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N-Methyl Groups in Bacterial Lipids

III. Phospholipids of Hyphomicrobia

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The phospholipids of *Hyphomicrobium vulgare* NQ-521 have been separated by preparative thin-layer chromatography and analyzed by paper chromotography of the water-soluble products of acid and mild alkaline hydrolysis. The principal phospholipids are phosphatidyl ethanolamine (23%), phosphatidyl *N*,*N'*-dimethylethanolamine (36%), lecithin (29%), and phosphatidyl glycerol (10%). Three other strains of *Hyphomicrobium* were found to have similar phospholipid compositions. Growing cells incorporated the methyl group of methionine into lipid-bound *N*,*N'*-dimethylethanolamine and choline. Experiments with sonic extracts of *H. vulgare* NQ-521 and ^{14}C (methyl) *S*-adenosylmethionine demonstrated the formation of phosphatidyl *N*-monomethylethanolamine in addition to the dimethylethanolamine and choline phosphatides.

The extensive examination of bacterial lipids in many laboratories has shown that lecithin, a widely distributed and quantitatively important phosphatide of higher organisms, is generally not present in bacteria (22, 26). There are, however, exceptions to this generalization. Choline was found in hydrolysates of the lipids of *Agrobacterium tumefaciens* by Geiger and Anderson (9), and the presence of lecithin in this organism was confirmed by Kaneshiro amd Marr (25). An investigation of the incorporation of the methyl group of methionine into the lipids of 21 species of bacteria revealed the formation of lecithin in two other species of *Agrobacterium*, *A. radiobacter* and *A. rhizogenes* (14). None of the other species, mostly of the order *Eubacteriales*, appeared to be capable of synthesizing lipid-bound choline de novo, but the incorporation of the methyl group of methionine into lipid-bound *N*-methylethanolamine was noted in *Clostridum butyricum* (11, 14) and *Proteus vulgaris* (14).

Substantial amounts of lecithin have been found in photosynthetic bacteria. *Rhodospeudomonas spheroides* contains approximately 25% lecithin among its phosphotides (15, 27); *R. capsulata* and *R. palustris* also contain this lipid (37). A similar amount of lecithin (27%) was recently found in *Rhodomicrobium vannielii* (31).

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Photosynthetic bacteria are noted for their content of complex intracytoplasmic membrane systems, in contrast to the paucity of these membranes in heterotrophic bacteria (29, 34). We undertook an examination of the phospholipids of two species of nitrifying bacteria and of *Hyphomicrobium*, all known to contain elaborate intracellular membranes (6, 30). Lecithin was found in one of the nitrifiers, *Nitrosocystis oceanus*, and in several strains of *Hyphomicrobium* (18). The present paper describes in detail our investigations on the lipid composition of hyphomicrobia and on the formation of lecithin in these organisms.

MATERIALS AND METHODS

Cultures and growth conditions. Two strains of *H. vulgare*, NQ-521 and ZV-580, and two unnamed *Hyphomicrobium* strains, H-526 and M-552, were obtained from P. Hirsch. They were grown on medium no. 337 containing methylamine hydrochloride, 0.135%, as the carbon source (21). The cells were grown for 4 days at 30 C in the dark in 2-liter indented Erlenmeyer flasks containing 500 ml of medium per flask, shaken rapidly on a Brunswick rotary shaker in order to provide good aeration. Attempts to grow the cells in standard 2-liter Erlenmeyer flasks under the same conditions yielded much less cell mass, even with strong aeration. Growth was found to proceed in the manner described by Hirsch and Conti, i.e., a lag phase of 1 day, followed by rapid growth during the next 24 to 48 hr, followed by a rapid cessation of growth (20). Cultures were monitored by microscopic examination in wet mounts and after staining with crystal violet. The cells showed the morphology described by Hirsch and Conti (20).

Isolation of lipids. The cells, harvested by centrif-

ugation, were washed once with cold 0.9% saline solution and suspended in a volume of chloroform-methanol (2:1, v/v) sufficient to form a single-phase system with the water from the cells, i.e., greater than 15 ml of chloroform-methanol per g of wet cells. The cells were then extracted as described previously (13). To remove nonlipid contaminants, the lipids were washed by the procedure of Folch et al. (8) with 0.05 N sodium chloride.

Since some strains were found to contain substantial amounts of a material slightly soluble in chloroform and insoluble in benzene [presumably poly-β-hydroxybutyric acid (20, 21)], the lipids were dried and taken up in benzene to remove this material prior to column chromatography.

Thin-layer chromatography. As a first step in identification, and in order to separate the individual polar lipids, the phospholipids were anaylzed by preparative thin-layer chromatography as described previously (2). The chromatograms on silica gel G were developed in chloroform-methanol-7 N NH_4OH (60:35:5, v/v/v).

Acid hydrolysis of lipids and paper chromatography of water-soluble products. The total phosphatides and fractions isolated from thin-layer plates were hydrolyzed overnight at 100 C in 1 N HCl in tightly closed, Teflon-lined, screw-cap tubes. The water-soluble products were obtained after petroleum ether extraction by evaporation of the aqueous phase. Ascending paper chromatography, on Whatman no. 1 filter paper, was carried out in the following solvent systems: solvent 1, phenol-*n*-butyl alcohol-formic acid (80%)-water (50:50:3:10, w/v/v/v), saturated with solid KCl and used with paper which had been previously dipped into 1 N KCl and dried (5); solvent 2, ethyl alcohol-ammonia-water (90:5:5, v/v/v); solvent 3, ethyl alcohol-acetic acid-water (90:5:5, v/v/v).

The ninhydrin spray reagent was purchased from Sigma Chemical Co. (St. Louis, Mo.). Choline and dimethlyethanolamine were revealed by exposing the chromatograms to iodine vapors (4) and, after the iodine stain had disappeared, by spraying with Dragendorff's reagent (3).

Deacylation and paper chromatography of water-soluble products. The total phosphatides and fractions isolated from thin-layer plates were hydrolyzed in dilute alkali according to the procedure of Tarlov and Kennedy (33). The water-soluble products thus obtained were chromatographed on Whatman no. 1 filter paper, ascending, in the following solvent systems: solvent 4, *n*-butyl alcohol-acetic acid-water (5:4:1, v/v/v); solvent 5, phenol-water-acetic acid-ethyl alcohol (50:22:3:3, w/v/v/v) (17). Phosphate esters on paper chromatograms were revealed by dipping in either the reagent of Hanes and Isherwood (19) or that of Wade and Morgan (35).

Analytical procedures. The technique used for phosphorus analysis of lipids has been described (14). Protein was measured by a biuret assay (16).

Radioactivity assays. Lipids and water-soluble materials were counted in a Nuclear-Chicago Corp. (Des Plaines, Ill.) Mark I scintillation spectrometer by methods previously described (13). Paper and thin-layer chromatograms were scanned for radioactivity in a Packard Radiochromatogram scanner.

Preparation of cell-free extracts. H. vulgare NQ-521 was grown, harvested, and washed as described above. The cells were then suspended in 30 ml of tris(hydroxymethyl)aminomethane (Tris)-chloride, *p*H 7.5 (0.05 M) per 5 g of wet cells and subjected to full power in a Raytheon 10-kc sonic oscillator for a total of 6 min, using cycles of 1.5 min (on) and 1.0 min (off) with ice water constantly running, in order to avoid overheating the extracts. The suspension was then centrifuged once at 6,000 $\times$ *g* for 15 min. The supernatant solution was withdrawn and recentrifuged at 12,000 $\times$ *g* to minimize whole-cell contamination. The supernatant extracts were stored at -15 C.

Assay for incorporation of ^{14}C (methyl) S-adenosylmethionine. This was performed in most experiments by extraction of lipid with chloroform-methanol (2:1) after acidification with HCl as described by Kaneshiro and Law (24). In the time-course experiment, a less specific assay, involving precipitation of the product on discs of filter paper with cold trichloroacetic acid followed by washing with cold water, was utilized (12).

Materials. Silica gel G was obtained from Brinkmann Instruments, Inc. ^{14}C-methyl L-methionine was obtained from New England Nuclear Corp. (Boston, Mass.). ^{14}C-methyl *S*-adenosyl L-methionine was the generous gift of John H. Law. Phosphatidyl ethanolamine was isolated from *Escherichia coli* (2). Phosphatidyl choline was obtained from several commercial sources. Phosphatidyl *N*,*N'*-dimethylethanolamine was the generous gift of Erich Baer.

RESULTS

Growth of Hyphomicrobium strains in the presence of ^{14}C (methyl) L-methionine. To detect the formation of lecithin, or its immediate precursors in the pathway from phosphatidyl ethanolamine, the incorporation of the labeled methyl group of methionine into the phospholipid bases of growing cells was examined (14). Table 1 shows the results of such an experiment with four strains of *Hyphomicrobium*. Of the label added to the medium, 10 to 15% was incorporated into the water-soluble fraction isolated after acid hydrolysis of the lipids. The four strains contained varying amounts of chloroform-methanol soluble

TABLE 1. *Incorporation of ^{14}C (methyl) L-methionine into hyphomicrobia lipids*

Hyphomicrobium strain[a]	Dry wt of cells	^{14}C in water-soluble hydrolysis products
	mg	*counts/min*
H-526	55.8	26,200
M-552	64.8	28,000
NQ-521	112.3	26,000
ZV-580	109.1	40,100

[a] Cells were grown in 500 ml of medium containing 275,000 counts/min of ^{14}C (methyl) L-methionine, 13.2 μc/μmole.

material that was insoluble in benzene. This was presumed to be poly-β-hydroxybutyric acid. After correction for this material, the benzene-soluble lipid represented from 7.1 to 10.5% of the dry weight of these cells.

The identity of these labeled bases was determined by paper chromatography in three solvent systems (Table 2). The radioactivity, as revealed by radioautography, migrated with the same R_F as choline and *N,N'*-dimethylethanolamine in all three solvent systems. Only in solvent 1 was there a separation of the two labeled bases. These results indicated that the lipids contained phosphatidyl *N,N'*-dimethylethanolamine in addition to phosphatidyl choline. Sufficient material was present for these radioactive bases to be visualized by staining with both iodine vapor and Dragendorff's reagent. Ethanolamine was also seen in the chromatograms of the bases from all four strains, on staining with ninhydrin.

Isolation and identification of individual lipids from H. vulgare NQ-521. For these analyses, cells were grown in 11 cultures of 500 ml each. The cells were harvested after 4 days of growth and the lipids were extracted from the wet cells. Dry weight of the extracted cells plus lipid was 1.094 g. (This does not include the material removed by dilute salt solution from the chloroform-methanol extracts.) Dry weight of the chloroform-methanol extractable lipid was 0.113 g, or 10.3% of the total dry weight. Of this, 0.086 g was soluble in benzene (7.9% of the total dry weight).

The total benzene-soluble lipid was chromatographed on a 4.5-g column of silicic acid in order to separate the lipids into groups (Table 3). The fraction eluted with chloroform-methanol, 2:1, was presumed to contain most of the phospholipid and was analyzed by preparative thin-layer chromatography. Some phospholipid does come through with acetone, but this fraction was not analyzed further since it does not contain phosphatidyl choline or phosphatidyl ethanolamine. The presence of glycolipids, which would ordinarily appear in this fraction, has not been studied.

Preparative thin-layer chromatography. When the phospholipid fraction from the column chromatogram was streaked along the origins of several plates of silica gel G and chromatographed in chloroform-methanol-ammonia, three principal bands were detected by staining with iodine vapors. These bands, I, III, and IV, were found to contain 99% of the total phospholipid phosphorus recovered from the plates (Table 4). Band I chromatographed alongside a standard of phosphatidyl choline and Band III alongside a standard of phosphatidyl ethanolamine. The lipids separated by this procedure were subjected to deacylation by mild alkaline hydrolysis and to acid hydrolysis, and the resulting water-soluble products were chromatographed on paper (Tables 5 and 6).

Alkaline hydrolysis of Band I yielded a phosphodiester which chromatographed with the same R_F as glycerylphosphorylcholine in both solvent systems. In solvent 5, some material migrating with the R_F of glycerylphosphorylethanolamine was also observed. Some inorganic phosphorus

TABLE 2. *Paper chromatography of water-soluble products of acid hydrolysis: lipids from hyphomicrobia grown with ^{14}C (methyl) L-methionine*

Solvent	Standard	R_F of standard	R_F of hydrolysis products			
			H	M	NQ	ZV
1[a]	Ethanolamine-HCl[b]	.09	.09	.09	.08	.08
	N-methylethanolamine-HCl	.23	—	—	—	—
	N,N'-dimethylethanolamine-HCl[c]	.40	.42	.43	.41	.42
	Choline-Cl[c]	.52	.55	.55	.55	.55
2[d]	Choline-Cl[c]	.47	.47	.46	.46	.47
3	Ethanolamine-HCl	.42	.4[e]	.4[e]	.4[e]	.4[e]
	N-methylethanolamine-HCl	.43	—	—	—	—
	N,N'-dimethylethanolamine-HCl[c]	.51				
	Choline-Cl[c]	.50	.55	.54	.54	.50

[a] The R_F values in this run were lower than usually observed with this solvent system (5). See Table 6 for usual R_F values in this system.

[b] R_F values obtained by staining lower half of chromatograms with ninhydrin.

[c] For comparison with the standard, R_F values of hydrolysis products were obtained by staining upper halves of chromatograms with iodine vapors followed by Dragendorff's reagent; all of these were shown to be radioactive by radioautography.

[d] Bases other than choline-Cl tend to be volatile in this system.

[e] R_F approximate because spots were cut in half in order to stain as described above.

also appeared to be present (Table 5). Acid hydrolysis yielded an iodine-staining compound which migrated with the same R_F as choline. Thus, Band I is identified as phosphatidyl choline with some contaminating phosphatidyl ethanolamine.

Alkaline hydrolysis of Band III yielded a phosphodiester which chromatographed alongside glycerylphosphorylethanolamine in both solvent systems (Table 5). In both cases, it was stained by ninhydrin as well as by the phosphate stain. Acid hydrolysis of Band III yielded a compound which stained with ninhydrin and had the same R_F as ethanolamine in solvent 1 (Table 6). Thus, band III is identified as phosphatidyl ethanolamine.

Alkaline hydrolysis of Band IV yielded two phosphodiesters which chromatographed with the same R_F as glycerylphosphorylglycerol and glycerylphosphorylcholine or glycerylphosphoryldimethylethanolamine in solvent 4. In addition, some phosphate-staining material was present which migrated with the R_F of inorganic phosphate. In solvent 5, in addition to the inorganic phosphate spot, only one phosphodiester was revealed and it had the same R_F as either glycerylphosphorylcholine or glycerylphosphoryldimethylethanolamine (Table 5). On acid hydrolysis the lipid in Band IV gave an iodine-staining water-soluble product with the R_F of dimethylethanolamine in solvent 1 (Table 6). It also gave the same R_F as dimethylethanolamine in solvent 3; however, choline has the same R_F in this system (R_F = 0.62). On the basis of the result obtained on acid hydrolysis, we tentatively concluded that Band IV contained the dimethylethanolamine which we observed to be labeled by ^{14}C-(methyl) methionine in the lipids of cells grown with this compound. The alkaline deacylation products did not help to distinguish between phosphatidyl choline and phosphatidyl *N,N'*-dimethylethanolamine; however, phosphatidyl choline (Band I) is well separated from Band IV on the thin-layer

TABLE 3. *Separation of Hyphimicrobium vulgare NQ-521 lipids on silicic acid*

Eluant	Lipid	Recovered lipid
	mg	%
Chloroform (350 ml)	10.2	10.8
Acetone (200 ml)	9.9	10.5
Chloroform-methanol (300 ml), 2:1, v/v	71.1	75.3
Methanol	3.2	3.4
Total	94.4	

TABLE 4. *Preparative thin-layer chromatography of Hyphomicrobium vulgare NQ 521 phospholipids*[a]

Fraction	Approximate R_F	Phosphorus	Phospholipid phosphorus
		μmoles	%
I	0.3	12.3	28.7
II	0.37	0.36	0.8
III	0.45	9.9	23.1
IV	0.6	20.3	47.3
Total		42.86	

[a] A total of 44 μmoles of lipid phosphorus was put on four thin-layer plates. Recovery = 97%. Standards of phosphatidyl ethanolamine, R_F = 0.41, and phosphatidyl choline, R_F = 0.29, were run alongside the unknowns.

TABLE 5. *Paper chromatography of deacylation products of lipids separated by thin-layer chromatography*

Deacylation, product[b]	Standard	Solvent 4, deacylation of fraction:[a]			Standard	Solvent 5, deacylation of fraction:[a]		
		I	III	IV		I	III	IV
GP	1.0				1.0			
GPG	0.87			0.87	1.21			
GPGPG	0.40				0.68			
GPE	0.57[c]		0.54[c]		1.62	1.66	1.70[c]	
GPDME	1.40	1.36		1.36	~2.0[d]	~2.0[d]		1.95
GPC	1.36							
P_i	1.05	1.12		1.18	0.59	0.57		0.58

[a] All values relative to glycerophosphate.

[b] GP = α-glycerophosphate; GPG = glycerylphosphorylglycerol; GPGPG = deacylation product of cardiolipin; GPE = glycerylphosphorylethanolamine; GPDME = glycerylphosphoryldimethylethanolamine; GPC = glycerylphosphorylcholine.

[c] Stained with ninhydrin and phosphate reagent. All other spots revealed by phosphate reagent alone.

[d] Streak.

TABLE 6. *Paper chromatography in Solvent 1 of acid-hydrolysis products of lipids from thin-layer chromatograms*

Standard	R_F of standard	R_F of product fraction		
		I	III	IV
Ethanolamine-HCl	0.13[a]			
N-methylethanolamine-HCl	0.38[a]		0.13[a]	
N,N'-dimethylethanolamine-HCl	0.61[b]			0.61[b]
Choline-Cl	0.73[b]	0.74[b]		

[a] Revealed by staining with ninhydrin.
[b] Revealed by staining with iodine vapor.

plate. In addition, it appeared that Band IV also contained phosphatidyl glycerol. This phosphatide is known to move faster than phosphatidyl ethanolamine in the thin-layer chromatography system used (2).

To determine the proportion of phosphatidyl glycerol and phosphatidyl *N,N'*-dimethylethanolamine in Band IV, the remaining lipid (14 μmoles of P) was streaked on two thin-layer plates of silica gel G and developed in benzene-pyridine-water, 60:60:10, v/v (2). Approximately 90% of the lipid phosphorus was recovered from the plate. Of the recovered phosphorus, 21.4% migrated with an R_F of 0.5, corresponding to that of phosphatidyl glycerol, and 75.5% migrated with an R_F of 0.28, corresponding to that of phosphatidyl *N,N'*-dimethylethanolamine.

Analysis of the total phosphatides of *H. vulgare* NQ-521 for plasmalogen (36) did not reveal detectable amounts of vinyl ether-containing phosphoglycerides.

Analysis of phospholipids of other hyphomicrobia strains. Cells of the other three strains of *Hyphomicrobium* were grown in two 500-ml lots each, as described above. The lipids were extracted and chromatographed by preparative thin-layer chromatography. Each band stained by iodine was eluted and analyzed for lipid phosphorus. The recovery of lipid phosphorus in the bands analyzed ranged from 88 to 96%. The results of these analyses are given in Table 7.

Methylation of lipids by S-adenosylmethionine in extracts. The formation of lecithin by the stepwise methylation of phosphatidylethanolamine has been shown to occur with enzymes from liver (5, 10), fungi (32), protozoa (28), and bacteria (*A. tumefaciens*; reference 24). In each case, *S*-adenosylmethionine has been shown to serve as the methyl donor. We examined the ability of sonic extracts of *H. vulgare* NQ-521 to carry out these reactions. Incorporation of ^{14}C from ^{14}C (methyl) *S*-adenosyl L-methionine into lipid during 40-min incubations was found to be proportional to the amount of extract added over the range 2.0 to 10.5 mg of protein per ml of incubation mixture. Sonic extracts were centrifuged twice, once at 6,000 × *g*, and once at 12,000 × *g*, in order to minimize whole-cell contamination. The extracts were rountinely examined microscopically.

The time-course of incorporation of label from *S*-adenosylmethionine was examined by use of a filter-paper disc assay (12) and was found to be linear for up to 120 min. This method measures incorporation of label into the total cold trichloroacetic acid- and cold water-insoluble fraction. Control experiments after 30-min incubations showed that 53% of the incorporation measured by the filter-paper disc assay was into chloroform-methanol-extractable lipid.

The dependence of the incorporation of label into lipid on added *S*-adenosylmethionine is shown in Fig. 1. A double-reciprocal plot shows the apparent K_m for *S*-adenosylmethionine to be 1.9×10^{-5} M.

Products of in vitro methylation of lipid. The chloroform - methanol - extractable radioactive products were examined by thin-layer chromatography (Fig. 2). There was radioactivity in each of the major lipid fractions, and two of these coincided with phosphatidyl choline and phosphatidyl ethanolamine standards. The middle radioactive peak, which migrated alongside phosphatidyl ethanolamine, was assumed to be phosphatidyl *N*-methylethanolamine, which has the same migration behavior as phosphatidyl ethanolamine in this solvent system (2). More than half of the radioactivity coincided with the lipid fraction that migrates faster than phosphatidyl ethanolamine. This fraction has been shown to contain a mixture of phosphatidyl glycerol and phosphatidyl *N,N'*-dimethylethanolamine (*see above*). Since phosphatidyl glycerol is

TABLE 7. *Thin-layer chromatographic separation of hyphomicrobia phospholipids*

Strain	Percentage of phospholipid phosphorus		
	Band I[a]	Band III[a]	Band IV[a]
H-526	33	19	41
M-552	35, 36	19, 20	43, 44
ZV-580	31, 33	11, 13	51, 55
NQ-521	29	23	10[b], 36[c]

[a] Band I, phosphatidyl choline; Band III, phosphatidyl ethanolamine; Band IV, phosphatidyl glycerol plus phosphatidyl dimethylethanolamine.
[b] Phosphatidyl glycerol.
[c] Phosphatidyl dimethylethanolamine.

unlikely to have become radioactive in this experiment, we tentatively concluded that the major labeled product was phosphatidyl *N,N'*-dimethylethanolamine.

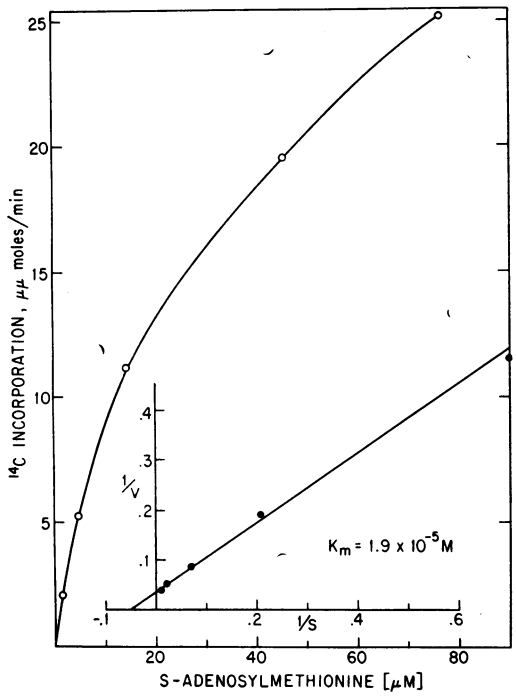

FIG. 1. *Dependence of lipid methylation on S-adenosyl methionine concentration. Reaction mixtures consisted of: Tris-chloride, pH 8.0, 0.075 M; cell-free extract, 4.2 mg of protein;* ^{14}C *(methyl) S-adenosyl methionine, 53 μc/μmole, as indicated; in a total volume of 0.40 ml. Incubated in air at 30 C for 45 min. Assayed by chloroform-methanol extraction.*

The identity of these labeled lipids was studied further by hydrolyzing the products of a similar incubation in 1 N HCl followed by paper chromatography of the water-soluble products, which contained about 90% of the total counts. Figure 3 shows that the radioactive bases are coincident with the three standards, *N*-methylethanolamine, *N,N'*-dimethylethanolamine, and choline, and that the major radioactive peak coincides with the dimethylethanolamine standard in agreement with the conclusion reached from thin-layer chromatograms of the intact lipids. In a number of experiments with different times of incubation, and with unlabeled phosphatidylethanolamine added in a micellar dispersion (7) as a potential precursor, phosphatidyl *N,N'*-dimethylethanolamine was consistently seen to be the major radioactive product. However, it should be noted that determination of the actual molar ratios of these products is complicated by the possibility of two labeled methyl groups in dimethylethanolamine and three labeled methyl groups in choline, as compared to not more than one labeled methyl group in monomethylethanolamine.

Radioactive lipids from similar incubations were also subjected to deacylation by mild alkali, and the water-soluble products were chromatographed on paper. In solvent 4, two radioactive peaks were observed. The major one, R_{GP} = 1.5, was coincident with a standard of glycerylphosphorylcholine. However, it should be recalled (Table 5) that the deacylation product of phosphatidyl dimethylethanolamine migrates with the same R_{GP} in this solvent system. The tail of this major peak extended into the zone occupied by the deacylation product of phosphatidyl *N*-methylethanolamine, R_{GP} = 1.3. The minor

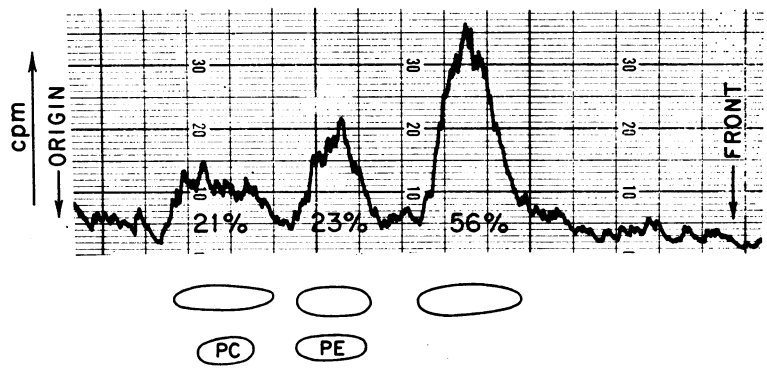

FIG. 2. *Thin-layer chromatography of products of methylation of lipids. Incubations were carried out as described in the legend for Fig. 1. Each incubation mixture contained 4.7 mg of protein. The lipid products were isolated by chloroform-methanol extraction and chromatographed on silica gel G in chloroform-methanol-7 N* NH_4OH *(60:35:5, v/v). Standards are: PC (phosphatidyl choline) and PE (phosphatidyl ethanolamine). Numbers refer to percentage of total radioactivity in individual peaks. The extracted lipids were revealed by staining with iodine.*

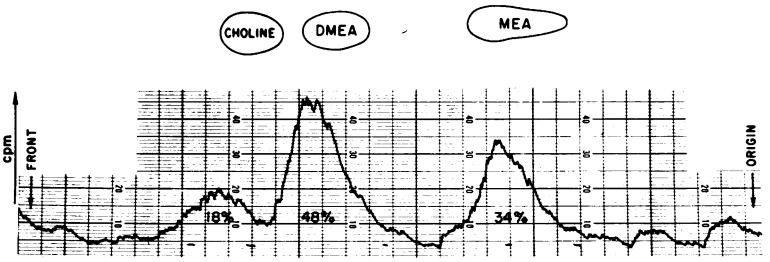

FIG. 3. *Examination of labeled bases from enzymatically synthesized radioactive lipids. The lipids from an experiment similar to that described in the legend to Fig. 2 were hydrolyzed in 1 N HCl and the water-soluble products were chromatographed on paper in solvent 1. In addition to choline, the other standards were MEA (N-methylethanolamine) and DMEA (N,N'-dimethylethanolamine). Numbers refer to percentage of total radioactivity in individual peaks.*

peak at R_{GP} = 2.4 has not been identified; it had one-fifth as much ^{14}C as the major peak.

Paper chromatography of the deacylation products in solvent 5 (PWAE) showed a double radioactive peak, which was not well separated, at R_{GP} = 2.2 and 2.4. These are in the region occupied by the deacylation products of phosphatidyl choline and phosphatidyl dimethylethanolamine (Table 5).

Stimulation of lipid methylation by added phosphatidyl ethanolamine. In two experiments with a dialyzed sonic extract, stimulation of lipid methylation was seen when a micellar dispersion of phosphatidyl ethanolamine (7) isolated from *E. coli* was added to the incubations (Table 8).

TABLE 8. *Stimulation of methyl transfer to lipid by added phosphatidyl ethanolamine*[a]

Expt	Addition	^{14}C incorporated
		mμmoles
1	None	0.397
	Phosphatidyl ethanolamine, 0.11 mM	0.417
	Phosphatidyl ethanolamine, 0.56 mM	0.628
2	None	0.396
	Phosphatidyl ethanolamine, 0.56 mM	0.850
	Phosphatidyl ethanolamine, 1.7 mM	0.882

[a] Incubation mixtures consisted of: Tris-chloride, *p*H 8.0, 0.075 M; ^{14}C (methyl) *S*-adenosylmethionine, 27 μM, 9.5 μc/μmole (experiment 1) and 76 μM, 9.9 μc/μmole (experiment 2); dialyzed cell-free extract, 3.4 mg of protein; in a total volume of 0.4 ml. Incubations were carried out in air, at 30 C, for 45 min. Assayed by chloroform-methanol extraction.

DISCUSSION

Lecithin is a rare component among bacterial phosphoglycerides, with the exception of photosynthetic bacteria (22, 26). Among nonphotosynthetic bacteria, substantial amounts of lecithin have been found only in the genus *Agrobacterium* (9, 14, 25), in *Thiobacillus thiooxidans* (23), and in the genus *Hyphomicrobium*, as demonstrated in this report. Small amounts of lecithin were also found in *N. oceanus* (18). There are a few reports of lecithin in other genera, but in these cases there are other conflicting reports (22).

We have discussed a possible interpretation of these findings in terms of the presence or absence of intracytoplasmic membranes (18). Photosynthetic bacteria have been found to possess complex intracytoplasmic membranes (29, 34), and a number of these organisms, though not all, have been found to contain lecithin (22). Complex intracytoplasmic membrane systems have also been seen in *Hyphomicrobium* (6), *N. oceanus*, and *N. europaea* (30), and we have found lecithin in all strains of *Hyphomicrobium* examined and in *N. oceanus* but not in *N. europaea* (18). In the cells of higher organisms, which have organelles characterized by complex membrane systems, lecithin is always found, and is often the most abundant phosphoglyceride (1). If these chemical and structural correlations are valid, it may be worthwhile to ask if the unique charge or size (or both) of the polar head group of lecithin somehow facilitates the folding necessary for the elaboration of intracytoplasmic membranes.

Ikawa, in a recent review of bacterial phosphatides, has taken another view of the distribution of lecithin in bacteria (22). He has focused on the association between lecithin and efficient electron transporting systems. From this point of view the photosynthetic bacteria, the sulfur-oxidizing pseudomonad, *T. thiooxidans*, the nitrifier, *N. oceanus*, and the aerobic organotroph, *Hyphomicrobium*, could all be grouped together.

Murray and Watson have pointed out that energy-yielding activity involving coupled metabolic reactions is usually membrane-linked, and that elaborate membrane systems are often associated with efficient electron transport in bacteria (30). From this point of view, the grouping of elaborate membranes and efficient electron transport could be extended to include lecithin. Whether lecithin is important for its structural features or because it may play a more active role in electron transport, as suggested by Ikawa, remains to be seen.

A third point of view is that lecithin represents a more advanced form of the phospholipid molecule (22) since its synthesis requires at least two enzymes in addition to those required for the synthesis of phosphatidyl ethanolamine, and that the ability to synthesize it is a feature of "advanced" groups of bacteria. But without some physiological or structural basis, this type of classification is not illuminating.

The most unusual feature of the lipids of *Hyphomicrobium* is the presence of phosphatidyl *N*,*N'*-dimethylethanolamine, which comprises 36% of the total phosphatides of strain NQ-521. This phosphatide has been shown to be an intermediate in the stepwise methylation leading from phosphatidyl ethanolamine to lecithin in mammalian liver (5, 10), *Neurospora* (32), agrobacteria (24), and protozoa (28). In none of these cases is there a substantial accumulation of the dimethylethanolamine phosphatide. Based on genetic and enzymatic evidence, it is thought that a single enzyme is responsible for the first methylation of phosphatidyl ethanolamine and that a separate enzyme is responsible for the subsequent two methylation steps leading to phosphatidyl choline (32). The enzyme responsible for the first methylation leading to phosphatidyl *N*-methylethanolamine has been separated from the second and third methylating steps in a soluble form from extracts of *A. tumefaciens* (24).

In work with cell-free extracts, some accumulation of the dimethylethanolamine phosphatide has been seen, but not to the extent observed in our experiments with extracts from *H. vulgare* NQ-521. Gibson et al. observed more radioactivity in dimethylethanolamine than in choline in brief incubations of microsomes with ^{14}C-*S*-adenosylmethionine. After 5 to 10 min, choline had more radioactivity than dimethylethanolamine (10). It appears that the final methylation leading to phosphatidyl choline is the slower step relative to the first two methylations in *H. vulgare* NQ-521. The basis of this difference from other organisms is not understood.

Only *S*-adenosylmethionine was tested as a methyl donor in the experiments with cell-free extracts. It was shown to be the most effective methyl donor in lecithin biosynthesis in the systems previously studied. It should be noted that the apparent K_m for *S*-adenosylmethionine is an order of magnitude lower in extracts of *Hyphomicrobium* than found by Kaneshiro and Law in their experiments with extracts from *A. tumefaciens* (24). The apparent K_m for adenosylmethionine with wild-type *Neurospora* extracts was recently shown to be similar to that for *H. vulgare* NQ-521 (32).

The presence of phosphatidyl ethanolamine and phosphatidyl glycerol in *Hyphomicrobium* is not unusual. Most groups of bacteria, with the exception of the *Lactobacillaceae* and *Micrococcaceae*, contain phosphatidyl ethanolamine (22). Phosphatidyl glycerol, its *O*-amino acid esters, and diphosphatidyl glycerol (cardiolipin) are even more widely distributed among bacterial lipids (22).

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