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Metabolism of the Alkane Analogue *n*-Dioctyl Ether by *Acinetobacter* Species

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Metabolism of *n*-dioctyl ether by *Acinetobacter* species HO1-N resulted in formation of 8-*n*-octoxy-1-octanoic acid and 2-*n*-octoxy-1-acetic acid. The 16-carbon ether acid was incorporated into the cellular lipids, whereas the 10-carbon ether acid accumulated in the growth medium. Qualitative and quantitative characteristics of the cellular phospholipids were similar to hexadecane-grown cells. The growth of *Acinetobacter* on dioctyl ether occurred at the expense of six-carbon atoms of dioctyl ether.

Considerable information has accumulated concerning specific substrate-product interrelationships that occur in the metabolism of diverse alkanes by microorganisms. Summarily, these studies have demonstrated a correlation between the cellular fatty acid composition and the carbon number of the growth alkane (2, 7, 9, 10). In addition, some bacteria exhibit repressed de novo fatty acid biosynthesis when grown at the expense of alkanes, deriving essentially all of their cellular fatty acids from direct oxidation of the alkane (5, 15). A comparative analysis of the lipid composition and metabolism in hydrocarbon-oxidizing bacteria has further demonstrated basic physiological differences (11-14). Interestingly, few studies have focused on the metabolism of alkane analogues by microorganisms. Of related interest, *Acinetobacter* species converted 1-*n*-hexadecylchloride to 16-chlorohexadecyl-16-chlorohexadecanoate and 16-chlorohexadecyl-hexadecanoate, with 80% of the cellular phospholipids consisting of ω-chloro fatty acids (8). Microbial oxidation of oxygenated derivatives of alkanes such as methyl ketones provides a further example (3, 15). This report describes the oxidation of *n*-dioctyl ether by *Acinetobacter* species HO1-N.

MATERIALS AND METHODS

Organism and culture conditions. All experiments were carried out with *Acinetobacter* sp. HO1-N. The organism was grown in a mineral salts medium with the following composition (in grams per liter): $(NH_4)_2SO_4$, 2; KH_2PO_4, 4; $Na_2H\ PO_4 \cdot 7H_2O$, 4; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.001; and $FeSO_4 \cdot 7H_2O$, 0.001, pH 7.8, which was supplemented with presterilized *n*-dioctyl ether (Humphrey Chemical Co., New Haven, Conn.) to a final concentration of 0.3% (vol/vol). Cultures were grown on a gyratory shaker at 28°C and harvested during the late exponential growth phase. Growth was followed with a Klett-Summerson colorimeter equipped with a 540-nm filter.

Extraction and fractionation of lipids. Extraction of total cellular lipids as well as separation of neutral lipids from the phospholipids by silicic acid column chromatography have been reported previously (13). Extraction and fractionation of extracellular lipids from the culture medium have also been previously described (14).

Thin-layer chromatography. (i) Separation of phospholipids. Thin-layer plates (0.4 mm thick) prepared with Silica Gel H containing 1 mM sodium tetraborate were activated at 100°C and used within 30 min. Phospholipids were separated with a solvent system of chloroform-methanol-water (95:35:5, vol/vol). The phospholipids were quantitated by visualizing individual phospholipids in iodine, subliming the iodine, transferring silica gels containing individual phospholipids to test tubes, and determining the amount of lipid phosphorus. All samples were done in triplicate with suitable silica gel blanks. Recovery of the lipid phosphorus applied to plates was greater than 97%. Identification of the phospholipids was accomplished by co-chromatography with authentic known standards described previously from this organism (11).

(ii) Separation of neutral lipids. Thin-layer plates prepared with Silica Gel G (0.4 mm thick) were activated at 100°C and used within 30 min. The neutral lipids were separated with a solvent system of petroleum ether-diethyl ether-glacial acetic acid (85:15:1, vol/vol). Lipids were detected by iodine vapors and identified by comparison with known standards of neutral lipids identified previously in this organism (14).

Lipid phosphorus determination. Lipid samples were evaporated under a stream of nitrogen and hydrolyzed in 0.5 ml of 70% perchloric acid at 100°C for 60 min. Phosphorus was estimated by the procedure of Bartlett (1), with KH_2PO_4 as the standard.

Identification and quantitation of fatty acids. Fatty acids were methylated as described previously (9), and the fatty acid methyl esters were identified with a Tracor model 560 gas chromatograph consist-

ing of a dual-column oven with coiled glass columns (4.0-mm inside diameter, 2.44 m long). A hydrogen flame ionization detector was employed, with column support systems consisting of 10% Apiezon L and 20% diethylene glycol succinate. All fatty acid identifications were substantiated by this two-column system. Operating conditions were: column temperature, 150 or 170°C; detector temperature, 250°C; injection port temperature, 200°C; nitrogen flow rate, 30 cm^3/min; chart speed, 4 min/inch (ca. 4 min/2.54 cm).

Fatty acids were quantitated with a Columbia Scientific Industries automatic digital integrator model CSI-208 interfaced to the gas chromatograph. Methyl palmitate served as a reference standard. Ether fatty acid structures were analyzed by mass spectrometry on a Dupont Instrument model 21-490 single-focusing mass spectrometer equipped with a direct-inlet-solids probe and variable-ionizing voltage control. Infrared spectra were determined on a Beckman model 33 infrared spectrometer.

Isolation of ether fatty acids. Methyl esters of the ether fatty acids were chromatographed on Silica Gel G thin-layer plates in a solvent system of benzene-diethyl ether-absolute ethanol-glacial acetic acid (50:40:2:0.2, vol/vol) (4). The area corresponding to ether fatty acid, after visualization with iodine vapors, was removed from the glass plate, and the methyl esters were eluted from the silica gel with hexane. The hexane layer was filtered through Whatman no. 1 filter paper, dried over anhydrous Na_2SO_4, and reduced to dryness under a stream of nitrogen. The residue was dissolved in hexane and analyzed by gas chromatography to assess purity prior to hydriodic acid (HI) treatment.

HI treatment. Location of the ether oxygen was determined by HI cleavage of the ether bond and subsequent gas chromatographic identification of the alkyl iodide product. A modified procedure of Kates et al. (6) was used for cleaving the methyl esters of the ether fatty acids to their alkyl iodide derivatives. Methyl esters of the ether fatty acids were heated under reflux in 55% HI for 8 h. The mixture was cooled to room temperature and extracted with diethyl ether. The ethyl ether extract was washed in succession with water, a saturated solution of Na_2CO_3, and 50% $Na_2S_2O_3$. The ethyl ether extract was reduced to dryness in vacuo, and the resulting residue was dissolved in methanol-water (9:1, vol/vol). The alkyl halides were extracted with petroleum ether, dried over anhydrous Na_2SO_4, and concentrated to dryness in vacuo. This product was dissolved in anhydrous hexane and analyzed by gas chromatography. Alkyl iodide standards were constructed by the same procedure with alkyl alcohols of differing chain lengths.

RESULTS

The various lipid classes derived from *Acinetobacter* HO1-N were examined for evidence of the direct conversion of oxidation products of dioctyl ether to cellular lipids. The composition of fatty acids derived from the neutral lipids of *Acinetobacter* HO1-N grown on dioctyl ether as the sole source of carbon and energy varied in chain length from C_{10} to C_{18}, comprising 64% of the total fatty acids (Table 1). Those remaining lipid components consisted of fatty acids containing the substrate ether functional group. These fatty acids were identified as a C_{10} fatty acid with the ether oxygen in the Δ^2 position, 2-*n*-octoxy-1-acetic acid, and a C_{16} fatty acid with the ether oxygen in the Δ^8 position, 8-*n*-octoxy-1-*n*-octanoic acid (Fig. 1). The lipid component (Fig. 1, D) was tentatively identified as an unsaturated fatty acid. This fatty acid migrated with the normal fatty acids and not with the ether-containing fatty acids on thin-layer chromatographs. The normal fatty acids were eluted from the silica gel, treated with bromine dissolved in CCl_4, and analyzed by gas chromatography. Lipid D, along with $C_{16:1}$ and $C_{18:1}$, was quantitatively removed from the fatty acid profile. Lipid E migrated with the normal fatty acid fraction on thin-layer chromatographs and was not affected by the bromine-addition reaction. Treatment of the neutral lipid fatty acid with HI eliminated only the designated ether fatty acids, B and C. Plots constructed with known standards to establish the log of the retention time versus the carbon number of the fatty acid eliminated *n*-fatty acids increasing in chain length to 20 carbons, 2-hydroxy fatty acids, and the iso- or anteiso-branched-chain fatty acids. The structure of lipid E remains undetermined.

Ether-acid structure determination. The structures of the fatty acids containing the ether functional group were confirmed by infrared and mass spectrometry. Absorption bands at 2,800 to 3,000 cm^{-1} and 1,700 cm^{-1} indicate the carboxyl functional group in each fatty acid (Fig. 2). The C-O stretch at 1,050 to 1,150 cm^{-1} substantiated the presence of an

TABLE 1. *Composition of fatty acids obtained from the neutral lipid fraction of Acinetobacter HO1-N grown on n-dioctyl ether*

Fatty acid	Amt in lipid fraction (%)
$C_{10:0}$	1.2
$C_{12:0}$	2.8
$C_{14:0}$	1.1
$C_{16:0}$	27.4
$C_{16:1}$	12.7
$C_{18:1}$	17.8
2-*n*-Octoxy-1-acetic acid	12.2
8-*n*-Octoxy-1-octanoic acid	20.2
Lipid D	1.0
Lipid E	3.4
Total fatty acids (μmol/g [dry cell wt])	82.8

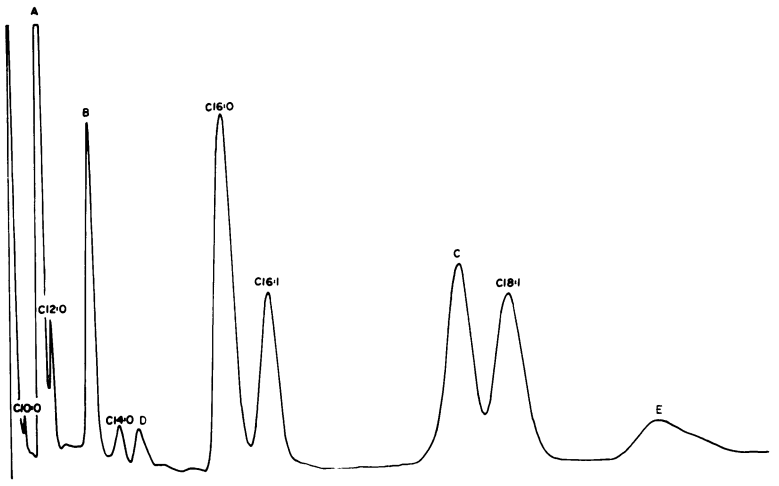

FIG. 1. *Fatty acids derived from cellular neutral lipids of n-dioctyl ether-grown cells. Results shown were obtained by analysis on 20% diethylene glycol succinate. A, n-Dioctyl ether; B, 2-n-octoxy-1-acetic acid methyl ester; C, 8-n-octoxy-1-octanoic acid methyl ester; D, unsaturated fatty acid methyl ester; E, unknown fatty acid methyl ester.*

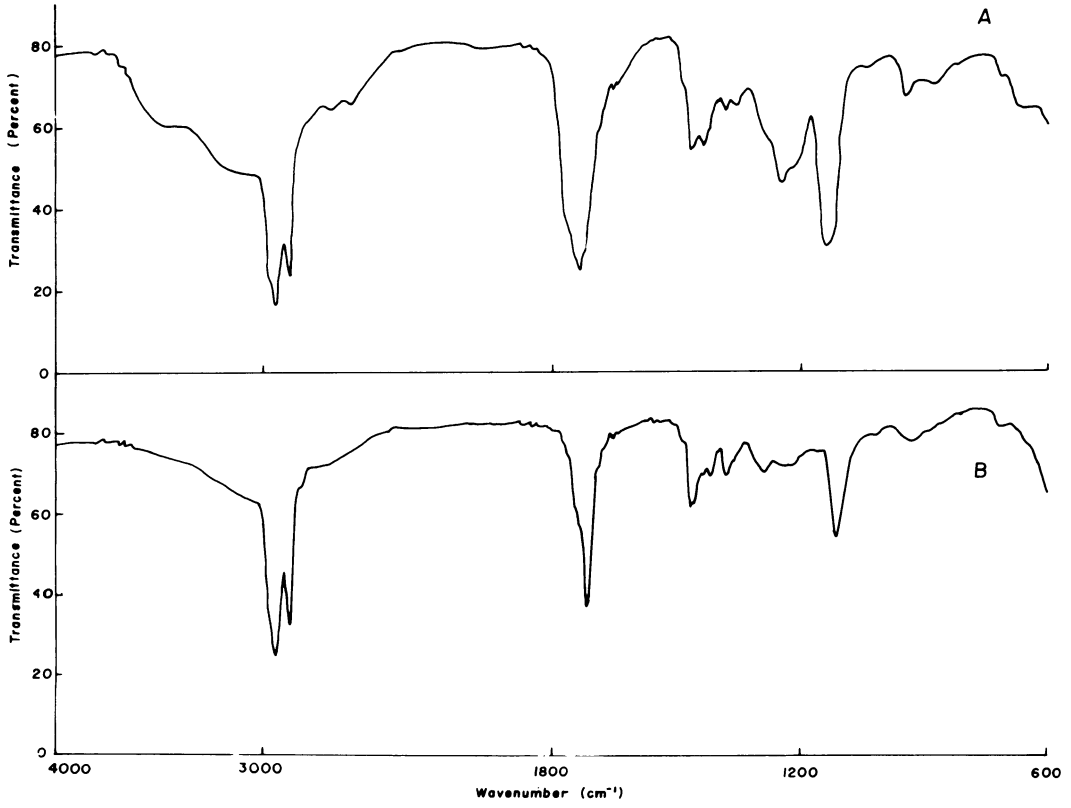

FIG. 2. *Infrared spectra of (A) 2-n-octoxy-1-acetic acid and (B) 8-n-octoxy-1-n-octanoic acid.*

ether functional group. The mass spectrum of the methyl ester of the C_{10} ether fatty acid, 2-*n*-octoxy-1-acetic acid, showed a molecular ion at 203, confirming the molecular weight of the methyl ester fatty acid (Fig. 3). Mass ions at 143, 129, and 74 established the ether oxygen at

the Δ^2 position. The mass spectrum of the methyl ester of the C_{16} ether fatty acid, 8-*n*-octoxy-1-*n*-octanoic acid, showed a molecular ion of 287, establishing the molecular weight of the C_{16} ether fatty acid (Fig. 4). Mass ions at 255 and 227 confirmed the methyl ester functional group of the fatty acid, whereas ions at 129 and 157 were evidence for an ether oxygen at the Δ^8 position in the aliphatic chain.

HI treatment of the methyl ester of 2-*n*-octoxy-1-acetic acid yielded octyl iodide as the cleavage product. Octyl iodide prepared from the HI treatment of 1-octanol was used as a standard, and identification was based on comparative retention times on 20% diethylene glycol succinate and 10% Apiezon L columns. The methyl ester of 8-*n*-octoxy-1-*n*-octanoic acid yielded octyl iodide after HI treatment. These data represent corroborative evidence that the ether oxygen bond was located at the Δ^2 position for the C_{10} ether fatty acid and at the Δ^8 position for C_{16} ether fatty acid.

Cellular phospholipds. The phospholipids derived from dioctyl ether-grown cells were essentially identical to that determined for hexadecane-grown cells (Table 2). Identifications of phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and lysocardiolipin were substantiated by co-chromatography with authentic standards (17). Fatty acids of chain length $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ characterized the phospholipids as well as the C_{16} ether fatty acid, 8-*n*-octoxy-1-octanoic acid (Fig. 5). 8-*n*-Octoxy-1-*n*-octanoic acid comprised 12.2% of the total phospholipid fatty acids (Table 3) and was distributed in all phospholipid species. The $C_{10:0}$ ether acid, 2-*n*-octoxy-1-acetic acid, was not present in the phospholipid fatty acids.

Extracellular lipids. Examination of the culture medium showed that 2-*n*-octoxy-1-acetic

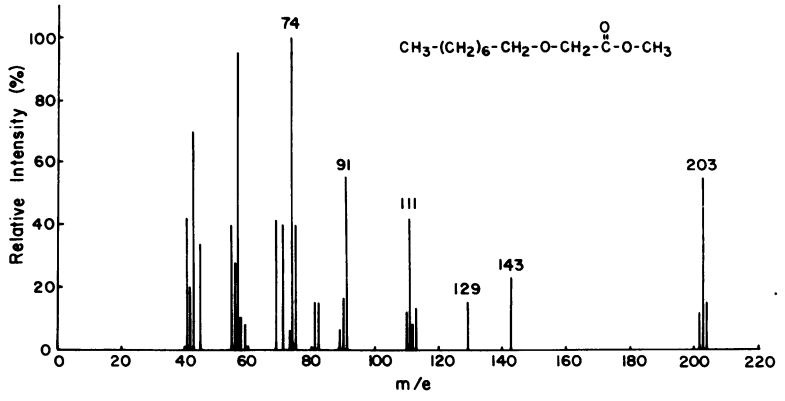

FIG. 3. *Mass spectrum of the methyl ester of 2-n-octoxy-1-acetic acid.*

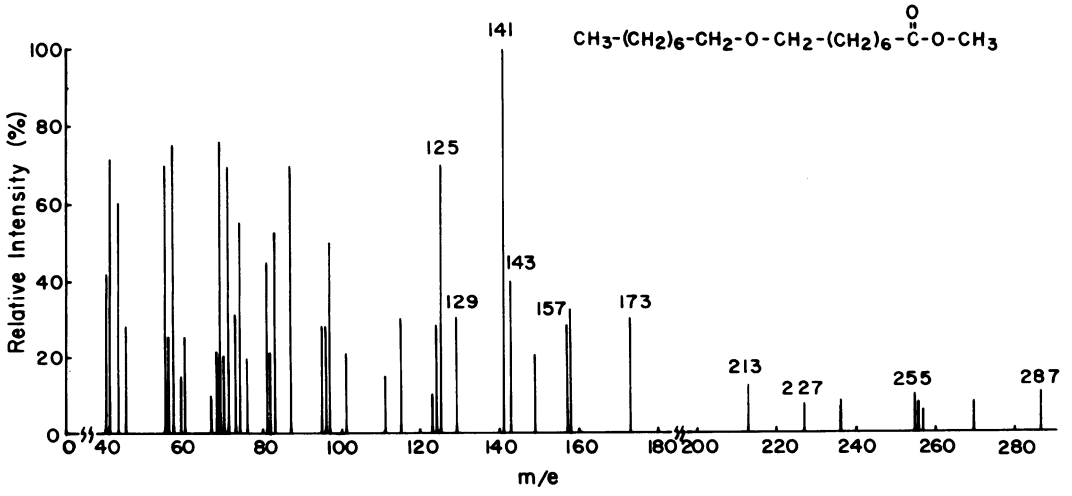

FIG. 4. *Mass spectrum of the methyl ester of 8-n-octoxy-1-n-octanoic acid.*

acid was the only extracellular lipid present. After extraction and purification, it was found that 2-*n*-octoxy-1-acetic acid was not metabolized by whole cells and appeared to represent an end product of *n*-dioctyl ether metabolism.

DISCUSSION

The oxidation of *n*-dioctyl ether appears to occur through methyl group attack with the formation of 8-*n*-octoxy-1-octanoic acid. This fatty acid, in turn, becomes oxidized to 2-*n*-octoxy-1-acetic acid, which accumulates in the growth medium. The sole source of carbon and energy for cellular metabolism and growth appears to be derived from only six carbon atoms of dioctyl ether. The C_{10} ether acid end product accumulates in the growth medium in an inverse relationship to the disappearance of dioctyl ether. This single extracellular product resulting from dioctyl ether oxidation represents a metabolite incapable of being further oxidized through ether bond hydrolysis or ω-oxidation.

TABLE 2. *Phospholipid composition of n-dioctyl ether-grown cells*

Phospholipid	Dioctyl ether-grown cells (%)	Hexadecane-grown cells[a] (%)
Phosphatidylglycerol	17.2	19.9
Phosphatidylethanolamine	57.7	61.5
Cardiolipin	15.0	12.9
Lysocardiolipin	5.6	5.4
Total phospholipid (μmol/g [dry cell wt])	127.5	110.0

[a] Reference 11.

TABLE 3. *Composition of fatty acids obtained from the cellular phospholipids of dioctyl ether-grown cells*

Fatty acid	Amt in cellular phospholipids (%)
$C_{16:0}$	48.6
$C_{16:1}$	15.7
$C_{18:1}$	23.4
8-*n*-Octoxy-1-*n*-octanoic acid	12.2
Total fatty acid (μmol/g [dry cell wt])	153.7

Acinetobacter grown at the expense of hexadecane exhibits repressed de novo fatty acid biosynthesis (16) with palmitate and palmitoleate, representing 98 to 99% of the total cellular fatty acid (9). The metabolism of dioctyl ether by *Acinetobacter* does not appear to repress de novo fatty acid biosynthesis. The fatty acids containing the ether functional group either were derived directly through the oxidation of dioctyl ether or resulted from the oxidation of 8-*n*-octoxy-1-octanoic acid. The metabolism of dioctyl ether by *Acinetobacter* species HO1-N exhibits a distinctly different metabolic pattern than that determined for hexadecane, in that dioctyl ether-grown cells accumulate only a single extracellular product, 2-*n*-octoxy-1-acetic acid, whereas hexadecane-grown cells ac-

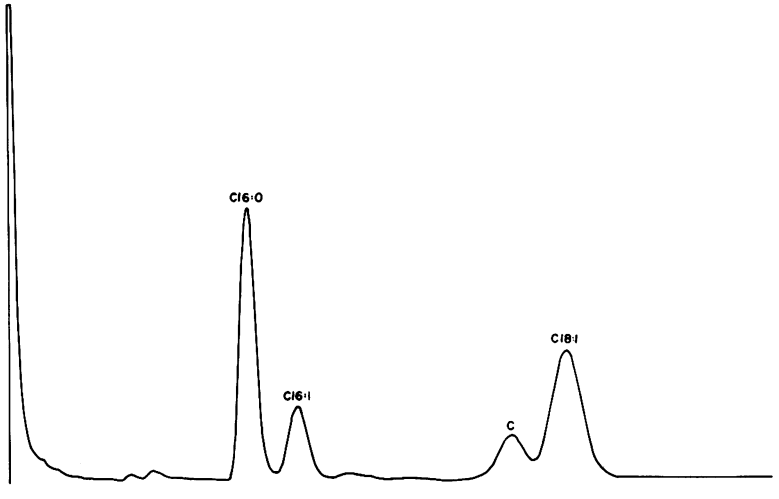

FIG. 5. *Fatty acids derived from the cellular phospholipids of Acinetobacter HO1-N grown on n-dioctyl ether. Results shown were obtained by analysis on 20% diethylene glycol succinate. C, 8-n-Octoxy-1-n-octanoic acid methyl ester.*

cumulate a variety of extracellular products (14).

Additional characteristics observed were that the polar lipids of dioctyl ether-grown cells were quantitatively and qualitatively similar to cells grown on hexadecane. Comparison of the phospholipids individually further shows no significant percentage differences between cells grown on either hexadecane or *n*-dioctyl ether.

The metabolism of dioctyl ether by *Acinetobacter* species HO1-N represents an example of restricted metabolism of parent substrate. The carbon and energy required for cellular growth resides in the terminal six carbon atoms of dioctyl ether. Whether these six carbon atoms are metabolized through β-oxidation of 8-*n*-octoxy-1-octanoic acid or represent metabolism of the six-carbon metabolite, adipic acid, generated from 8-*n*-octoxy-1-octanoic acid remains undetermined. Preliminary analyses support the latter mechanism in the intermediary metabolism of the 16-carbon ether acid. The growth characteristics of this microorganism on dioctyl ether are similar to those reported for growth on hexadecane (13). The oxygen-dependent, terminal oxidation of dioctyl ether and hexadecane by *Acinetobacter* allows for a comparative assessment of the oxidation mechanism in two structurally similar substrates.

LITERATURE CITED

1. **Bartlett, G. B.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234:466.
2. **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.
3. **Fredricks, K. M.** 1967. Products of the oxidation of n-decane by *Pseudomonas aeruginosa* and *Mycobacterium rhodochrous*. Antonie van Leeuwenhoek J. Microbiol. Serol. 33:41–48.
4. **Freeman, C. P., and D. West.** 1966. Complete separation of lipid classes on a single thin-layer plate. J. Lipid Res. 7:324–327.
5. **Gill, C. O., and C. Ratledge.** 1973. Regulation of *de novo* fatty acid and biosynthesis in the n-alkane-utilizing yeast, *Candida* 107. J. Gen. Microbiol. 78:338–347.
6. **Kates, M. L., S. Yengoyan, and P. S. Saster.** 1965. A diether analog of phosphatidyl glycerophosphate in *Halobacterium cutirubrum*. Biochim. Biophys. Acta 98:252–268.
7. **Klug, Y. J., and A. J. Markovetz.** 1967. Degradation of hydrocarbons by members of the genus *Candida*. II. Oxidation of *n*-alkanes and l-alkenes by *Candida lipolytica*. J. Bacteriol. 93:1847–1852.
8. **Kolattukudy, P. E., and L. Hankin.** 1968. Metabolism of a plant wax paraffin (n-nonacosane) by a soil bacterium (*Micrococcus aerificans*). J. Gen. Microbiol. 51:457–463.
9. **Makula, R. A., and W. R. Finnerty.** 1968. Microbial assimilation of hydrocarbons. I. Fatty acids derived from normal alkanes. J. Bacteriol. 95:2102–2107.
10. **Makula, R. A., and W. R. Finnerty.** 1968. Microbial assimilation of hydrocarbons. II. Fatty acids derived from l-alkenes. J. Bacteriol. 95:2108–2111.
11. **Makula, R. A., and W. R. Finnerty.** 1970. Microbial assimilation of hydrocarbons: identification of phospholipids. J. Bacteriol. 103:348–355.
12. **Makula, R. A., and W. R. Finnerty.** 1971. Microbial assimilation of hydrocarbons: phospholipid metabolism. J. Bacteriol. 107:806–814.
13. **Makula, R. A., and W. R. Finnerty.** 1972. Microbial assimilation of hydrocarbons: cellular distribution of fatty acids. J. Bacteriol. 112:398–407.
14. **Makula, R. A., P. J. Lockwood, and W. R. Finnerty.** 1975. Comparative analysis of the lipids of *Acinetobacter* species grown on hexadecane. J. Bacteriol. 121:250–258.
15. **Markovetz, A. J.** 1972. Subterminal oxidation of aliphatic hydrocarbons by microorganisms. Crit. Rev. Microbiol. 1:225–237.
16. **Sampson, K. L., and W. R. Finnerty.** 1974. Regulation of fatty acid biosynthesis in the hydrocarbon oxidizing microorganism, *Acinetobacter sp.* Arch. Microbiol. 99:203–220.
17. **Scott, C. C. L., R. A. Makula, and W. R. Finnerty.** 1976. Isolation and characterization of membranes from a hydrocarbon-oxidizing *Acinetobacter* sp. J. Bacteriol. 127:469–480.