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Cellular Lipids of a *Nocardia* Grown on Propane and *n*-Butane

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ABSTRACT

DAVIS, J. B. (Socony Mobil Oil Co., Inc., Dallas, Tex.). Cellular lipids of a *Nocardia* grown on propane and *n*-butane. Appl. Microbiol. **12**:301-304. 1964.—Lipid fractions of propane- and *n*-butane-grown nocardial cells each contain a chloroform-soluble, ether-insoluble polymer not observed previously in liquid *n*-alkane-grown cells. The polymer in propane-grown cells is poly-β-hydroxybutyrate. The polymer in *n*-butane-grown cells apparently contains unsaturation in the molecule, and is identified tentatively as a co-polymer of β-hydroxybutyric and β-hydroxybutenoic (specifically 3-hydroxy 2-butenoic) acids. The other major component of the lipid fraction consists of triglycerides containing principally palmitic and stearic acids. There seems to be little qualitative distinction in the glycerides of propane- or *n*-butane-grown cells. Oxidative assimilation of *n*-butane is described.

Nocardial cells cultivated in mineral salts media with liquid *n*-alkanes as the sole carbon source were found previously to contain large amounts of cellular lipid (Raymond and Davis, 1960). When relatively long-chain alkanes such as *n*-hexadecane or *n*-octadecane were used as substrates, aliphatic waxes accumulated in the cells. The rest of the lipid fraction consisted principally of glycerides.

In this paper, the results of an examination of the cellular lipid of *Nocardia* grown on gaseous alkanes, propane, and *n*-butane are reported. Rather than an accumulation of aliphatic waxes in the cells, there is evident a storage of poly-β-hydroxybutyrate by propane-grown cells and an even greater accumulation of a closely related polymer by *n*-butane-grown nocardial cells.

MATERIALS AND METHODS

Culture. Nocardia 107-332, described earlier by Davis and Raymond (1961), was cultivated in 4-liter impeller-agitated systems previously described (Raymond and Davis, 1960). Propane or *n*-butane was added to the culture systems in a 10 to 15% concentration in air with gas flow adjusted to about 250 ml/min. The culture medium used initially had the following composition (in g per liter of distilled water): $(NH_4)_2SO_4$, 1.0; Na_2HPO_4, 0.3; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; Na_2CO_3, 0.1; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.005; and $MnSO_4$, 0.002.

Growth was stimulated markedly by further addition of phosphate salts to effectively buffer the medium at about neutrality. The above phosphate salts were, therefore, often increased tenfold and consequently used at a total 0.5% concentration. When urea was used as the nitrogen source instead of ammonium sulfate, less phosphate was required to maintain neutral pH owing to the lack of accumulating acidic sulfate ion. To obviate the ultimate acidic effect of added ammonium sulfate, urea was adopted for addition to growing cultures to satisfy their nitrogen requirement.

Mineral salts medium (2.5 liters) was usually added to the 4-liter fermentors. These were inoculated with either a 50- or 100-ml culture of *Nocardia* previously grown on 0.4% *n*-octadecane in 250-ml bottles incubated on a rotary shaker at 30 C. Propane or *n*-butane was added in air to the culture systems through fritted steel spargers. The impellers were turned at about 1,500 rev/min. Growth of the *Nocardia* was followed by withdrawing approximately 100-ml samples at intervals, centrifuging them, and then drying the collected cells over P_2O_5 and weighing the cells. Table 1 shows the cell weights obtained in an experiment in which *Nocardia* cells were grown on propane and *n*-butane.

RESULTS

Cellular protein and lipid. Maximal growth obtained on *n*-butane was 17.9 g (dry weight) of cells per liter of culture. This yield was obtained after 17 days, during which time 1 g of urea per liter was added at 5 days and again at 13 days. The original medium was in accordance with the formula given above, but with the phosphate salts in a 0.5% concentration.

The total amount of nitrogen employed in the culture medium was 1.14 g per liter. The cells harvested at 17 days (17.9 g per liter) contained 4.4% nitrogen; this accounts for only about 0.79 g of nitrogen, indicating that about 69% of the nitrogen added to the medium had been utilized. At 13 days, the cell concentration was 15 g per liter. These cells contained only 3.5% nitrogen or 0.53 g on a per liter basis. At this time, 0.68 g of nitrogen had been added to the culture; utilization at this point was 78%. Determination of available nitrogen in solution was not made during or at the termination of the experiment; hence, possible loss of nitrogen from the medium in the form of ammonia was not ascertained.

The amount of protein in microbial cells was estimated on the basis of cellular nitrogen. (Kjeldahl nitrogen values were multiplied by the factor 6.25 to yield per cent protein.) Table 2 gives the per cent nitrogen and protein values of the nocardial cells at progressive stages of cell de-

velopment. Actually, the protein content of the cells is quite low, but, in general, limited available nitrogen results in low protein values and high lipid values. Also given in Table 2 are the cellular lipid values.

Ether-insoluble lipid fraction. The lipid of *n*-butane-grown cells yielded a fairly large amount (20 to 25 %) of an ether-insoluble material. The material was extracted from the nocardial cells with a mixture of chloroform-methanol (2:1, v/v) and was readily soluble in chloroform but not in methanol, ethanol, ethyl or petroleum ether, benzene, or carbon tetrachloride. When separated from the lipid fraction by washing with ethyl ether, and dried, the material looks and feels somewhat like a cloth fabric, but it is rather fragile. When dissolved in chloroform and dried, a rather strong film was obtained. The material may be purified by precipitation from chloroform solution with excess ether.

The material has a neutral pH and is not hydrolyzed by concentrated HCl even when autoclaved. It is hydrolyzed by strong, hot H_2SO_4 and the hydrolysate, which is not distillable and not ether-soluble, appears to be crotonic acid on the basis of its infrared spectrum. Saponification of the polymer in alcoholic potassium hydroxide gives a saponification number of about 600. Tests for phosphorus and nitrogen were negative.

The best characterization of the ether-insoluble fraction from *n*-butane-grown cells was based upon its infrared spectrum, made of a thin film of the material on a silver chloride disc. This spectrum is similar with one exception to that published by Blackwood and Epp (1957) and Haynes et al. (1958), for a β-hydroxybutyrate polymer isolated from various bacteria. The exception is absorption of infrared at 6.3 μ, indicating unsaturated bonds in the molecule. This distinctive feature is noted in Fig. 1, which shows comparative spectra of the polymer isolated from the lipid of *n*-butane-, propane-, and *n*-hexadecane-grown nocardial cells. The spectra of the polymer from propane- and *n*-hexadecane-grown cells are identical to that of poly-β-hydroxybutyrate, but the polymer from *n*-butane-grown cells is consistently different.

Figure 2 shows spectra of the depolymerized products of the polymer from propane- and *n*-butane-grown cells compared with β-hydroxybutyric acid. A distinctive infrared absorption is noted at 6.1 μ for the *n*-butane-grown cellular product. Absorption at this point occurs also with acetoacetic acid of which β-hydroxybutenoic acid constitutes either of two enol forms. Acetoacetic was tested as the ethyl ester. Absorption of infrared is also noted for the de-

TABLE 1. *Growth of Nocardia on propane and n-butane**

Time of incubation	Propane	*n*-Butane
days		
4	6.5	4.0
6	9.4	—
7	12.0	9.5
10	12.8	12.3

* Results are expressed as dry weight of cells in grams per liter.

TABLE 2. *Protein and lipid in Nocardia growing on n-butane*

Incubation	Cells (dry wt)	Kjeldahl N	Protein	Lipid*
days	*g/liter*	%	%	%
5	3.0	4.0	25.0	45.0
10	9.0	4.1	25.6	50.3
13	15.0	3.5	21.9	—
17	17.9	4.4	27.4	56.2

* Material extracted from cells with chloroform-methanol (2:1).

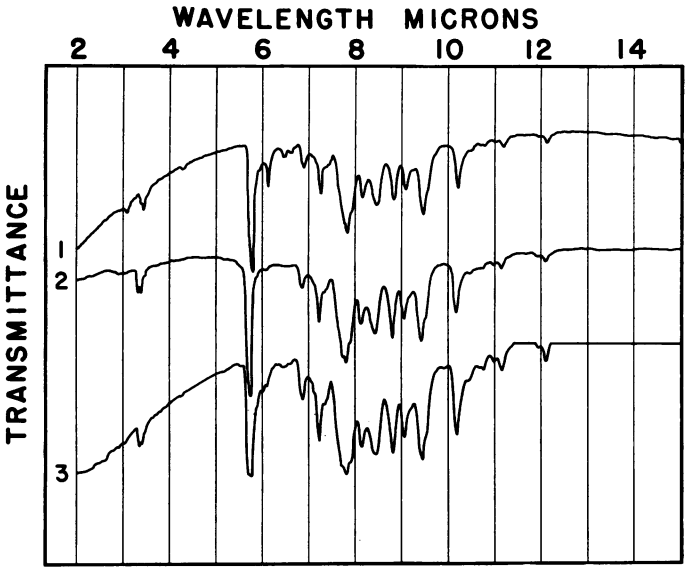

FIG. 1. *Infrared spectra of chloroform-soluble, ether-insoluble fraction of the cellular lipid of Nocardia 107–332. (1) Grown on n-butane. (2) Grown on propane. (3) Grown on n-hexadecane.*

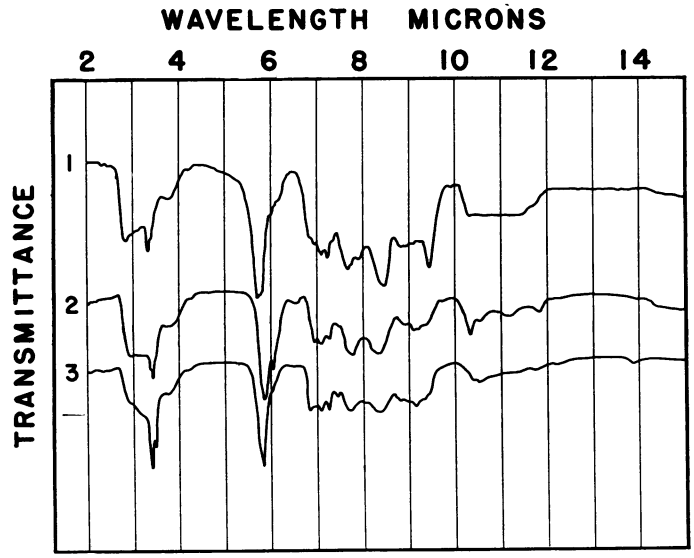

FIG. 2. *Infrared spectra of the depolymerized chloroform-soluble, ether-insoluble lipid fraction of Nocardia 107–332 grown on n-butane (2) and propane (3), compared with β-hydroxybutyric acid (1).*

polymerized material from *n*-butane-grown cells at about 10.35 μ, indicative of unsaturation (Fig. 2). From the available data, it is tentatively proposed that the new polymer is a co-polymer of β-hydroxybutyric and β-hydroxybutenoic (specifically 3-hydroxy 2-butenoic) acids, based principally upon infrared spectra.

The polymer in *n*-butane-grown cells constitutes about 12 to 14% of the cell weight, whereas in propane-grown cells the polymer is about 4% and in *n*-hexadecane-grown cells only 0.1% of the cell weight.

Ether-soluble lipid fraction. The saponification number of the ether-soluble lipid fraction was high for both propane- and *n*-butane-grown cells, and the glycerol content was low, based upon the identity of the fatty acids (Table 3). These respective values may be explained on the basis of other components in the ether-soluble fraction. That is, the presence of either a small amount of the polymer or an acidic impurity in the sample would cause a high saponification number, and any component (other than glycerides) would cause a lower glycerol value. The fatty acids were identified after their conversion to methyl esters and fractionation by gas chromatography. The fatty acid fractions were almost identical for both propane- and *n*-butane-grown cells. Oleic acid could have been present and not readily detected by the method employed. However, the iodine numbers were rather low, indicating only a small degree of unsaturation in the ether-soluble lipid.

Oxidative assimilation. Washed cells of *Nocardia* 107-332 were suspended in 0.5 M phosphate buffered at pH 7. Quantities (50 ml) of the cell suspensions containing 12 mg (dry weight) of cells per ml were added to bottles sealed with Neoprene septums. Duplicate systems were evacuated through a syringe needle and gassed with 3% *n*-butane in air and 3% helium in air, respectively. The gas volume in each case was 200 ml. *n*-Butane and oxygen uptake were measured by an analysis of the original gas composition of the systems and again after 16 hr. Gas analyses were conveniently performed by fractionating the samples on a column of Linde 10× molecular sieve pellets ($\frac{1}{16}$ in. or 1.6 mm; Linde Co., Division of Union Carbide Corp., New York, N.Y.) Oxygen and nitrogen were fractionated at 50 C; *n*-butane and carbon dioxide at 250 C (model 300 gas chromatograph, F & M Scientific Corp., Wilmington, Del.). Prior to the final analysis, the cell suspensions were acidified to pH 2 to release any carbon dioxide absorbed by the phosphate buffer. Only a very small amount of carbon dioxide was apparently produced in either the test systems or the controls, both being approximately the same, with an average of 2 ml. Addition of 3-ml quantities of tank carbon dioxide to similar cell suspensions, followed by acidification, showed good recovery, indicating that the unexpectedly low recovery of produced carbon dioxide was reasonably accurate. All *n*-butane (6 ml per test system) was consumed, and the average difference in oxygen uptake between the controls and the test systems was 13.2 ml. This difference is attributed to *n*-butane oxidation. Since there was no carbon dioxide formation attributable to *n*-butane oxidation, it is suggested that the cells oxidatively assimilated the *n*-butane. No molecular hydrogen and no extracellular acidic or neutral products were detected in the systems.

TABLE 3. *Ether-soluble lipid fraction of propane- and n-butane-grown nocardial cells*

Cells grown on	Saponification no.[a]	Glycerol[b]	Hanus iodine no.[c]	Methyl esters of fatty acids[d] (approx %)
		%		
Propane.....	321	7.3	39.2	Myristate (5) Palmitate (50) Stearate (45)
n-Butane.....	275	7.9	24.1	Myristate (5) Palmitate (50) Stearate (45)

[a] Milligrams of KOH required to neutralize acids in 1 g of lipid.
[b] According to method of Neish (1952).
[c] Grams of I_2 reacting with 100 g of lipid.
[d] Prepared with boron trifluoride as catalyst; some oleate probably in stearate fraction.

In the absence of oxidative assimilation, the complete oxidation of *n*-butane would result in the following molar balance:

$$C_4H_{10} + 6.5O_2 \rightarrow 4CO_2 + 5H_2O$$

The data obtained, indicating a consumption of 6 ml of *n*-butane and an uptake of 13.2 ml of oxygen, support the following approximate molar relationship:

$$C_4H_{10} + 2O_2 \xrightarrow[Nocardia]{\text{nongrowing}} (C_4H_6O_2) + 2H_2O$$

Thus, the data tentatively are interpreted to indicate the oxidative assimilation of *n*-butane by *Nocardia* and the intracellular formation of a polymer with an empirical formula approximating $(C_4H_6O_2)n$, the unit monomer of poly-β-hydroxybutyrate.

DISCUSSION

Although the presence in microbial cells of poly-β-hydroxybutyrate was observed many years ago by Lemoigne (1927), its role as a storage material in microbial cells was discovered only recently. Its presence in cells was undetected by workers ordinarily, because of its solubility characteristics. Lipid extraction by ordinary solvents, other than chloroform, will not extract the polymer. Williamson and Wilkinson (1958) pointed out the accumulation of poly-β-hydroxybutyrate in bacilli, particularly, and showed that the polymer is actually a reserve (storage) material. Doudoroff and Stanier (1959) then described the function of poly-β-hydroxybutyrate as reserve material in bacteria. They demonstrated with C^{14} that the polymer $(C_4H_6O_2)n$ is stored immediately in cells as a product of either photosynthetic assimilation (with *Rhodospirillum rubrum*) or oxidative assimilation (with *Pseudomonas saccharophila*). Subsequently utilized by the bacteria for en-

ergy, the polymer may also be used as a source of other cellular components such as amino acids if nitrogen is added in usable form. Referring to poly-β-hydroxybutyrate, Stanier (1961) stated in his paper on a unitary concept of photosynthesis in bacteria and plants: "On a physiological level, polymer synthesis can be regarded as a mechanism for the intracellular storage of large quantities of fatty acid carbon; as a result of polymerization, the acidic substrate is neutralized and made osmotically inert. Upon removal of the external carbon source, the cell can draw on this internal carbon store for general biosynthesis."

As far as *Nocardia* is concerned, there are at least three lipid products which accumulate in the cells, dependent qualitatively upon the substrate and quantitatively upon the conditions of culture. These products are common glycerides, aliphatic waxes, and a polymer either identical to or very closely related to poly-β-hydroxybutyrate. All of these materials may be considered as reserve materials. Common to the cells regardless of the substrate are the glycerides. Aliphatic waxes accumulate in cells grown on long-chain *n*-alkanes virtually to the exclusion of polymer, whereas polymer material accumulates in cells grown on short-chain *n*-alkanes to the exclusion of aliphatic waxes.

The accumulation in *n*-butane-grown cells of a polymer which appears to be a co-polymer of β-hydroxybutyric acid and an enol form of acetoacetic acid (β-hydroxybutenoic acid) indicates that both C_4-oxidized units are readily derived from *n*-butane since oxidation involves the formation first of β-hydroxybutyric acid, and then of acetoacetic acid. Ordinary C_2 condensation forms acetoacetic acid, which is reduced to form β-hydroxybutyric acid on the way to polymerization of storage product.

The oxidative assimilation of *n*-butane is interesting, since the energy required for polymerization of the oxidized products must come from the oxidative reactions leading to polymer units without apparent carbon-chain rupture; i.e., carbon dioxide is not produced. *R. rubrum* was found by Stanier (1961) to photoassimilate β-hydroxybutyrate (anaerobically) without production of carbon dioxide; here, the energy was derived and was required from light.

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