown peaks encountered during GLC analysis of fatty acid composition of organisms after growth on n-alkyl-substituted cyclohexanes constituted a family of compounds (Fig. 1). GLC mass spectral analysis provided confirmation of the molecular structure of these compounds. The mass spectra of components proposed to be the esters of cyclohexyldodecanoic and cyclohexyldecanoic acid (Fig. 2) confirm the presence of the methyl ester function (m/e = 74; 73 + 1 for McLafferty rearrangement).

TABLE 1. *Ability of microorganisms isolated on n-alkanes to utilize n-alkyl-substituted cycloparaffins and related substrates*[a]

Substrate	Organism				
	M. convolutum R-22	*M. vaccae* JOB5	*M. rhodochrous* 7E1C	*M. rhodochrous* OFS	*N. asteroides* A-116
Methylcyclohexane	−	−	−	−	−
Ethylcyclohexane	−	−	−	−	−
Dodecylcyclohexane	+	+	+	+	+
Heptadecylcyclohexane	+	+	+	+	+
1-Cyclohexylmethanoic acid	+	+	+	+	+
Cyclohexane acetic acid	−	−	−	−	−
1-Cyclohexylpropionic acid	+	+	+	+	+
1-Cyclohexylbutyric acid	−	−	−	−	−
Tetradecane	+	+	+	+	+

[a] Culture conditions: 30 ml of L-salts medium with 0.2% substrate in 125-ml Erlenmeyer flask; incubated at 26 C for 7 days on a rotary shaker. Symbols: −, no growth; +, growth in excess of 0.5 mg/ml.

TABLE 2. *ω-Cyclohexyl-substituted fatty acids released into the medium by strain R-22 during growth on n-alkyl-substituted cyclohexane substrates*

Substrate	Supernatant fatty acid			
				
Dodecylcyclohexane	TR[a]	79.0[b]	19.1	1.5
Heptadecylcyclohexane	ND[c]	15.1	65.2	19.7

[a] TR, Trace (less than 1%).

[b] Recorded as percentage of the total cyclohexyl-substituted fatty acids isolated from supernatant. The organism was grown on a rotary shaker at 26 C for 6 days. The substrates were added at 0.2% (wt/vol or vol/vol).

[c] ND, None detected.

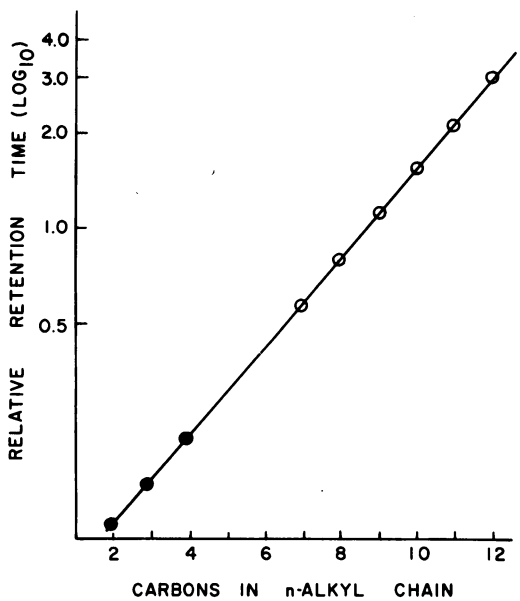


FIG. 1. *James plot of methyl esters of ω-cyclohexyl-substituted fatty acids. Retention times are relative to methyl ester of hexadecanoic acid. Symbols: ●, authentic standard; ○, compounds derived from hydrocarbon-utilizing microorganisms after growth on dodecylcyclohexane.*

The cyclohexyl function (m/e = 83) is present in both spectra along with sequential fragments of 97, 111, etc., representative of predictable chain fragments. The parent molecular ion (PMI + 1) at 297 for cyclohexyldodecanoate is confirmed by ion peaks at 325 (PMI + 29) and at 263 (PMI − 33). The parent molecular ion at 269 (PMI + 1) for cyclohexyldecanoate is also confirmed by ion peaks at 297 (PMI + 29) and at 235 (PMI − 33).

The ω-cyclohexyl-substituted fatty acids comprised an appreciable portion of the total cellular fatty acids of several hydrocarbon-utilizing organisms after growth on dodecylcyclohexane. Cyclohexyl acids containing carbon side chains of 7 to 12 carbons were incorporated (Table 3) into the microorganism. The predominant acids were 1-cyclohexyldodecanoic acid and the corresponding acid with a side chain two carbons shorter in length, 1-cyclohexyldecanoic acid.

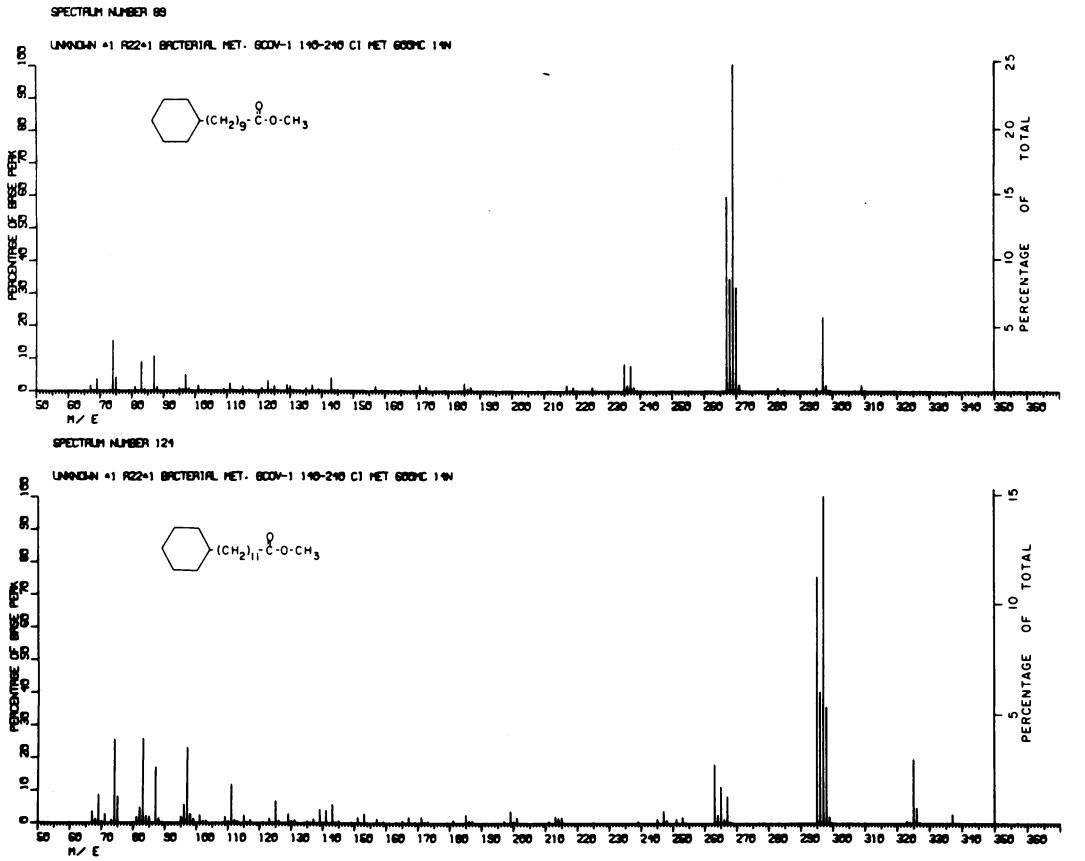


FIG. 2. *Mass spectra of the methyl ester of cyclohexyldecanoic acid and cyclohexyldodecanoic acid recovered from M. convolutum strain R-22 after growth on dodecylcyclohexane.*

Analysis of the phospholipid fraction of strain R-22 after growth on dodecylcyclohexane revealed that ω-cyclohexyl-substituted fatty acids comprised a major portion (50%) of the total fatty acid profile (Table 4) in this cellular fraction. Hexadecanoic acid and 1-cyclohexyldodecanoic acid were present in approximately the same percentage, ~25%.

DISCUSSION

The resistance of cyclohexylacetic acid to microbial attack has been reported (6). The quantity that accumulated during growth of strain R-22 at the expense of dodecylcyclohexane (Table 2) suggests incomplete metabolism of this *n*-alkyl-substituted cycloparaffin. Conversely, data regarding the biodegradability and accumulation of cyclohexylmethanoic and 1-cyclohexylpropionic acids (6; Table 1 and 2) suggest a higher degree of mineralization of heptadecylcyclohexane by hydrocarbon-utilizing microorganisms.

Identification of an intermediate of beta-oxidation of the *n*-alkyl side chain of dodecylcyclohexane (Table 3) confirms previous work (6, 28, 29) concerning beta-oxidation of *n*-alkyl-substituted cyclic hydrocarbons. Although initial monoterminal oxidation and subsequent beta-oxidation of the *n*-alkyl substituent predominate, the data also suggest participation of alpha-oxidation in the degradation process (Table 2-4). The yeasts *Candida tropicalis* and *C. lipolytica* have been reported to attack *n*-alkanes by both alpha- and beta-oxidation (20).

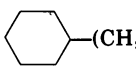
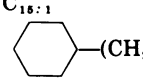
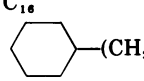
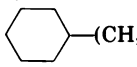
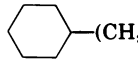
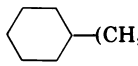
The majority of the cellular fatty acids of strains R-22, OFS, and JOB5 grown at the expense of dodecylcyclohexane were directly derived from the *n*-alkyl-substituted cyclohexane substrate rather than synthesized via the classic C_2-condensation route. The predominate fatty acid (Table 3) contained the same number of carbons as the substrate dodecylcyclohexane. The major fatty acid of *M. vaccae* strain JOB5

(8), *Micrococcus cerificans* (16), a *Nocardia* (4), and several genera of yeasts (21) grown on *n*-alkanes is also of the same chain length as the substrate. Dodecylcyclohexane apparently reacts physicochemically much as do the *n*-alkanes C_{13}-C_{17} (9) and can be directly incorporated into cellular lipids after monoterminal oxidation.

The results confirm that 1-cyclohexyldodecanoic acid was incorporated into the phospholipids of strain R-22 (Table 4). The 1-cyclohexyldodecanoic acid can serve as an important structural component of cellular membrane since it made up about 25% of the total phospholipid fatty acid. Manipulation of membrane structure function by varying growth substrate of microorganisms has previously been reported (17, 23, 27).

Microbial incorporation of compounds having undergone no appreciable structural alteration raises a point of ecological significance. Since *n*-alkyl-substituted cycloparaffins are constituents of certain crude oils (5), they must be regarded as environmentally important molecules. These compounds, although attacked by microorganisms, may be as significant, ecologically, as are compounds resistant to microbial degradation. The incorporation of these *n*-alkyl-substituted cycloparaffins into lipoidal material of microorganisms demonstrates the entrance of an environmental pollutant into the lowest level of many potential food chains.

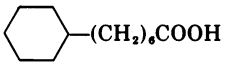
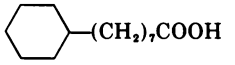
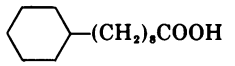
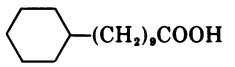
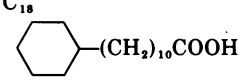
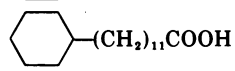
TABLE 3. *Fatty acid composition of hydrocarbon-utilizing microorganisms after growth on dodecylcyclohexane*

Fatty acid	Organism		
	M. vaccae JOB5	*M. rhodochrous* OFS	Strain R-22
	TR[a]	TR	TR
$C_{15:1}$	TR	1.03	TR
	1.23[b]	TR	1.33
$C_{16:1}$	TR	1.66	TR
C_{16}	2.07	4.75	1.99
	TR	TR	TR
$C_{17:1}$	TR	TR	TR
	11.35	10.06	13.33
$C_{18:1}$	2.62	6.16	2.57
	1.00	4.93	TR
	77.07	69.02	77.51

[a] TR, Trace (less than 1%).

[b] Recorded as percent of the total fatty acids present. The cells were grown for 6 days at 26 C on a rotary shaker. The substrate was added at 0.2% (vol/vol).

TABLE 4. *Fatty acid composition of phospholipid fraction of strain R-22 after growth on dodecylcyclohexane*[a]

Fatty acid	Total fatty acids present (%)
	6.10
$C_{15:1}$	2.04
	10.06
$C_{16:1}$	4.33
C_{16}	22.71
	3.55
	2.69
$C_{18:1}$	9.03
C_{18}	9.06
	3.35
	26.42

[a] Cells were grown for 6 days at 26 C on a rotary shaker. Substrate was added at 0.2% (vol/vol) in 500 ml of L-salts medium.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant GB-23815 from the National Science Foundation and a grant from the North Carolina Board of Science and Technology.

We are grateful to E. O. Oswald at the National Institute of Environmental Health Sciences, Research Triangle Park, N.C., for determining and interpreting the mass spectra.

LITERATURE CITED

1. Arai, Y., and K. Yamada. 1969. Studies on the utilization of hydrocarbons by microorganisms. XII. Screening of alicyclic hydrocarbon-assimilating microorganisms and *trans*-4-ethylcyclohexanol formation from ethylcyclohexane. Agr. Biol. Chem. **33**:63-68.
2. Baggi, G., D. Catelani, E. Galli, and B. Treccani. 1972. The microbial degradation of phenylalkanes. Biochem. J. **126**:1091-1097.

3. Blevins, W. T., and J. J. Perry. 1972. Metabolism of propane, n-propylamine, and propionate by hydrocarbon-utilizing bacteria. J. Bacteriol. **112**:513-518.
4. Davis, J. B. 1964. Microbial incorporation of fatty acids derived from n-alkanes into glycerides and waxes. Appl. Microbiol. **12**:210-214.
5. Davis, J. B. 1967. Petroleum microbiology. Elsevier Publishing Co., New York.
6. Davis, J. B., and R. L. Raymond. 1961. Oxidation of alkyl-substituted cyclic hydrocarbons by a nocardia during growth on n-alkanes. Appl. Microbiol. **9**:383-388.
7. Douros, J. D., Jr., and J. W. Frankenfeld. 1968. Oxidation of alkylbenzenes by a strain of *Micrococcus cerificans* growing on n-paraffins. Appl. Microbiol. **16**:532-533.
8. Dunlap, K. R., and J. J. Perry. 1967. Effect of substrate on the fatty acid composition of hydrocarbon-utilizing microorganisms. J. Bacteriol. **94**:1919-1923.
9. Dunlap, K. R., and J. J. Perry. 1968. Effect of substrate on the fatty acid composition of hydrocarbon- and ketone-utilizing microorganisms. J. Bacteriol. **96**:318-321.
10. Horning, E. C., E. H. Ahrens, Jr., S. R. Lipsky, F. H. Mattson, J. F. Mead, D. A. Turner, and W. H. Goldwater. 1964. Quantitative analysis of fatty acids by gas-liquid chromatography. J. Lipid Res. **5**:20-27.
11. Imelik, B. 1948. Oxydation de cyclohexane par *Pseudomonas aeruginosa*. C.R. Soc. Biol. **226**:2082-2083.
12. James, A. T. 1960. Qualitative and quantitative determination of the fatty acids by gas-liquid chromatography. Methods Biochem. Anal. **8**:1-59.
13. Johnson, F. H., W. E. Goodale, and J. Turkevich. 1942. The bacterial oxidation of hydrocarbons. J. Cell. Comp. Physiol. **19**:163-172.
14. Jones, J. G., and M. A. Edington. 1968. An ecological survey of hydrocarbon oxidizing microorganisms. J. Gen. Microbiol. **52**:381-390.
15. Leadbetter, E. R., and J. W. Foster. 1958. Studies on some methane-utilizing bacteria. Arch. Mikrobiol. **30**:91-118.
16. Makula, R., and W. R. Finnerty. 1968. Microbial assimilation of hydrocarbons. I. Fatty acids derived from normal alkanes. J. Bacteriol. **95**:2102-2107.
17. Makula, R. A., and W. R. Finnerty. 1970. Microbial assimilation of hydrocarbons: identification of phospholipids. J. Bacteriol. **103**:348-355.
18. Makula, R. A., and W. R. Finnerty. 1972. Microbial assimilation of hydrocarbons. Cellular distribution of fatty acids. J. Bacteriol. **112**:398-407.
19. McKenna, E. J., and R. E. Kallio. 1964. Hydrocarbon structure: its effects on bacterial utilization of alkanes, p. 1-14. *In* H. Heukelikian and N. C. Dondero (ed.), Principles and applications of aquatic microbiology. John Wiley & Sons, Inc., New York.
20. Mishima, M., S. Yanagawa, A. Tanaka, and S. Fukui. 1973. Effects of chain-length of alkane substrate on fatty acid composition and biosynthetic pathway in some *Candida* yeasts. Agr. Biol. Chem. **37**:863-870.
21. Pelechova, J., V. Krumphanzl, J. Uher, and J. Dyr. 1971. Assimilation of hydrocarbons. I. Proportion of fatty acids in the cell fat. Folia Microbiol. **16**:103-109.
22. Randerath, K. 1966. Thin-layer chromatography, p. 177, 2nd ed. Academic Press Inc., New York.
23. Raymond, R. L., and J. B. Davis, 1960. n-Alkane utilization and lipid formation by a nocardia. Appl. Microbiol. **8**:329-334.
24. Skarzynski, B., and J. W. Czekalowski. 1946. Utilization of phenols and related compounds by *Achromobacter*. Nature (London) **158**:304-305.
25. van Ravenswaay Claasen, J. C., and A. C. van der Linden. 1971. Substrate specificity of the paraffin hydroxylase of *Pseudomonas aeruginosa*. Antonie von Leeuwenhoek J. Microbiol. Serol. **37**:339-352.
26. Vestal, J. R., and J. J. Perry. 1969. Divergent metabolic pathways for propane and propionate utilization by a soil isolate. J. Bacteriol. **99**:216-221.
27. Vestal, J. R., and J. J. Perry. 1971. Effect of substrate on the lipids of the hydrocarbon-utilizing *Mycobacterium vaccae*. Can. J. Microbiol. **17**:445-449.
28. Webley, D. M., R. B. Duff, and V. C. Farmer. 1956. Evidence for β-oxidation in the metabolism of saturated hydrocarbons by soil nocardias. Nature (London) **178**:1467-1468.
29. Willetts, A. J., and R. B. Cain. 1972. Microbial metabolism of alkylbenzene sulphonates. Enzyme system of a *Bacillus* species responsible for β-oxidation of the alkyl side chain of alkylbenzene sulphonates. Antonie von Leeuwenhoek J. Microbiol. Serol. **38**:543-555.