Microbial Assimilation of Hydrocarbons: Phospholipid Metabolism

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An analysis of the turnover of the major phospholipids of Micrococcus cerificans growing or nongrowing cultures. The turnover rates of 14C-PE and 14C-PE were 61.5% of the total phospholipid, exhibited no significant rate of turnover in either growing or nongrowing cultures. The turnover rates of PE-14C and PE-32P were 3.2% per hr and 1.2% per hr, respectively. Phosphatidylglycerol (PG) exhibited a turnover rate of 11% and 7.7% per hr for 14C and 32P, respectively, indicating an extremely slow metabolism. PG metabolism was examined in greater detail, and the data indicated a preferential 75% incorporation of glycerol-1,3-14C into the unacylated portion of the PG molecule. The turnover of cardiolipin (CL) was extremely slow in growing cells whereas nongrowing cells exhibited a 30% and 36% increase per hr for 14C-CL and 14C-CL, respectively. Glycerol-1,3-14C was not converted to phospholipid fatty acid carbon; all radioactivity appeared only in the water-soluble backbone of the phospholipids. The kinetics of assimilation of hexadecane-1-14C into cellular lipids is presented. Radioactivity in neutral lipid increased approximately sevenfold over the growth cycle, whereas radioactivity in phospholipid increased 50-fold during the same time period. The incorporation of radioactive fatty acids derived from the direct oxidation of hexadecane-1-14C demonstrated differential kinetics of assimilation into PE, PG, and CL. The results indicated a rapid turnover of phospholipid fatty acids in M. cerificans growing at the expense of hexadecane.

Little information is available concerning phospholipid metabolism in those microorganisms capable of hydrocarbon assimilation. A previous report characterized the phospholipid composition of Micrococcus cerificans grown at the expense of long-chain n-alkanes and described the tentative biosynthetic pathway for these phospholipids (11).

In current membrane models, lipid forms an integral part of a protein-lipid network for which a fundamental structural role is attributed to phospholipids (10). Natural membranes have been observed to be metabolically dynamic entities with the phospholipids contributing to the dynamic character of such processes as active transport of cations (1, 5, 14) and mitochondrial function (3, 4, 7). Phospholipids as metabolically active constituents of membranes have been studied with respect to turnover of phospholipids (18), polar end-group conversions (8, 12, 17), and phospholipid fatty acyltransferase activities (6, 17).

Most proponents believe that interactions of lipids and proteins within membranes give these biointerphases their characteristic and vital properties. These lipid-lipid and lipid-protein interactions provide a hydrophobic environment for certain processes that would not occur under more hydrophilic conditions. The role of lipid metabolism, therefore, becomes particularly germane to hydrocarbon assimilation. A relationship between phospholipid biosynthesis and hydrocarbon oxidation has already been suggested (11). Two basic questions proposed at the outset of this work were as follows: (i) Do membrane phospholipids exhibit specific turnover properties that correlate with alkane oxidation? (ii) What relationships exist between cellular lipid composition and hydrocarbon assimilatory processes? These studies detail answers to certain aspects of the problem in addition to implicating heretofore unrecognized relationships.

MATERIALS AND METHODS

A description of culture conditions, extraction of lipids, thin-layer chromatography, and silicic acid column chromatography has been reported (11).

Recovery of phospholipids. Phospholipids were recovered from silica gel thin-layer plates after one-dimensional development in chloroform-methanol-glacial
acetic acid (65:25:8, v/v/v). The silica gel area containing each phospholipid was removed and successively extracted with five volumes of chloroform-methanol (1:1, v/v) and five volumes of methanol, each solvent system containing 2% water. The solutions were filtered through glass wool and partitioned with 0.3% NaCl, and the sample was reduced to dryness under nitrogen. Purified phospholipids were dissolved in chloroform for subsequent analyses.

Acid hydrolysis of lipids and paper chromatography of water-soluble products. Phosphatidylethanolamine (PE) was hydrolyzed for 12 hr in 3 N HCl at 100°C in sealed glass ampoules. The water-soluble products, ethanolamine-hydrochloride and α-glycerophosphate, were obtained by evaporation of the aqueous phase after ether extraction. Ascending paper chromatography on Whatman no. 1 filter paper employing ethanol-acetic acid-water (90:5:5, v/v/v) as the solvent system was used to separate these hydrolysis products. Co-chromatography with known standards established chromatographic identity. Ethanolamine-hydrochloride was revealed with 0.2% ninhydrin in aceton, and α-glycerophosphate was revealed by the phosphate reagent of Wade and Morgan (as described in reference 11).

Deacylation of phospholipids. Mild alkaline methanolysis was performed by a modification of the procedure of White (16). The phospholipid was dissolved in 1 ml of anhydrous methanol-toluene (1:1, v/v), and 1 ml of freshly prepared 0.2 M methanolic KOH was added. The sample was held for 2 hr at 4°C, after which the solvents were removed by nitrogen evaporation. The sample was suspended in acidified distilled water (15% HCl) and exhaustively extracted with ether.

Materials. Carrier-free 32P-orthophosphate and glyceral-1,3,4C (specific activity, 12.5 mCi/m mole) were purchased from New England Nuclear Corp. N-hexadecane-1-14C (specific activity, 47.2 mCi/m mole) was purchased from Amersham/Searle Co. and diluted with carrier hexadecane to specific activity 24.71 μCi/m mole. All experiments with hexadecane-1-14C used 2.05 mmoles containing 3 x 106 total counts per min.

RESULTS

Phospholipid turnover-growing cells. The decrease of radioactivity from phospholipids after a period of growth in 14C and 32P offers a measure of phospholipid metabolism during bacterial growth. M. cerificans was grown for 2 hr in the presence of 1 mCi of H232PO4 and 25 μCi of glycerol-1,3,414C with hexadecane as the sole carbon and energy source. The cells were harvested by centrifugation, washed with basal growth medium minus requisite salt amendments (MgSO4, CaCl2, FeSO4), and suspended in complete growth medium with hexadecane as the carbon source. Samples (60 ml) were removed at time zero and at hourly intervals for 5 hr, they were extracted with chloroform-methanol at a ratio of 2:1:0.2 (v/v/v) yielding a monophasic system, and the phospholipids were separated as previously described (11). Phosphorus analyses on the crude lipid samples verified that phospholipid synthesis occurred during growth (Fig. 1A).

The turnover of crude lipid radioactivity (Fig. 1B) indicated a slow metabolism of the cellular phospholipids. The turnover of 14C-glycerol is faster than the turnover of 32P (30% for 14C and 20% for 32P) over the 5-hr incubation. Although the amount of phospholipid tripled during this period, the slow turnover rate of crude lipid radioactivity indicates that the cellular phospholipids are stable.

The phospholipids present as predominant species, i.e., PE, phosphatidylglycerol (PG), and cardiolipin (CL), were isolated by thin-layer chromatography, and each sample was analyzed for 14C and 32P. Figure 1C shows a 16% and 6% decrease of 14C and 32P, respectively, in PE over the 5-hr growth period. Turnover data for PG (Fig. 1D) show a decrease of 14C and 32P of 55% and 38.5%, respectively. CL exhibited a slight decrease in 14C (10%), with 32P remaining constant for 4 hr. A 35% increased incorporation of both 14C and 32P into CL was noted during the last hour.

Phospholipid turnover-nongrowing cells. Phospholipid turnover in a nongrowing culture was analyzed under previously described conditions, with the exception that cells were suspended in 0.05 M phosphate buffer (pH 7.8). Figure 2A shows that neither growth nor an increase in lipid phosphorus occurred during the 5-hr incubation period in nonradioactive phosphate buffer. The conditions, therefore, enable an assessment of phospholipid metabolism under nongrowing conditions for direct comparison to growing conditions.

The turnover of 14C and 32P from the crude lipid fractions is shown in Fig. 2B. An initial decrease in 14C and 32P was observed for the first 2 hr, followed by an increase to the initial level. The individual phospholipids, PE, PG, and CL, were purified and their turnover kinetics were examined. PE did not exhibit turnover of either 14C and 32P throughout the 5-hr incubation, indicating this phospholipid to be stable under these conditions (Fig. 2C). PG, however, exhibited turnover kinetics similar to crude lipid, showing an initial decrease in both 14C and 32P and an increase to initial levels at later time points (Fig. 2D). Analysis of CL showed a 150% increase in 14C in CL over the 5-hr incubation, whereas 32P increased 110% over 3 hr and then remained constant (Fig. 2E). Two major phospholipids of M. cerificans, PG and CL, appear to possess differential turnover rates in nongrowing cells.
Metabolism of PG. The lipids of M. cerificans were labeled with 25 μCi of glycerol-1,3-14C added to the medium at mid-exponential growth, and samples were removed at 0.5, 1, 5, 10, 20, 30, and 60 min. All samples were pipetted into chloroform-methanol (2:1, v/v) to yield a mono-phasic system from which the crude lipid extract was obtained. A sample of crude lipid from each sample was assayed for radioactivity. Figure 3 shows the kinetics of incorporation of glycerol-1,3-14C label into crude lipid. A constant rate of incorporation was observed for the initial 10 min. This represents assimilation of 3.4% of available 14C-glycerol into phospholipid. The crude lipid extracts were fractionated into neutral lipid and phospholipid by silicic acid column fractionation.
PHOSPHOLIPID METABOLISM IN M. CERIFICANS

chromatography. Less than 5% of the radioactivity was found in neutral lipids whereas the remainder of the 14C label from glycerol-1,3-14C was in the phospholipid fraction. Since the phospholipid fraction contained essentially all the 14C-label, further analysis was performed only with the phospholipids.

Analysis for incorporation kinetics of glycerol-1,3-14C into each individual phospholipid is shown in Fig. 4. The curve designated PG1 represents the total incorporation of glycerol-1,3-14C into PG without reference to either diglyceride glycerol or headgroup glycerol. PG exhibited the highest labeling of all phospholipids analyzed and was, therefore, subject to further analysis. Two dimensional thin-layer chromatography was employed to determine the contribution of phosphatidylglycerol phosphate (PGP) to total 14C-glycerol incorporation in PG. PG and PGP were separated on Silica Gel G thin-layer plates (0.25 mm) with the following solvent systems: first dimension, chloroform-methanol-5 N NH4OH (65:30:5, v/v/v); second dimension, chloroform-methanol-glacial acetic acid (70:20:5, v/v/v). The contribution of 14C-PGP to PG varied between 1.5 and 3% during the course of the reaction, so that subsequent analyses to be discussed do not involve specific procedures for the removal of PGP from purified PG.

PG1 was purified from all samples by preparative thin-layer chromatography in an acidic solvent system (chloroform-methanol-glacial acetic acid; 70:20:5, v/v/v) and hydrolyzed in 90% acetic acid for 20 min. The products, diglyceride and α-glycerophosphate, were separated by ether extraction, and radioactivity was determined in each fraction. The distribution of total radioactivity was 25% in the ether-soluble diglyceride moiety and 75% in α-glycerophosphate. This fact suggests a difference in the metabolism of the unacylated and diacyl glycerols of PG. The curve designated PG2 represents a plot of glycerol-1,3-14C incorporation into the diacylglycerol portion

![Fig. 3. Incorporation of glycerol-1,3-14C into the chloroform-soluble lipids of Micrococcus cerificans.](image)

![Fig. 4. Glycerol-1,3-14C incorporation into the phospholipids of Micrococcus cerificans. PG1 represents the total incorporation of glycerol-1,3-14C into PG. PG2 represents the incorporation of glycerol-1,3-14C into the diglyceride backbone of PG.](image)
of PG based on the radioactivity distribution analysis of 25%. Assuming this 25% represents de novo synthesis of phospholipid from requisite precursors, it is possible to calculate percentage composition of phospholipid. Table 1 compares the percentage composition of *M. cerificans* phospholipids by two independent methods. An extremely close approximation of percentage composition is obtained.

A portion of PG, from all samples was treated by mild alkaline hydrolysis to determine the amount of $^{14}$C-glycerol converted to fatty acid carbon. These results demonstrated no radioactivity in fatty acids, indicating that all glycerol incorporated into phospholipid was localized in the water-soluble backbone, glycerylphosphorylglycerol. The data further indicate the unacylated glycerol of PG to be metabolically active for reasons unknown.

PE was purified by thin-layer chromatography and hydrolyzed in 3 N HCl. The water-soluble products, ethanolamine-hydrochloride and α-glycerophosphate, were analyzed by paper chromatography in ethanol-acetic acid-water (90:5:5, v/v/v) and compared to authentic standards. All radioactivity in the sample co-chromatographed with α-glycerophosphate. Mild alkaline hydrolysis of PE demonstrated no conversion of glycerol carbon to fatty acid carbon.

CL was not appreciably labeled with $^{14}$C during the course of the experiment. This result was unexpected, since CL contains three glycerol moieties per molecule, and PG, which is thought to be a precursor of CL, was highly labeled with $^{14}$C. A possible explanation for the PG-CL labeling pattern relates to an alternate biosynthetic pathway for CL. The biosynthetic pathway described in *E. coli* involves a condensation reaction between cytidine-diphosphoglyceride and PG to yield CL (15). If this reaction mechanism was operative in *M. cerificans*, a highly labeled CL molecule would be expected. An alternative possibility is that, under conditions of the experiment, CL biosynthesis is limited.

$^{14}$C-Hexadecane assimilation into cellular lipid. The assimilation of hexadecane carbon into cellular lipid was assessed by growing *M. cerificans* in the presence of 49.4 μCi of hexadecane-$^{1-14}$C. Samples (25 ml) were removed at 2-, 4-, 8-, and 12-hr intervals, and sodium azide (1 ml of 10% solution) was added immediately to each sample. The cells were collected by centrifugation, washed twice with basal medium, lyophilized, and extracted for total lipid. The crude lipid was fractionated by silicic acid chromatography into nonmetabolized hexadecane, neutral lipid, and phospholipid by hexane, chloroform, and methanol elution to obtain the respective lipid fractions.

A 7.92% increase of radioactivity from hexadecane-$^{1-14}$C or products of hexadecane-$^{1-14}$C assimilation was observed over the time course (Table 2, A). Extraction of total lipid (Table 2, B) by chloroform-methanol (2:1, v/v) yields approximately 50% of the total radioactivity in the chloroform-soluble fraction. Since it has been impossible to remove hexadecane from cells by aqueous washing procedures, these determinations

### Table 1. Comparison of phospholipid composition by two independent criteria

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Percentage composition based on $^{14}$C-glycerol incorporation</th>
<th>Percentage composition based on phosphorus analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>60</td>
<td>61.5</td>
</tr>
<tr>
<td>PG</td>
<td>23</td>
<td>19.9</td>
</tr>
<tr>
<td>CL</td>
<td>10</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

* See reference 11.

### Table 2. Distribution of hexadecane-$^{1-14}$C assimilation products in the lipids of *Micrococcus cerificans*

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Cell mass (mg dry wt)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total* (10⁶</td>
<td>Chloroform-soluble</td>
<td>Hexadecane</td>
<td>Neutrals</td>
<td>Phospholipids</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>counts/min)</td>
<td>(10⁶ counts/min)</td>
<td>(10⁶ counts/min)</td>
<td>(10⁶ counts/min)</td>
<td>(10⁶ counts/min)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>250.0 (0.83)*</td>
<td>100.0 (40)*</td>
<td>80.0 (80)*</td>
<td>7.5 (37.5)*</td>
<td>2.2 (11)*</td>
<td>90*</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>407.0 (1.36)*</td>
<td>210.0 (52)</td>
<td>172.0 (82)</td>
<td>10.8 (28.4)</td>
<td>6.4 (17)</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>150</td>
<td>975.0 (3.25)*</td>
<td>485.0 (50)</td>
<td>338.0 (70)</td>
<td>18.0 (12.2)</td>
<td>31.0 (21)</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>2,378.0 (7.92)</td>
<td>665.0 (28)</td>
<td>394.0 (59)</td>
<td>52.0 (19)</td>
<td>104.0 (38.4)</td>
<td>82</td>
</tr>
</tbody>
</table>

* Total counts per minute were determined by solubilizing 5 mg of dry cells in hyamine hydroxide.

* See reference 11.

* A, Total counts per minute/counts per minute of hexadecane-$^{1-14}$C added × 100; B, B/A × 100; C, C/B × 100; D, D/B − C × 100; E, E/B − C × 100.

* F, C + D + E/B × 100.
are influenced by the presence of hexadecane-\(^{14}\)C adsorbed to cell surfaces in an unspecified manner. Therefore, fractionation of the total crude lipid was necessary to determine the amount of hexadecane-\(^{14}\)C in each sample. Hexadecane was removed from the crude lipid samples by silicic acid chromatography eluting with 20-column volumes of hexane. Thin-layer chromatography and gas chromatography of the hexane fraction indicated only hexadecane-\(^{14}\)C to be present. Hexadecane-\(^{14}\)C accounted for 80% of the total chloroform-soluble radioactivity at 2 hr and decreased to 59% at 12 hr (Table 2, C). The neutral lipid was removed by elution of the silicic acid column with 10-column volumes of chloroform with total radioactivity increasing throughout the time course (Table 2, D). The percentage of radioactivity derived from hexadecane-\(^{14}\)C decreased during the time samplings. Neutral lipid samples were analyzed by thin-layer chromatography for free fatty acid and free fatty alcohol. Commercial thin-layer chromatography silic acid sheets (Eastman chromograms) employing two independent solvent systems gave identical results [(i) petroleum ether-diethyl ether (85:15, v/v); (ii) petroleum ether-diethyl ether-glacial acetic acid (90:10:1, v/v/v)]. Free fatty acid contributed 8.0, 9.0, 12.0, and 10.0% of the total radioactivity to the neutral lipid samples whereas free fatty alcohol accounted for 9.0, 8.3, 8.0, and 6.0% in the 2-, 4-, 8-, and 12-hr samples, respectively. The ratios of total phospholipid radioactivity to total neutral lipid radioactivity for the samples were 0.29, 0.59, 1.72, and 2.0, indicating progressively greater labeling of phospholipids through the growth cycle. The results demonstrate that hexadecane-\(^{14}\)C was converted to radioactive intermediates which can be isolated in the neutral lipid fraction. Since only 1 hexadecane carbon atom in 16 contains radioactivity, interpretation becomes tenuous with respect to total neutral lipid and conversion efficiency. The phospholipid fraction was eluted with 20-column volumes of methanol and concentrated to a known small volume. The phospholipid fraction reflects an increasing percentage of radioactivity derived from \(^{14}\)C-hexadecane metabolism over the time course (Table 2, E). Radioactivity appearing in this fraction represents assimilation of metabolic intermediates derived from the substrate by direct incorporation (i.e., fatty acids) or indirect incorporation of carbon moieties derived from hexadecane metabolism (i.e., glycerophosphoryl esters).

**Phospholipid labeling.** The phospholipid fraction was analyzed by thin-layer chromatography to determine the radioactivity in each phospholipid species throughout the time course (Table 3). Phosphatidylserine (PS) never exceeded 1.6% of the total phospholipid radioactivity, whereas radioactivity contained in PE was 41% of the total at 2 hr and increased to 63% at 8 hr. PE exhibited the greatest change among the phospholipids, with the exception of CL, which decreased from 25.9% (2 hr) to 6.4% (12 hr). Radioactivity in PG and PGP remained relatively constant in all samples. PG and PGP are combined since separate analyses showed PGP to contribute only 4% to the total radioactivity for all samples. PE, PG, and CL were purified, hydrolyzed by mild alkaline hydrolysis, and separated into ether-soluble fatty acids and water-soluble glycerolphosphoryl esters. Figure 5 shows the distribution of radioactivity into each of these derived moieties for PE, PG, and CL. PE contained 75% of its radioactivity in the fatty acids at 2 hr, increasing to 90% at 4 hr. The water-soluble glycerolphosphoryl ester did not exhibit significant radioactivity at any time point throughout the experiment. For radioactivity to appear in the water-soluble portion of the phospholipid molecule, extensive degradation and resynthesis of hexadecane carbon into requisite intermediates must occur. In experiments using terminally labeled substrates, the factor of metabolic dilution of carbon becomes of critical importance. Therefore, low labeling patterns may reflect either extensive turnover or minimal incorporation of relevant precursors into specific phospholipids.

The comparison of radioactivity distribution

<table>
<thead>
<tr>
<th>Phospholipid*</th>
<th>Total counts/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr</td>
</tr>
<tr>
<td>PS</td>
<td>0 (0)*</td>
</tr>
<tr>
<td>PE</td>
<td>864 (41)</td>
</tr>
<tr>
<td>PG-PGP</td>
<td>736 (36)</td>
</tr>
<tr>
<td>CL</td>
<td>560 (25.9)</td>
</tr>
</tbody>
</table>

*PS, phosphatidylerine; PE, phosphatidylethanolamine; PG-PGP, phosphatidylglycerol-phosphatidylglycerol phosphate; CL, cardiolipin.

*Numbers in parentheses represent percentage of radioactivity for each designated phospholipid of total phospholipid radioactivity recovered. All recoveries of phospholipid radioactivity exceeded 95%.
Fig. 5. Distribution of phospholipid radioactivity derived from hexadecane-1-14C oxidation by Micrococcus cerificans. Symbols: ether soluble (x); water soluble (○).

patterns of PE with PG and CL reveals a distinct difference. PG and CL exhibit extensive radioactivity labeling of the water-soluble backbone early in the time course, with radioactivity decreasing with time. The fatty acids comprising the makeup of PG and CL show an inverse labeling pattern to labeling of the water-soluble glycerolphosphoryl esters.

**DISCUSSION**

The purpose of these studies was to investigate a possible relationship between the metabolism of *M. cerificans* phospholipids and hydrocarbon assimilation. The turnover kinetics of the major phospholipids were examined in context of defining a functional rather than structural role for individual phospholipids. Since total phospholipid was 50% higher in hydrocarbon-grown cells (11), it seemed reasonable that one or more phospholipid species would exhibit rapid turnover properties.

PE is the major component; it represents 61.5% of the total phospholipid and exhibited no significant rate of turnover in either growing or nongrowing cultures. The turnover rate of 14C-PE was 3.2% per hr and for 32P-PE it was 1.2% per hr (approximate doubling time, 60 min). Ames showed similar results with *Salmonella typhimurium* (2), and Kanfer and Kennedy with *Escherichia coli* B (9). White and Tucker reported that PE of *Haemophilus parainfluenzae* exhibited no turnover of the glycerol moiety, whereas the ethanolamine and phosphate moieties turn over more rapidly (17).

PG exhibited a turnover rate of 11 and 7.7% per hr for 14C and 32P, respectively, indicating an extremely slow metabolism of PG in growing cells. These data differ significantly from those reported by Ames (2) and Kanfer and Kennedy (9), where approximately 50% of the PG turned over in excess of one generation. White and Tucker detailed the turnover of PG from *H. parainfluenzae*, demonstrating disproportionate turnover kinetics for the diacyl glycerol, unacylated glycerol, and the phosphate moieties of PG (17). These workers found that the unacylated glycerol and phosphate of PG lose radioactivity at identical rates, whereas the loss of radioactivity from the diacyl glycerol moiety of PG is 2.7 times slower. The turnover data for PG in nongrowing *M. cerificans* cultures was characterized by an initial decrease in the amount of radioactivity followed by an increased incorporation of radioactivity back into PG. This pattern might result from the action of phospholipase D on PG yielding phosphatidic acid and glycerol, which are reincorporated into PG without dilution through a pool. Since cardiolipin exhibited an increase in incorporation of radioactivity, it is suggestive that PG is a precursor of CL and, further, that CL synthesis is stimulated under nongrowing conditions. Evidence concerning the biosynthetic pathway of CL has indicated that intact PG is converted to CL by addition of a molecule of phosphatidic acid (15). Our data concerning metabolism of PG indicate a preferential 75% incorporation of glycerol-1,3-14C into the unacylated portion of the PG molecule over incorporation into the acylated portion. The significance remains obscure but suggests a role for specific phospholipases in the metabolism of membrane phospholipids. A phospholipase D specific for CL has been described in *H. parainfluenzae* yielding phosphatidic acid (PA) and PG as prod-
products (13). This CL-specific phospholipase D has been implicated in the active degradation of CL to PA and PG with a corresponding resynthesis of CL from the hydrolysis products.

The turnover of CL was extremely slow in growing *M. cerificans*, whereas nongrowing cells exhibited a 30 and 36% increase per hr for $^{14}$C-CL and $^{32}$P-CL, respectively, for the initial 3 hr. Since CL represents 12.9% of the total phospholipids and PG contains 62% of the glycerol-1,3-$^{14}$C incorporated into all phospholipids, it was surprising that CL contained only approximately 5% of all radioactivity incorporated into the phospholipids. If PG constitutes the cellular precursor for CL biosynthesis, more extensively labeled CL would be expected. This conclusion is further supported by consideration of the turnover data from growing cells indicating slow metabolism of CL. Alternatively, PG could be independently turning over exclusive of its conversion to CL. Our data indicate that the headgroup glycerol of PG is metabolically labile and that a significant percentage of the PG content is continuously changing with respect to specific activity.

These studies have revealed that glycerol-1,3-$^{14}$C is not converted to phospholipid fatty acid. Mild alkaline hydrolyses of PE, PG, and CL showed all radioactivity in the water-soluble backbone of the phospholipid. A comparison with *H. parainfluenzae* (17), which converts 63% of the total $^{14}$C-glycerol incorporated into lipid to phospholipid fatty acid, demonstrates a striking difference between these two microorganisms. Of several compounds tested for conversion to lipid carbon, only acetate, palmitate, cetyl alcohol, and hexadecane have demonstrated significant results with hexadecane-grown cells. Negative compounds tested were methionine-methyl-$^{14}$C, $^{14}$C-serine, $^{14}$C-ethanolamine and $^{14}$C-glycerol.

The analysis of hexadecane-$^{14}$C conversion to cellular lipids represents an attempt to define functional lipid metabolic patterns in *M. cerificans*. Owing to inherent and obvious difficulties associated with studying the assimilation of a terminally labeled alkane, the data point to several pertinent observations concerning hydrocarbon metabolism in this microorganism. Total radioactivity associated with cells increased linearly, paralleling cellular growth. Fractionation of total cellular lipid indicated that the percentage of available hexadecane, specifically or nonspecifically bound to cells, decreased throughout the growth period. Total neutral lipid radioactivity increased approximately sevenfold, although the proportion of the radioactivity in total neutral lipid decreased when compared to chloroform-soluble radioactivity minus hexadecane radioactivity (see Table 2). The percentage composition of free fatty acid in neutral lipid remained relatively constant in all samples. Phospholipid radioactivity increased 50-fold over the growth cycle, in addition to increasing in percentage of radioactivity. These results indirectly support our conclusions concerning a direct role for fatty acid metabolism in *M. cerificans* (Makula and Finnerty, *submitted for publication*).

Phospholipid fatty acid incorporation of radioactivity derived from hexadecane-$^{14}$C oxidation demonstrated distinct differences between PE and PG-CL. PE was extensively labeled with fatty acids early in the growth cycle (2 hr). PG and CL fatty acid labeling patterns were initially low, increasing with growth. These data suggest rapid turnover properties for fatty acyl residues in *M. cerificans* phospholipids. Data presented demonstrated that 98% of the phospholipid fatty acids from hexadecane-grown cells were 16 carbons. Since no lysophospholipids have been detected in *M. cerificans* (11), an active acylase is implicated.

Initial objectives imposed at the outset of this study have been partially accomplished. The question of relevancy for membrane phospholipids in hydrocarbon oxidation remains unanswered. Structural relationships of fatty acids to hydrocarbon chain length suggest a functional correlation (*submitted for publication*). Current data presented demonstrate that the polar portions of *M. cerificans* are relatively stable whereas the apolar side chains appear to be metabolically labile. Further analyses are in progress detailing the turnover parameters of fatty acids in individual phospholipids with respect to positional specificity and structural changes.

Novel membrane properties appear characteristic of this microorganism. Enzymatic properties associated with these membranes become relevant to structure-function relationships. Membrane-bound enzymes demonstrated in *M. cerificans* are cytidine triphosphate: phosphatidic acid cytidyl transferase, $\alpha$-glycerol phosphate-cytidine monophosphate-phosphatidyl transferase, L-serine-cytidine monophosphate-phosphatidyl transferase, phosphatidyleserine decarboxylase, and phosphatidic acid phosphatase (W. R. Finnerty, American Chemical Society SE-SW Meeting, Dec. 1970, p. 31). Membrane fractionation plus further characterization of these enzymes offer probes into membrane structure and lipid involvement in membrane function.

ACKNOWLEDGMENTS

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LITERATURE CITED


ERRATUM

Microbial Assimilation of Hydrocarbons: Phospholipid Metabolism

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Volume 107, no. 3, p. 806, abstract, line 2: Change entire line to read “grown on hexadecane is reported. Phosphatidylethanolamine (PE) representing…”
p. 813, column 2, line 33: Add “phospholipids” after “M. cerificans.”