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Use of Lipid Composition and Metabolism to Examine Structure and Activity of Estuarine Detrital Microflora

JOHN D. KING, DAVID C. WHITE,* and CECILE W. TAYLOR
Department of Biological Science, Florida State University, Tallahassee, Florida 32306

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Earlier studies have shown that the activity of the estuarine detrital microflora measured by various enzyme activities, muramic acid and adenosine 5'-triphosphate (ATP) content, heterotrophic potentials, and respiratory activities correlates with the incorporation of ^{14}C and ^{32}P into the microbial lipids. In this study, these lipids were reproducibly fractionated into neutral lipid, glycolipid, and phospholipid classes. Distinct differences between the active microflora of oak leaves, sweet gum leaves, and pine needles were evidenced both in the rate of lipid synthesis and in the proportions of neutral lipids, glycolipids, and phospholipids. Successional changes in the microflora of leaves incubated in a semitropical estuary, previously suggested by ATP-to-muramic acid ratios and scanning electron micrography, were reflected in changes in the proportions of ^{14}C in major lipid classes when analyzed from the same type of detritus. Short incubation times with ^{14}C gave lipid compositions rich in phospholipids that are typical for the faster-growing bacterial populations; longer incubation with ^{14}C gave lipid compositions richer in neutral and glycolipids, more characteristic of slower-growing eukaryotes or morphologically more complex prokaryotes. The metabolism of the lipids of the estuarine detrital microflora was examined by a pulse-chase experiment with ^{14}C. Glycolipids lost ^{14}C at a rate equal to the loss of ^{14}C of the slow component of muramic acid. Individual phospholipids lost ^{14}C from their backbone glycerol esters at different rates.

One of the pressing needs of microbial ecology is for methods to assess the composition, activities, and interactions of complex natural microfloral communities. These methods must be designed to disturb the complex associations between components of the microbiosphere and the various microenvironments as little as possible. Classical techniques of plating in selective media with studies of isolates under laboratory conditions have provided valuable information, especially about specific organisms, but clearly do not reflect the activities of the microbial communities in nature (1, 11). Greater insights into the microfloral activities can be gained with measurements of mass and activity such as extractible adenine nucleotides (3, 9), muramic acid (15, 16), respiratory activities, heterotrophic potentials, or various enzymatic activities (18). Comparison of these assays with rates of lipid synthesis from ^{14}C- or ^{32}P-labeled precursors has shown that lipid biosynthesis also reflects the microfloral activity (25).

Use of rates of lipid synthesis as measures of microfloral activities could provide an additional insight into the microbial community. The great volume of work on the lipid composition of microorganisms (7, 20) has shown some classes of lipids to be restricted to certain species or to be formed only under certain conditions of growth (5-7, 20, 23). Since the lipids synthesized during exposure to isotopes are relatively easily extractible from environmental samples (25), the composition of these labeled lipids was examined to see whether insights into community structure and activities could be gained.

MATERIALS AND METHODS

Materials. [1-^{14}C]sodium acetate and $H_3{}^{32}PO_4$ were supplied by New England Nuclear Corp., Boston, Mass. Chloroform and methanol were supplied by Fisher Scientific Co., St. Louis, Mo., and silicic acid Unisil, 100 to 200 mesh, was obtained from Clarkson Chemical Co., Williamsport, Pa. Other reagents have been described previously (15, 22, 24).

Samples. Sweet gum leaves (*Liquidamber styraciflua*), oak leaves [*Quercus virginiana* (Mill)], and pine needles (*Pinus elliottii*) were collected on the Florida State University campus, Tallahassee, after winter leaf fall and stored in plastic bags. Approximately 200 g of plant material was placed in baskets and incubated in East Bay, Apalachicola Bay, Fla. (29°43.73'N, 84°57.3'W) during an 11-week period beginning 21 October 1975. In this period the tem-

perature varied between 23.5 and 9°C, the salinity between 0 and 1.7 g/liter, the dissolved oxygen between 9.8 and 6.7 mg/liter, the pH between 7.6 and 6.5, and the depth between 0.2 to 0.9 m. Baskets (25) were removed at specified intervals, and the detritus was transported to the laboratory in estuarine water at ambient temperature with aeration to maintain an oxygen tension of 6 to 7 mg/liter. The experiments were initiated immediately on return to the laboratory (3 to 4 h after sampling). For the pulse-chase experiments, oak leaf detritus was incubated in the East River, St. Marks National Wildlife Refuge, Newport, Fla., for 3 weeks beginning 22 June 1976 (temperature, 27°C; salinity, 0 to 5.0 g/liter; and dissolved oxygen, 7 mg/liter).

Oak and sweet gum leaves were cut into 6.5-mm disks with a paper punch, and the pine needles were cut into 15-mm segments. Sampling methods with statistical justification have been described (25).

Lipid synthesis. Leaf samples (25 to 43 mg, dry weight) were suspended in 2 ml of estuarine water containing 10 μCi of $H_3{}^{32}PO_4$ or 4 μCi of [1-^{14}C]sodium acetate and incubated with aeration for 2 to 6 h at 25°C. For the pulse-chase experiments, a total of 5,000 6.5-mm oak leaf disks were incubated for 6 h in 500 ml of estuarine water in which the [1-^{14}C]sodium acetate was maintained between 2.0×10^9 and 1.56×10^9 cpm by hourly additions. Washing by decantation five times reduced the ^{14}C in the water at least three orders of magnitude, and the incubation was continued with aeration for 140 h. At intervals, 200 disks were removed, and the lipids were extracted, fractionated by silicic acid chromatography, deacylated by mild alkaline methanolysis, and separated chromatographically.

Lipid extraction. Samples of leaves, 0.5 to 2.0 g, were extracted by a modified Bligh and Dyer procedure (4).

The leaves were suspended in a solution containing 40 ml of absolute methanol, 20 ml of chloroform, and 16 ml of aqueous 0.24 N HCl and shaken vigorously. The single-phase extraction mixture was allowed to stand at room temperature for at least 2 h. An additional 20 ml of chloroform and 20 ml water were then added, and the phases were allowed to separate overnight. The lower (chloroform) layer was collected for lipid analysis. The disks were dried and hydrolyzed, and the muramic acid was separated chromatographically and assayed colorimetrically after mild alkaline hydrolysis as described (15).

Separation of the lipid classes. The chloroform phase of the extraction was taken to dryness with reduced pressure, dehydrated by resuspension in benzene-absolute ethyl alcohol (4:1) and evaporation, and resuspended in chloroform. The lipids (less than 2.5 mg) were added to a column of silicic acid (Unisil; 100 to 200 mesh, 29 by 0.54 cm [containing 7 g]), and solvents were pumped through the column as illustrated in Fig. 1. The effectiveness of these procedures in separating the lipid classes has been documented (26). The reproducibility of this separation using solvents added in both reservoirs on top of the column is illustrated in Table 1. Neutral lipids, glycolipids, and phospholipids were fractionated with standard errors of 13, 17, and 5% of the means, respectively. The recovery of ^{14}C was 91 ± 2% by this procedure. Lipid glucose (measured after hydrolysis by the phenol method [13]) was fractionated (84.6 ± 6% found in the acetone fraction) with a recovery of 96.8 ± 4%.

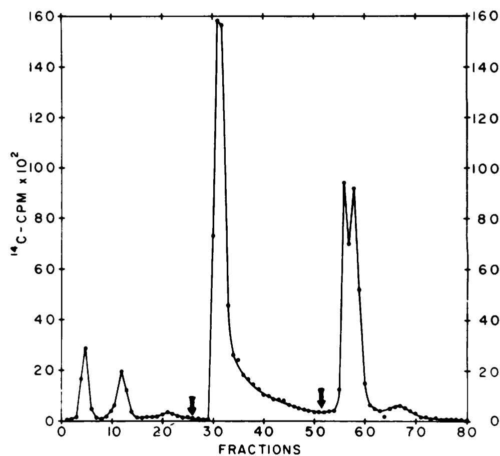

FIG. 1. *Separation of microbial lipids synthesized in [1-^{14}C]sodium acetate by silicic acid chromatography. A column of silicic acid (Unisil; 7 g, 29 by 0.54 cm) was loaded with 2.5 mg of lipid and eluted with 50-ml portions each of chloroform (neutral lipid), acetone, begun at arrow I (glycolipid), and methanol, begun at arrow II (phospholipid) at a flow rate of 1.1 ml/min. The 2-ml fractions were collected, the solvents were removed, and the ^{14}C was determined in the scintillation spectrophotometer. The recovery of ^{14}C-labeled lipid added to the column was 92%.*

TABLE 1. *Reproducibility of lipid fractionation*[a]

Lipid class	^{14}C cpm/ml ± standard error	Lipid glucose (μg ± standard error)
Neutral lipid	72,400 ± 9,300	2.37 ± 0.09
Glycolipid	160,000 ± 26,900	16.7 ± 1.4
Phospholipid	23,000 ± 1,060	<0.01
Total lipid recovered	255,000 ± 18,200	19.2 ± 0.08

[a] A total of 1,000 6.5-mm disks punched randomly from leaves of *Quercus virginiana* incubated for 1 week in Apalachicola Bay were suspended in 20 ml of estuarine water containing 50 μCi of [1-^{14}C]sodium acetate for 24 h at 25°C. The disks were collected, the lipids were extracted, dehydrated, and divided into five equal portions, and each portion was applied to 1.5-g silicic acid columns (1 by 10 cm). Fractions were eluted with 50 ml of chloroform (neutral lipids), 200 ml of acetone (glycolipids and acidic phospholipids), and 10 ml of methanol (phospholipids). Each fraction was recovered, and the solvent was evaporated and resuspended in 10 ml of methanol-toluene (1:1). The radioactivity was determined in the scintillation spectrometer and reported as counts per minute per milliliter. A total of 287,000 cpm of ^{14}C-labeled lipid was added to each of the five columns. Lipid glucose was measured with phenol (13) on each fraction. A total of 19.7 μg of lipid glucose was added to the column.

Phospholipid analysis. The methanol fraction containing the phospholipids was deacylated by mild alkaline methanolysis (24), and the water-soluble glycerol esters were separated by two-dimensional chromatography on cellulose thin-layer plates (Eastman 6064), using solvents described by Short et al. (22). ^{14}C-labeled esters were located by radioautography using Kodak NoScreen X-ray film; the corresponding areas cut out, and the radioactivity was determined as described below. Tentative identification of the glycerol esters was made by comparison of chromatographic mobilities with lipids from *Micrococcus denitrificans* (28).

Determination of radioactivity. The lipids or the cellulose powder containing the lipids were dissolved or suspended in a scintillation fluid of 9.28 mM 2.5 bis-[tert-butyl-benzoxazolyl(2')]-thiophen (in toluene) and counted in a Packard Tri-Carb scintillation spectrophotometer. Correction for quenching was made by the channels ratio method.

RESULTS

Rates of lipid synthesis. Rates of lipid synthesis by the detrital microflora have been shown to correlate with measures of microbial activity, enzyme activities, oxygen respiration, and adenosine 5'-triphosphate (ATP) levels (25). Since different detrital substrates are degraded at rates (14) showing different levels of microbial activities, the rates of lipid synthesis by the microflora might be expected to show differences. In the data of Fig. 2, rates of lipid

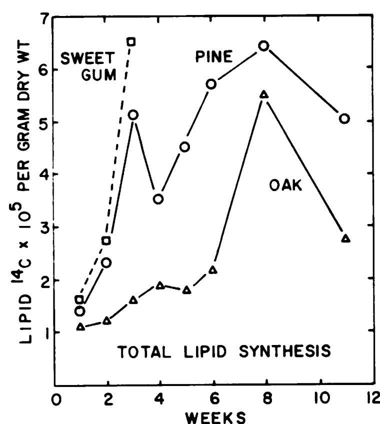

FIG. 2. *Rates of lipid synthesis by the detrital microflora on three types of detritus. Leaf material (58 to 70 mg, dry weight) or pine needle segments (64 to 107 mg, dry weight) were recovered from Apalachicola Bay at weekly intervals and were incubated with 4 μCi of [1^{14}C]sodium acetate at 25°C for 2 h in 2 ml of estuarine water. The lipids were extracted and recovered, and the chloroform was removed. The radioactivity of the lipids was measured in the scintillation spectrometer. The sweet gum leaves disintegrated after the third week of incubation in Apalachicola Bay.*

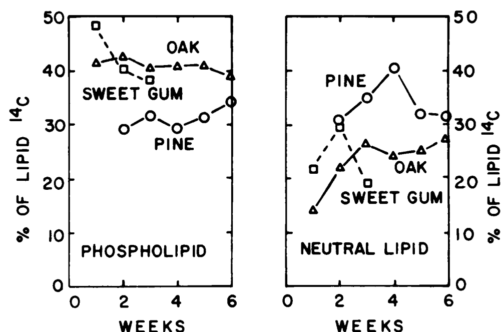

FIG. 3. *Proportions of the phospholipids and neutral lipids in the detrital microflora incubated in Apalachicola Bay. Detritus was recovered from the estuary and incubated with [1-^{14}C]sodium acetate for 2 h at 25°C, and the lipids were extracted and separated into lipid classes as in Fig. 1. The ^{14}C in each lipid class was determined. Recoveries of ^{14}C-labeled lipid added to the silicic acid columns ranged between 66 and 96%.*

synthesis were highest for sweet gum, intermediate for pine needles, and lowest for oak leaves incubated in Apalachicola Bay, Fla. The sweet gum leaves disintegrated beyond use after 3 weeks.

Lipid composition of the detrital microflora. The lipids recovered during the first 6 weeks of the experiments illustrated in Fig. 2 were fractionated into neutral lipids, glycolipids, and phospholipids by silicic acid chromatography as in Fig. 1.

The proportion of the lipids in the phospholipid and neutral lipid classes were distinctly different between the flora on the pine and the oak detritus, with the pine flora having a higher neutral lipid and lower phospholipid composition (Fig. 3). The glycolipid content of all three microfloral assemblies ranged between 30 and 40%. There were clear differences in lipid class proportions between the different types of detrital substrates. There were also significant changes in lipid composition on one type of detrital substrate as the microfloral populations changed with incubation in the bay. The proportion of phospholipid fell in the sweet gum microflora; the proportion of neutral lipids in the oak microflora rose from 15% to maintain a relatively constant 25 to 27% of the total lipids with longer incubation.

Lipid composition and microflora composition. If changes in the proportions of the major lipid classes in the detrital flora indicate changes in the microfloral population, one might predict that slower-growing, morphologically more complex microorganisms should change the proportions of major lipid classes if

the incubation period with the [1-^{14}C]sodium acetate is lengthened. Bacteria are generally considered the most rapidly growing component of the microflora, being the initial colonizers of new surfaces added to the environment (18), and the bacterial lipids are characterized by a very high proportion of phospholipids (12). Actinomycetes and fungi contain higher proportions of metabolically active glycolipids than do bacteria (2).

The proportions of ^{14}C in the major lipid classes in oak detritus incubated in St. Marks National Wildlife Refuge for 2 weeks and then allowed to incubate with [1-^{14}C]sodium acetate for 2 and 24 h were measured (Table 2). Even though there was only a doubling in the total ^{14}C-labeled lipid in the 24-h incubation, the proportion of phospholipid was decreased threefold. There was a slight increase in the proportions of glycolipids.

Metabolism of the major lipid classes. One of the most effective ways to study the metabolism of lipids in growing microorganisms is to examine the replacement of ^{14}C in the lipids by ^{12}C after a short period of growth in the presence of ^{14}C-labeled precursors (27). An experiment using a 6-h pulse with [1-^{14}C]sodium acetate followed by a chase for 140 h in the absence of radioactivity is illustrated in Fig. 4. The turnover of muramic acid is illustrated in the upper portion of Fig. 4. Data taken from King and White (15) show a biphasic turnover with a rapid-phase $t_{1/2}$ of 3.2 h and a slower phase with a $t_{1/2}$ of 78.3 h. In the lower figure, the loss of ^{14}C from the major classes of lipids is illustrated. The loss of ^{14}C from the glycolipids was exponential, with a $t_{1/2}$ of 72 h, remarkably like the muramic acid. Phospholipid ^{14}C disappeared during the chase period exponentially, with a $t_{1/2}$ of 120 h. The neutral lipid showed a biphasic exponential loss of ^{14}C with a rapid-phase $t_{1/2}$ of 33 h and a slow-phase $t_{1/2}$ of 190 h.

Metabolism of the phospholipids. The phospholipid portion of the total lipids from the experiment illustrated in Fig. 4 was recovered and deacylated, and the water-soluble glycerolphosphate (GP) esters were separated chromatographically. Glycerol esters with chromatographic mobilities corresponding to glycerolphosphorylglycerol (GPG) derived from phosphatidylglycerol (PG), 19.2%; glycerolphosphorylethanolamine (GPE) derived from phosphatidylethanolamine (PE), 10.9%; glycerolphosphoryldimethylethanolamine (GPDME) derived from phosphatidyldimethylethanolamine, 2.4%; bisglycerolphosphorylglycerol

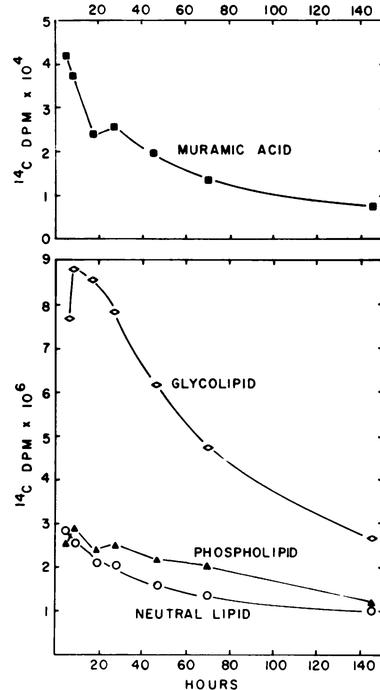

FIG. 4. *Loss of ^{14}C from muramic acid and the major classes of lipids by the estuarine detrital microflora incubated with [1-^{14}C]sodium acetate for 6 h and resuspended in estuarine water not containing ^{14}C. Approximately 230 g (dry weight) of 6.5-mm disks of oak leaves that had been incubating in the East River of the St. Marks National Wildlife Refuge for 3 weeks was recovered and then incubated with 1 mCi of [1-^{14}C]sodium acetate, and the radioactivity was maintained at 2 × 10^9 to 1.6 × 10^9 cpm for 6 h at 28°C. After this, the radioactivity was washed out with estuarine water and incubation was continued for 140 h. Periodic samples of 200 disks (about 0.86 g, dry weight) chosen randomly were recovered and extracted, and the lipid classes were separated chromatographically as in Fig. 1. The upper figure represents data taken from King and White (15).*

TABLE 2. *Effects of incubation time on the proportions of major lipid classes synthesized by oak leaf detrital microflora incubated in a North Florida estuary*

Lipid class	Percent of total lipid after:	
	2-h incubation	24-h incubation
Neutral lipid	9[a]	28
Glycolipid	57	63
Phospholipid	34	9

[a] Approximately 2.4 g (dry weight) of oak leaf disks was incubated in the presence of 50 μCi of [1-^{14}C]sodium acetate in 25 ml for 2 or 24 h at 25°C. The lipids were extracted and fractionated as described in Table 1. Recovery was 93% (2 h) and 90% (24 h). The total ^{14}C incorporated was 105,000 cpm/100 mg (dry weight) for 2 h and 179,000 cpm/100 mg (dry weight) for 24 h.

(GPGPG) derived from cardiolipin (CL), 1.6%; glycerolphosphorylcholine (GPC) derived from phosphatidylcholine (PC), 1.5%; glycerolphosphorylserine (GPS) derived from phosphatidylserine (PS), 1.1%; methylglycerolphosphate, usually an artifact of mild alkaline methylation, 0.6%; and GP derived from phosphatidic acid (PA), 0.4%, were found. These lipids accounted for 38% of the lipids spotted on the plate. A total of 56% of the ^{14}C moved with the solvent fronts, representing unhydrolyzed or partially methylated lipids; 0.7% remained at the origin, 6.5% moved with the first but not the second solvent, and 0.7 and 0.6% represented glycerol esters derived from unknown lipids. The deacylation by mild alkaline methanolysis removed 94.4% of the ^{14}C from the phospholipid fraction in the form of fatty acid methyl esters.

The metabolism of the water-soluble GP esters derived from the phospholipids in the pulse-chase experiment is illustrated in Fig. 5. PG showed a monophasic exponential loss of ^{14}C, with a $t_{1/2}$ of 110 h. GPE showed an early increase, followed by a loss of ^{14}C paralleling GPG. GPC and GPS both showed initial slow increases in ^{14}C which remained stable. GPGPG showed a 40-h lag before a just less than doubling of the ^{14}C content. The GPGPG then slowly lost ^{14}C.

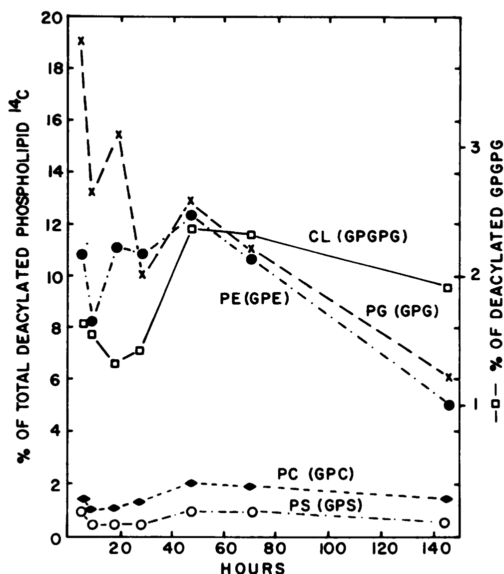

FIG. 5. *Loss of ^{14}C from the glycerolphosphoryl ester backbones of the phospholipids synthesized by the estuarine detrital microflora. The phospholipid portion of the lipids derived from the experiment illustrated in Fig. 4 were deacylated, and the water-soluble glycerol phosphoryl esters were separated by two-dimensional chromatography in cellulose thin-layer plates (22). The esters were localized by radioautography (24), and the radioactivity was determined in the scintillation spectrophotometer. Esters with the chromatographic mobilities of authentic lipids from Micrococcus denitrificans (28) are deacylated as GPG derived from PG, GPGPG derived from CL, (GPE) derived from PE, GPC derived from PC, and GPS derived from PS. The ^{14}C in the GPGPG is plotted using the right-hand ordinate for clarity. See text for abbreviations.*

DISCUSSION

Lipid composition as evidence of community structure. Since rates of lipid synthesis by the detrital microflora correlate with other measures of microbial activities (25), the differences in synthetic rates on different types shown in Fig. 2 suggest that different populations or at least different activities exist on the three types of detritus. Succession of the estuarine detrital microflora on one type of detritus has been suggested by changes in ATP levels, muramic acid content, respiratory activity, heterotrophic potential, esterase activity, and morphology by scanning electron microscopy (R. E. Bechtold, M.S. thesis, and R. J. Bobbie, M.S. thesis, Florida State University, Tallahassee, 1976; 18). The postulated succession is also reflected in the changes in the proportions of the neutral lipids and phospholipids synthesized during the incubation in the estuary (Fig. 3).

The prediction that labeling slower-growing components of the microflora would shift the major lipid class proportions toward that expected for the actinomycetes, fungi, or algae has been borne out (Table 2), again showing that even at the relatively crude level of lipid class separations, differences in the structure of the detrital microflora can be detected.

Examination of the phospholipid composition. The glycerolphosphoryl esters derived from the phospholipids are found in proportions typical of bacteria with high levels of GPE, GPG, and GPGPG. The GPDME suggests the presence of the typical bacterial methylation pathway of PC synthesis (7). The presence of GPC suggests the activity of bacteria with highly organized membrane systems (8) and efficient electron transport systems (10). The proportion of gram-positive organisms could be estimated from the presence of amino acid esters of PG (7). These aminoacyl PG lipids must be separated as intact lipids as the mild alkaline methanolysis splits off the amino acids, and these lipids would be detected in the GPG fraction (26).

An estimate of the gram-positive population

can be made by using the proportions of the glycosyldiglyceride content, since glycosyldiglycerides are characteristic of these microorganisms (20). The oak leaves used in these experiments contained about 80 μg of muramic acid per g (dry weight) of leaves (15). Assuming 6.4 mg of muramic acid per g (dry weight) of bacteria (16), the microbial population represented 12.5 mg of bacteria per g (dry weight) of leaves. *Staphylococcus aureus* contains 2.6 μg of lipid glucose per mg (dry weight) of bacteria (26). If half the 19.2 ± 0.08 μg of glycolipid per g (dry weight) of leaves were glycosyldiglyceride, the maximum *S. aureus*-like organisms would represent 3.4 mg or at most 30% of the 12.5 mg of bacteria present. Preliminary analysis of deacylated glycolipids from this microbial population show that this may represent a reasonable estimate.

The fact that 56.4% of the ^{14}C remained at least partially resistant to mild alkaline methanolysis indicates that glycerol ethers, plasmologens, and phosphosphingolipids may be present, which suggests that other types of bacteria are active (7, 12, 19).

Lipid metabolism and the state of the detrital microflora. Pulse-chase experiments can give insight into the detailed metabolism of microbial lipids (27). The data of Fig. 4 show that the turnover of ^{14}C in the phospholipids and neutral lipids is slower than in the glycolipids. The monophasic exponential rate of glycolipid turnover parallels the slow phase of the muramic acid turnover. The rate of neutral lipid turnover is biphasic and much slower than glycolipid turnover. In work with rapidly growing bacterial monocultures, correlations between physiological growth states and turnover of the GP esters derived from the phospholipids have been reported (27). The turnover of the GP esters derived from the phospholipids of the detrital microflora are illustrated in Fig. 5. Correlations between rapidly growing monocultures of gram-negative chemoheterotrophs and a slowly growing detrital microflora must be considered very tentative at this stage of the research. GPC shows little turnover, as in some gram-negative bacteria (22, 28); GPE in many gram-negative monocultures shows a slow metabolism (17, 21, 22, 27, 28); but in the detrital microflora after saturation of the precursor pool (40 h), the GPE turnover parallels that of GPG. The lipid with the most active metabolism in most eubacteria is GPG. Apparently this is true in the detrital microflora as well (Fig. 5). It is interesting that if the pulse is less than 25% of the generation time in *Haemophilus parainfluenzae* growing under different conditions with different rates of GPG turnover, there is a lag in the full saturation of the precursor GPGPG (27). The more rapid the turnover, the shorter the lag. With $t_{1/2}$ values for GPC turnover of 47, 118, and 150 min, the lag periods to saturation of GPGPG were <10, 70, and 90 min. With a pulse period of 1.3 generations, there was no detectible lag in the saturation of GPGPG. With the detrital microflora and a pulse period of 6 h, the $t_{1/2}$ of GPG turnover was 100 h and the lag to saturation of the GPGPG was 40 h, suggesting that organisms with metabolism like that of *H. parainfluenzae* in the detrital microflora had at least a 60-fold-slower response. The generation time thus must be more than 24 h. A slow generation time for a significant portion of the detrital microflora has been postulated from the rates of phoslipid synthesis, the rates of respiration per muramic acid or ATP content (18), and the slow rates of turnover of the muramic acid (15).

The enormous body of information on the changes in microbial lipids induced by changing cultural parameters in laboratory monocultures possibly can be utilized to help dissect the metabolic complexities of the microfloral communities as they exist in nature. Examination of the composition and metabolism of the lipids formed in intact microbial assemblies may well offer an increasingly precise understanding of the community structure, interactions, and succession, especially if studies can be directed at lipids unique to specific components of the microbial population that can be examined and correlated with other measures of microbial activities.

ACKNOWLEDGMENTS

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