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Degradation of Hydrocarbons by Members of the Genus *Candida*

II. Oxidation of *n*-Alkanes and 1-Alkenes by *Candida lipolytica*

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Candida lipolytica ATCC 8661 was grown in a mineral-salts hydrocarbon medium. *n*-Alkanes and 1-alkenes with 14 through 18 carbon atoms were used as substrates. Ether extracts of culture fluids and cells obtained from cultures grown on the various substrates were analyzed by thin-layer and gas-liquid chromatography. Analyses of fluids from cultures grown on *n*-alkanes indicated a predominance of fatty acids and alcohols of the same chain length as the substrate. In addition, numerous other fatty acids and alcohols were present. Analyses of saponifiable and nonsaponifiable material obtained from the cells revealed essentially the same products. The presence of primary and secondary alcohols, as well as fatty acids, of the same chain length as the *n*-alkane substrate suggested that attack on both the methyl and α-methylene group was occurring. The significance of these two mechanisms in the degradation of *n*-alkanes by this organism was not evident from the data presented. Analyses of fluids from cultures grown on 1-alkenes indicated the presence of 1,2-diols, as well as ω-unsaturated fatty acids, of the same chain length as the substrate. Alcohols present were all unsaturated. Saponifiable and nonsaponifiable material obtained from cells contained essentially the same products. The presence of 1,2-diols and ω-unsaturated fatty acids of the same chain length as the substrate from cultures grown on 1-alkenes indicated that both the terminal methyl group and the terminal double bond were being attacked.

Few of the reports, cited in another paper (M. J. Klug and A. J. Markovetz, Appl. Microbiol., *in press*), on the assimilation of hydrocarbons by members of the genus *Candida* have been concerned with the fate of the hydrocarbon substrate. Bruyn (1) demonstrated the formation of 1,2-hexadecanediol from 1-hexadecene in cultures of *C. lipolytica* growing in a defined medium at the expense of the alkene. This finding was confirmed (16), and, subsequently, atmospheric oxygen was implicated in the oxidation of the 1-alkene to the diol (6).

Mizuno et al. (13) reported the composition of cellular lipids of *C. petrophillum* grown on several *n*-alkanes and compared it with that found when the same organism was grown on glucose. The fatty acid composition was essentially the same whether the organism was grown on *n*-hexadecane or glucose. When *n*-tridecane served as growth substrate, an increase in number of odd chain-length fatty acids was seen.

Iizuka, Iida, and Unami (5) described a number of degradation products isolated from cultures of *C. rugosa* grown at the expense of *n*-decane. *n*-Decyl alcohol, *n*-decyl aldehyde, and *n*-decanoic acid were isolated, as well as a number of dicarboxylic acids. These products reflect a mono- and a diterminal attack on the *n*-alkane molecule.

Identification of products found in the fluids of cultures of *C. lipolytica* grown on *n*-alkanes and 1-alkenes possessing 14 through 18 carbon atoms is the subject of the present paper. The composition of saponifiable and nonsaponifiable material of the cellular lipids from these cultures is described. Culture fluids and cellular lipids from the yeast grown on glucose also were analyzed, and these results are compared with those obtained when hydrocarbons served as substrates.

MATERIALS AND METHODS

Organism and cultural methods. C. lipolytica ATCC 8661 was obtained from the American Type Culture Collection, Washington, D.C. Large-scale cultures were grown in a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) containing 10 liters of mineral-salts medium of the following composition (per liter of distilled water): KH_2PO_4, 7.0 g; Na_2HPO_4, 1.2 g; $MgSO_4 \cdot 7H_2O$, 200 mg; NH_4SO_4,

3.0 g; $CaCl_2 \cdot 2H_2O$, 50 mg; NaCl, 50 mg; $CuSO_4 \cdot 5H_2O$, 80 μg; KI, 200 μg; $FeCl_3 \cdot 6H_2O$, 1 mg; $MnSO_4 \cdot 2H_2O$, 30 μg; $Na_2MoO_4 \cdot H_2O$, 10 μg; $ZnSO_4$, 80 μg; boric acid, 500 μg. Filter-sterilized *n*-alkanes and 1-alkenes were added to a final concentration of 1% at the time of inoculation. Glucose, when used as substrate, was added aseptically to a final concentration of 2%. All cultures were agitated at 400 rev/min for 60 hr at 30 C, with an air flow of 200 to 300 ml/min.

Source of compounds. Hydrocarbons of 98 mole % purity were obtained from The Humphrey Chemical Co., North Haven, Conn. Oxygenated impurities were removed from the 1-alkenes by passing them through a column containing Adsorbosil I (Applied Science Laboratory, State College, Pa.).

Methyl esters of fatty acids used as standards were obtained from Applied Science Laboratory and The Hormel Institute, Austin, Minn. 1,2-Diols were synthesized according to the methods of Swern, Billen, and Scanlon (17). Alcohols were obtained from Aldrich Chemical Co., Milwaukee, Wis. 13-Tetradecenoic and 15-hexadecenoic acids were synthesized by the Chemistry Department of the University of Iowa.

Lipid extraction. Lipids were extracted (9) in an atmosphere of nitrogen with CH_3OH-$CHCl_3$ (2:1, v/v). Saponifiable and nonsaponifiable materials were separated by preparative thin-layer chromatography.

Recovery and detection of metabolic products. Cells were removed from the cultures by suction filtration, and the clarified fluid was extracted continuously with diethyl ether for 72 hr. Extracts were dried over Na_2SO_4 and reduced in volume by flash evaporation. Residual hydrocarbon was removed by adding the extracts to a column of Adsorbosil-1 and eluting with *n*-hexane. Oxygenated products were removed from the column with diethyl ether.

Extracts were assayed by thin-layer chromatography on layers of Adsorbosil 1 (250 μ) according to Stahl (15). Chromatograms were developed in a solvent of *n*-hexane-diethyl ether-acetic acid (85:15:2, v/v). Lipid material fluoresced yellow-green under ultraviolet light (254 mμ).

Preparative thin-layer chromatography was used to recover detected classes of compounds. Thin layers of Adsorbosil-5 (250 μ) were used, and *n*-hexane-diethyl ether-acetic acid (85:15:2, v/v) was employed as developing solvent. Developed plates were sprayed lightly with 2′,7′-dichlorofluorescein, and visible spots (bands) were marked. Silica gel from the marked areas was scraped from each plate, and the compounds were eluted from the silica gel with diethyl ether. Compounds were stored under nitrogen until they were analyzed. The saponifiable and nonsaponifiable materials obtained from the cellular lipids were assayed, separated, and stored in a similar manner.

Analyses of the components of each class of compounds generally were obtained by gas-liquid chromatography. Instruments used were an F & M model 700 (F & M Scientific Corp., Avondale, Pa.) and an Aerograph model 600 D (Varian-Aerograph, Walnut Creek, Calif.). Both instruments were equipped with flame ionization detectors. Helium was the carrier gas, and flow was measured by use of either a rotameter or a soap-bubble flowmeter.

Carboxylic acids were converted to their methyl esters (14) and chromatographed on two columns: (i) polar column—8 ft by 0.25 inch (outer diameter) copper tubing packed with 60/80 mesh Diatoport S (F & M Scientific Corp.) coated with 9.1% Lac-2R-446 (diethylene glycol pentaerythritol adipate, Applied Science Laboratory); operational conditions—column temperature, 150 C; detector temperature, 250 C; injector temperature, 250 C; flow rate, 60 ml/min; (ii) nonpolar column—8 ft by 0.25 inch (outer diameter) copper tubing packed with 60/80 mesh Diatoport S coated with 6% Apiezon L; operational conditions—column temperature, 197 C; detector temperature, 265 C; injector temperature, 280 C; flow rate, 60 ml/min.

Analyses of 1,2-diols were carried out on a polar column consisting of 6 ft by 0.125 inch (outer diameter) stainless-steel tubing packed with 60/80 mesh Chromasorb AW coated with 2% Versamid 900 (column packing obtained from F & M Scientific Corp.). Operational conditions employed were: column temperature, 185 C; detector temperature, 185 C; injector temperature, 220 C; flow rate, 25 ml/min.

Alcohols were analyzed on a nonpolar column consisting of 6 ft by 0.125 inch (outer diameter) copper tubing packed with 60/80 mesh Chromasorb AW coated with 5% SE-30 (column obtained from Varian-Aerograph). Operational conditions employed were: column temperature, 140 C; detector temperature, 140 C; injector temperature, 180 C; flow rate, 25 ml/min.

Tentative identification of methyl esters of isolated fatty acids was based on a comparison of retention times (relative to methyl hexadecanoate) with authentic compounds on both polar and nonpolar liquid phases. Unsaturation was determined by the bromination technique of James and Martin (7). Since authentic unsaturated fatty acids were not available in all chain lengths, another method was used to assign tentative designations for unsaturated peaks. This method involved plotting $\log_{10}$ values of the relative retention times of authentic fatty acids on Apiezon L against times obtained on Lac-2R-446. The number of double bonds and the number of carbon atoms in an unknown fatty acid can be determined by plotting its relative retention time on the grid formed by plotting the authentic compounds.

Peak areas were measured in square centimeters by use of a polar planimeter. In some cases, only approximate areas could be obtained because peaks were not completely resolved. Tentative identification of alcohols and diols was made by comparison of retention times of authentic compounds with those of unknowns.

RESULTS AND DISCUSSION

Thin-layer chromatograms of ether extracts of culture fluids obtained from cultures grown on the various *n*-alkanes indicated that the same classes of compounds were present regardless of

the substrate. Spots corresponding to primary and secondary alcohols and monocarboxylic acids were detected. A number of unidentified spots were also seen on the chromatograms. Spots corresponding to 1,2-diols, primary alcohols, and monocarboxylic acids were detected on all thin-layer chromatograms of ether extracts of culture fluids obtained from cultures grown on 1-alkene. No fluorescing classes of compounds were detected from fluids of cultures grown on glucose.

Chromatograms of the saponifiable and nonsaponifiable material from cells grown on *n*-alkanes and 1-alkenes exhibited the same classes of compounds as did chromatograms of the respective culture fluids. Thin-layer chromatograms of the saponifiable and nonsaponifiable material from cells grown on glucose indicated the presence of primary alcohols and monocarboxylic acids.

The presence of 1,2-diols in cultures grown on 1-alkenes, and the presence of secondary alcohols in cultures grown on *n*-alkanes, were the only notable differences between the classes of compounds ordinarily detected when 1-alkenes or *n*-alkanes served as growth substrates. Neither 1,2-diols nor secondary alcohols were found in cultures grown on glucose.

Individual classes of compounds were separated and recovered by preparative thin-layer chromatography and analyzed by gas-liquid chromatography. Further characterization of the components of each class was hindered by the small quantity of each sample available for analysis.

Gas-liquid chromatographic data on the 1,2-diol fractions from culture fluids and nonsaponifiable cellular material from the organism grown on all the 1-alkenes showed that each contained a 1,2-diol of the same chain length as the respective substrate. 1,2-Diols with the same carbon skeleton as the substrate were reported previously in the fluids of cultures grown on 1-hexadecene (1, 16) and 1-octadecene (6). The presence of these compounds in the cells and the fluids of cultures grown on all of the 1-alkenes suggests that diols may be intermediate products in the catabolism of 1-alkenes by the organism. This point will be discussed later.

Data on gas-liquid chromatographic analyses of alcohols from fluids of cultures grown on *n*-alkanes with 14 through 18 carbon atoms, and from the cellular lipids of cells grown on glucose, are presented in Table 1. Both primary and secondary alcohols of the same chain length as the hydrocarbon substrate were detected. Primary alcohols were detected in the cells, but not in the fluids, of cultures grown on glucose; no secondary alcohols were present.

Because of the lack of standards for unsaturated alcohols, analyses of alcohols from cultures grown on 1-alkenes will not be reported. All the alcohols detected appeared to be unsaturated.

Gas-liquid chromatographic data on fractions containing mono-carboxylic acids from fluids of cultures grown on *n*-alkanes and 1-alkenes with 14 through 18 carbon atoms, and from the cellular lipids of cells grown on glucose, are presented in Table 2. With the exception of minor variations, the profile of fatty acids found in the cells and in the respective culture fluid was essentially the same. For this reason, only data concerning fatty acids found in the culture fluids are presented.

Fatty acids in the fluids of cultures grown on *n*-tetradecane, *n*-hexadecane, and *n*-octadecane

TABLE 1. *Alcohols from cultures grown on n-alkanes[a] and glucose[b]*

Alcohol	Substrate					
	Glucose	*n*-Tetradecane	*n*-Pentadecane	*n*-Hexadecane	*n*-Heptadecane	*n*-Octadecane
1-Dodecanol	10.00[c]	6.12	13.60	11.10	—	—
1-Tetradecanol	10.00	40.80	—	11.10	—	—
2-Tetradecanol	—	28.50	—	—	—	—
1-Pentadecanol	20.00	—	59.30	—	34.50	—
2-Pentadecanol	—	—	27.30	—	—	—
1-Hexadecanol	45.00	24.50	—	48.70	—	10.00
2-Hexadecanol	—	—	—	28.70	—	—
1-Heptadecanol	—	—	—	—	41.30	—
2-Heptadecanol	—	—	—	—	24.00	—
1-Octadecanol	15.00	—	—	—	—	65.00
2-Octadecanol	—	—	—	—	—	25.00

[a] Alcohols from culture fluid.
[b] Alcohols from cells.
[c] Results expressed as percentage of total.

TABLE 2. *Fatty acids from cultures grown on n-alkanes[a], 1-alkenes[a], and glucose[b]*

Fatty acid	Substrate										
	Glucose	*n*-Tetradecane	1-Tetradecene	*n*-Pentadecane	1-Pentadecene	*n*-Hexadecane	1-Hexadecene	*n*-Heptadecane	1-Heptadecene	*n*-Octadecane	1-Octadecene
11:0[c]	Tr[d]	Tr	Tr	—	—	—	Tr	1.30	—	—	—
12:0	Tr	Tr	0.90	Tr	Tr	Tr	1.20	—	—	2.60	2.80
13:0	Tr	Tr	0.90	Tr	Tr	5.00	2.40	1.96	0.69	4.00	1.40
13:1	—	—	1.10	2.50	—	—	—	—	—	—	—
14:0	Tr	2.98	2.00	0.80	3.40	5.00	1.20	Tr	Tr	Tr	1.40
14:1	—	—	1.00	—	—	—	—	—	—	—	—
15:0	Tr	4.48	2.30	20.80	5.20	5.00	7.20	3.90	1.70	Tr	1.40
15:1	—	—	—	1.73	5.20	—	—	3.90	—	—	—
15:1	—	—	—	—	6.90	—	—	—	—	—	—
16:0	32.60	13.45	26.70	2.60	12.10	30.00	7.22	5.20	13.90	21.40	8.50
16:1	17.80	7.45	6.90	4.30	6.90	20.00	8.43	2.60	3.49	6.70	5.70
16:1	—	—	—	—	—	—	7.22	—	—	—	5.70
17:0	Tr	Tr	1.52	0.80	1.70	Tr	Tr	14.30	3.49	Tr	7.10
17:1	Tr	Tr	3.05	25.40	2.60	Tr	3.61	36.60	16.80	5.30	15.70
17:1	—	—	—	—	—	—	3.61	—	13.90	—	Tr
18:0	3.25	44.80	26.71	12.30	8.60	10.00	3.61	19.60	20.90	6.70	8.50
18:1	40.30	10.10	3.81	8.60	15.60	25.00	9.63	3.90	9.09	16.70	11.40
18:1	—	—	—	—	—	—	—	—	—	—	5.70
18:2	4.03	14.90	2.29	19.73	22.60	—	36.10	5.20	13.28	34.80	8.50
18:2	—	—	—	—	—	—	—	—	—	—	10.00

[a] Fatty acids from culture fluid.
[b] Cellular fatty acids.
[c] First figure denotes number of carbon atoms; second figure, number of double bonds.
[d] Results expressed as percentage of total; Tr, trace.

were similar in that the predominant chain lengths were 16 and 18 carbon atoms. This is contrasted with what was found in the fluids of cultures grown on *n*-pentadecane and *n*-heptadecane. Predominant fatty acids from cultures grown on *n*-pentadecane were pentadecanoic acid and heptadecenoic acid, whereas those from *n*-heptadecane-grown cultures were heptadecenoic acid and 18-carbon acids. Therefore, the utilization of *n*-alkanes with odd numbers of carbon atoms resulted in a predominance of fatty acids having an odd number of carbons; the utilization of *n*-alkanes with even numbers of carbon atoms resulted in a predominance of fatty acids with an even number of carbons. Further, some of these predominant fatty acids were of the same, or nearly the same, chain length as the substrate. The possibility arose that a high proportion of the acids of the same chain length as the substrate were derived from the direct oxidation of the hydrocarbon rather than by cellular resynthesis. This was supported by finding primary alcohols of the same chain length as the substrate in each of the fluids of cultures grown on the various *n*-alkanes. Other predominant acids may have been synthesized from two-carbon condensation on, or by β-oxidation of, the fatty acid arising from direct oxidation of the hydrocarbon.

Results somewhat similar to ours were reported by Davis (2) for the fatty acids from cells of *Nocardia* grown on a series of *n*-alkanes. Predominant fatty acids found in these cells reflected the chain length of the growth substrate. This was interpreted as evidence for direct incorporation of alkane-derived fatty acids into the glycerides. Furthermore, only acids with odd numbers of carbon were detected from glycerides of nocardial cells grown on *n*-alkanes with an odd number of carbons, and only acids with even numbers of carbon were seen from even-numbered *n*-alkanes. This "odd or even" observation differs from our data concerning fatty acids from *C. lipolytica*, since both odd- and even-numbered acids were detected regardless of the number of carbon atoms in the *n*-alkane. Mizuno et al. (13) also found that both odd- and even-numbered fatty acids were produced from *n*-tridecane and *n*-hexadecane by *C. petrophillum*.

A comparison of the fatty acid profiles obtained from cultures grown on *n*-alkane and 1-alkene showed an increase in number of unsaturated acids of the same chain length as the substrate when 1-alkenes served as substrates. Fur-

thermore, the predominant fatty acids were not necessarily of the same chain length as the substrate.

In cultures grown on 1-pentadecene, 1-hexadecene, 1-heptadecene, and 1-octadecene, two monounsaturated fatty acids of the same chain length as the substrate were seen. One of these acids can be compared with a *cis* Δ^9-unsaturated acid. However, double-bond position was not verified by oxidative cleavage. The other unsaturated acid present when 1-hexadecene served as growth substrate was tentatively identified as 15-hexadecenoic acid by comparison with a synthesized standard. On the basis of a logarithmic plot of the relative retention times of two authentic ω-unsaturated acids, 13-tetradecenoic, and 15-hexadecenoic acid, it was speculated that in all cases the second acid is an ω-unsaturated acid of the same chain length as the substrate. When 1-octadecene was the substrate, the 16-carbon ω-unsaturated acid, 15-hexadecenoic acid, was also present.

Alkanes. The presence of primary alcohols and monocarboxylic acids of the same chain length as the substrate in cultures grown on *n*-alkanes with 14 through 18 carbon atoms indicated that the oxidative attack was on one of the methyl groups of the hydrocarbon molecule. This mode of attack is similar to what has been observed in bacterial systems metabolizing *n*-alkanes (12).

Occurrence of a secondary alcohol of the same chain length as the substrate in these cultures may be explained by a mechanism involving the formation of an alkyl hydroperoxide on the penultimate carbon. This mechanism was proposed by Leadbetter and Foster (10) to explain ketone formation from saturated hydrocarbons. However, Kallio et al. (8) indicated that this mechanism appears to be operative only in systems involving *n*-alkanes with less than 10 carbon atoms. This was concluded from a survey of the literature concerning *n*-alkane oxidations which indicated that methyl ketones had not been detected in systems involving *n*-alkanes with more than 10 carbon atoms (except, *see* 11). The significance of the occurrence of secondary alcohols in *n*-alkane cultures is not clear at this time.

Alkenes. Accumulation of 1,2-diols of the same chain length as the substrate indicated an attack on the terminal double bond of the 1-alkene. Foster (3) proposed a mechanism for the degradation of 1,2-octadecanediol derived from 1-octadecene by *C. lipolytica*. This mechanism proceeds through the α-hydroxy acid and then to a fatty acid with one carbon less than the original substrate. In the present study, no α-hydroxy acids were seen; however, fatty acids with one carbon less than the substrate were present in a higher percentage in 1-alkene cultures than in the corresponding *n*-alkane cultures. These acids may have arisen through cleavage of a 1,2-diol

The presence of ω-unsaturated acids of the same chain length as the substrate demonstrated that an attack on the terminal methyl group of the 1-alkene also was occurring. It was indicated that ω-unsaturated acids were subject to further oxidation when 1-octadecene served as growth substrate and both 17-octadecenoic acid and 15-hexadecenoic acid were detected.

Huybregtse and van der Linden (4) concluded that the main pathway involved in the oxidation of 1-octene by a species of *Pseudomonas* was via the terminal methyl group. They also indicated that a number of minor reactions occur at the double bond: (i) epoxidation, (ii) proposed nonenzymatic rupture of the epoxide bond to yield 1,2-octanediol, and (iii) formation of a saturated acid possibly via the aldehyde. Further, these authors suggested that, on the basis of these observations, *C. lipolytica* may produce only small amounts of 1,2-diols from the corresponding 1-alkene, the major degradation proceeding by way of a primary attack on the methyl group.

Markovetz, Klug, and Forney (12*a*) have isolated 13-tetradecenoic acid from cultures of *Pseudomonas aeruginosa* grown on 1-tetradecene. No diols were detected, but 2-tetradecanol was present. Finding these compounds indicates that pathways are operative through both the terminal double bond and the methyl group. It may be that pathways involved in the oxidation of 1-alkenes by bacteria and *C. lipolytica* are essentially the same; i.e., oxidation occurs through both the terminal methyl group and the double bond. The relative significance of the two pathways in *C. lipolytica* remains to be determined.

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