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Extraction and Composition of Polar Lipids from the Archaebacterium, *Methanobacterium thermoautotrophicum*: Effective Extraction of Tetraether Lipids by an Acidified Solvent[1]

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The usual Bligh and Dyer method could extract only a small part of the lipids of *Methanobacterium thermoautotrophicum*. When the water in the solvent was replaced by 5% trichloroacetic acid, the lipid recovery reached the maximum level, which was 6 times higher than that by the former method. The use of HCl (2 M) or disruption of cells was also effective but prolonged extraction with the HCl-containing solvent caused degradation of some phosphoglycolipids. Twenty-three spots of polar lipids were detected on a thin-layer chromatogram of the total lipid. These were 10 phospholipids (18%), 6 aminophospholipids (17%), 3 aminophosphoglycolipids (15%), 2 phosphoglycolipids (31%), and 2 glycolipids (19%). The predominant polar lipids were a highly polar phosphoglycolipid (PGL1, 30%) and a glycolipid (GL1a, 16%). The other major lipids included an aminophospholipid (PNL1a, 9%), and an aminophosphoglycolipid (PNGL1, 7%). The complete structure determination of PNL1a, GL1a, and PNGL1 is described in the accompanying paper. Acetolysis of the total lipids followed by acid methanolysis was required for the complete cleavage of polar head groups, releasing core residues of diphytanyl glycerol diether (C_{20} diether) and dibiphytanyl diglycerol tetraether (C_{40} tetraether). A densitometric assay of a thin-layer chromatogram showed that the ratio of C_{20} diether and C_{40} tetraether was 1 : 14. GLC analysis of alkyl chlorides prepared from the total lipid by BCl_3 treatment showed that phytanyl (C_{20}), biphytanyl (C_{40}), and unidentified alkyl chains accounted for 10, 83, and 7 mol% of the total alkyl chains, respectively. Strong acid hydrolysis of the macromolecular residue obtained after lipid extraction gave a significant amount of C_{40} tetraether, which had probably been bound covalently to other substances in the cells.

[1] This study was supported in part by Grants-in-Aids for Scienctific Research (Nos. 59560094 and 61760088) from the Ministry of Education, Science and Culture of Japan, and a grant from the Agricultural Chemistry Foundation. Abbreviations: TCA, trichloroacetic acid; C_{20} diether, diphytanyl glycerol diether; C_{40} tetraether, dibiphytanyl diglycerol tetraether.

Methanobacterium thermoautotrophicum is a strictly anaerobic, thermophilic methanogen which can grow autotrophically (*1*). It was selected as the "organism of choice" for mass-culturing of a methanogen, as the appearance of contaminants in large fermentors was not a problem when an inorganic medium and a high temperature were used. In fact, many of the biochemical studies on methanogens have been carried out with this organism, for example, studies on the structures of unique coenzymes (*2*), methane biosynthesis (*3*), energetics coupled with methanogenesis (*4*), the new CO_2 fixation pathway (*5*), biosynthesis of amino acids and sugars (*6*), and pseudomurein structures (*7*). However, the investigations on the membrane polar lipids in this organism are still at a rather early stage (*8*, *9*), although it has been reported that the hydrophobic portion of polar lipids (core residues) of this organism consists of diphytanyl glycerol diether (C_{20} diether) and dibiphytanyl diglycerol tetraether (C_{40} tetraether) in a ratio of 26 : 74 (*8*) or 45 : 55 (*10*).

Recently, we have found and identified a novel aminophospholipid in *Methanobrevibacter arboriphilus* as a diphytanyl ether analog of phosphatidylserine (*11*), and this aminolipid was also detected in *M. thermoautotrophicum* (*12*). In a survey of aminolipids in methanogens by using two-dimensional TLC, a number of novel phospho-, aminophospho-, glyco-, phosphoglyco-, and aminophosphoglycolipids were also detected in *M. thermoautotrophicum*. In the course of the structure study of the lipids, the original Bligh and Dyer extraction method with a neutral solvent (*13*) was found to be inadequate for the complete extraction of lipids. Furthermore, the combination of acetolysis and acid methanolysis was known to be required to cleave all polar head groups completely. Because the earlier authors (*8–10*) had used the neutral solvent for lipid extraction and acid methanolysis for the removal of the polar groups, the reported ratio of core lipids (*8*, *10*) could be based on only a part of the polar lipids. In fact, the diether analog of phosphatidylserine was not methanolyzed (*11*). The present paper describes the conditions of lipid extraction with an acidified solvent adapted for *M. thermoautotrophicum* cells and the composition of polar lipids and hydrocarbon residues in the extracted lipids. The structures of three novel polar lipids extracted by the method are described in the accompanying paper (*14*).

MATERIALS AND METHODS

Growth of the Bacterium—Methanobacterium thermoautotrophicum ΔH (DSM1053) was obtained from the German Collection of Microorganisms (Göttingen, B.R.D.) and grown in 5 liters of the medium 2 described by Balch *et al.* (*15*) in a 10 liter carboy. Resazurin was omitted. A gas mixture of H_2+CO_2 (4 : 1) was continuously bubbled at a flow rate of 1,250 ml/min while the medium was stirred with a magnetic stirrer. After incubation at 65°C for 4 days, 4.5 liters of the culture (late log phase, 1.25 g dry cells/liter culture) was withdrawn and the cells were collected by centrifugation. Then 4.5 liters of fresh medium was added to the remaining culture (0.5 liter), and the incubation was continued. Thus, a semi-continuous cultivation was established and maintained for at least 18 months. During this period contamination was never observed.

Extraction of Lipids—Lipids were extracted essentially according to the method of Bligh and Dyer (*13*) modified as follows; cells (5.6 g) harvested from a 4.5-liter culture as above were suspended in 150 ml of water followed by the successive additions of 750 ml of methanol, 375 ml of chloroform, and 150 ml of 10% aqueous trichloroacetic acid solution (TCA-acid solvent). The mixture was stirred at room temperature for 2 h before the addition of 375 ml each of chloroform and water. After separation into two phases by low-speed centrifugation, the lower chloroform phase (1 volume) was washed with 1.9 volumes of methanol–water (1 : 0.8) to remove TCA. Phosphate or sugar content in the chloroform layer did not decrease during this washing. In some experiments on lipid extraction, cells were disrupted twice by passage through a French pressure cell (American Instrument Company, U.S.A.) at a pressure of 1,400 kg/cm² before lipid extraction by the usual Bligh-Dyer solvent.

Thin-Layer Chromatography—The following solvents (compositions in volume ratio) were used for TLC on Silica Gel 60 plates (Merck Art 5721); solvent A, chloroform–methanol–7 M aqueous ammonia (60 : 35 : 8); solvent B, chloroform–methanol–acetic acid–water (85 : 30 : 15 : 5), and

solvent C, light petroleum–ethyl ether–acetic acid (50 : 50 : 1). Polar lipids were separated by two-dimensional TLC with solvent A in the vertical direction and solvent B in the horizontal direction. Diether and tetraether core lipids devoid of polar head groups were chromatographed with solvent C. Lipid spots on TLC plates were detected by using the following spray reagents: ninhydrin for amino groups, acid molybdate (*16*) for phospholipids, 0.5% α-naphthol reagent (*17*) for glycolipids, periodate-Schiff reagent (*18*) for vicinal hydroxyl groups, and 30% H_2SO_4 for all lipids.

Determination of Lipid Composition—The ratio of non-polar and polar lipids and the composition of C_{20} diether and C_{40} tetraether cores were determined by densitometric tracing of the thin-layer chromatogram with a microdensitometer, model 3CS (Joyce-Loebl, U.K.) as described previously (*11*). Polar lipid composition was determined by the measurement of phosphorus (*19*) and/or sugar (*20*) of individual lipid spots on a two-dimensional thin-layer chromatogram. Composition of alkyl chains was determined by GLC of alkyl chlorides prepared by BCl_3 treatment (*21*) of total lipid as described in the accompanying paper (*14*). Lipid content was determined gravimetrically after drying to a constant weight at room temperature *in vacuo*.

Degradative Procedures—Acid methanolysis or acid butanolysis was carried out in anhydrous 5% methanolic or butanolic HCl at 100°C for 3 h. Acetolysis was performed in a mixture of acetic acid and acetic anhydride (3 : 2, v/v) for 16 h at 160°C. Chloroform-unextractable C_{40} tetraether-containing material in the residual macromolecular fraction obtained after the TCA-acid extraction was hydrolyzed by heating at 100°C in 4 M HCl for 16 h. The methanolyzate, acetolyzate or acid hydrolyzate was subjected to Bligh and Dyer partitioning between chloroform and aqueous-methanol. In the case of strong acid hydrolysis, the C_{40} tetraether-containing material was extracted 5 times with petroleum ether to remove brownish degradation products after evaporation of the chloroform solution.

Fatty Acid Analysis—Total lipid was methanolyzed and fatty acid methyl esters, if present, were purified by TLC and analyzed by GLC. Standard fatty acid methyl esters prepared from authentic diacyl phosphatidylethanolamine was used as R_f standards. To estimate fatty acid content in polar lipids, polar lipids were isolated by TLC with solvent C, and subjected to methanolysis or butanolysis without removal of the silica gel. The resultant chloroform-soluble products were analyzed by GLC. GLC analysis of fatty acid methyl or butyl esters was carried out with a 2.0 m glass column packed with 2% OV-17 on Chromosorb W at a temperature increasing from 100 to 330°C at a rate of 4°C/min. Chromatograms of methanolysis and butanolysis products were compared. Butyl esters of standard fatty acids showed 4 min longer retention times than the methyl esters. This shift of retention time on going from methyl ester to butyl ester was used as the basis of detection of fatty acids. For quantitative analysis, methyl or butyl pentadecanoate which was not present in the total lipid was used as an internal standard (the detection limit was 0.5 μg/mg of original lipid).

Materials—Authentic samples of C_{20} diether and C_{40} tetraether were prepared from *Methanospirillum hungatei* GP1 (DSM 1101) as described in the previous paper (*11*). The lipids of this organism have been completely analyzed by Kushwaha *et al.* (*22*). Authentic diacyl phosphatidylethanolamine (beef heart) was a product of Serdary. BCl_3 (99.999% pure) was obtained from Wako Pure Chemicals Inc., Japan.

RESULTS

Extraction of Total Lipid from M. thermoautotrophicum Cells with Acidified Solvents—Table I summarizes the results of lipid extraction from cells of *M. thermoautotrophicum*. At first, the cells were treated according to the usual method of Bligh and Dyer (*13*), but only 4.3 nmol of lipid-phosphorus and 5.8 nmol of lipid-sugar per mg of dry cells or 0.98% lipid with respect to dry cell weight were recovered in the chloroform phase (neutral extract). Because this value was so small compared to the lipid contents of other bacteria (*23*, *24*), it was expected that more lipid remained unextracted in the cells. In fact, when the water in the extraction solvent was replaced by 2 M HCl (HCl-acid solvent), the recovery was greatly increased as shown in Fig. 1. That is, the yield of lipid-phosphorus and lipid-sugar reached maximum at 5 h after the acidification of the monophasic

TABLE I. Recovery of lipids extracted from *M. thermoautotrophicum* cells by the Bligh and Dyer method with various aqueous phases.

Source	Aqueous phase[a]	Recovery		Lipid content of dry cell (%)	Nonpolar lipid in total lipid[b] (%)
		CH_3Cl soluble			
		Phosphorus (nmol/mg dry cell wt.)	Sugar		
Cells	H_2O	4.3	5.8	0.98	31
	2 M HCl	22.5	35.7	5.12	ND
	5% TCA	25.3	36.8	5.58	15
	2 M KCl	4.3	6.4	ND[c]	ND
Neutral extraction residue	5% TCA	19.1	29.1	4.33	14
Disrupted cells	H_2O	25.6	30.3	5.33	ND

[a] The lipid extraction method was essentially as described by Bligh and Dyer (*13*) except that the aqueous phase was modified as indicated. The monophasic extracting suspension was stirred for 12, 5, 2, and 12 h in the cases of H_2O, HCl, TCA, and KCl, respectively. [b] Determined by densitometry of a thin-layer chromatogram. [c] ND, not determined.

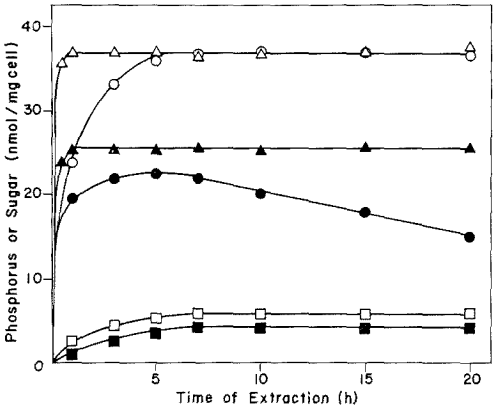

Fig. 1. Time courses of lipid extraction with the neutral and acidified solvents. The aqueous phase of the monophasic suspension in the Bligh and Dyer extraction method (*13*) was water (□,■), 5% TCA (△,▲), or 2 M HCl (○,●). The suspension was stirred at room temperature for the indicated times before separation into two phases by the addition of chloroform and the aqueous solution. Phosphate (■,▲,●) and sugar (□, △,○) in the chloroform layer were determined.

extraction suspension. The amount of lipid in this extract (HCl-acid extract) was 5.2 (phospholipids) and 6.2 (glycolipids) times higher than that of the usual Bligh-Dyer extract. After 5 h of extraction, lipid-phosphorus recovered in the HCl-acid extract gradually declined while lipid-sugar in the same fraction remained constant up to 20 h (Fig. 1). This phenomenon was due to the degradative conversion of a phosphoglycolipid to a glycolipid during prolonged extraction under the acidic conditions, as described below. The problem of the phosphoglycolipid degradation during HCl-acid extraction was overcome by the use of 5% TCA solution instead of 2 M HCl (TCA-acid extraction). The amount of the extracted lipid-phosphorus and lipid-sugar reached a plateau at 1 h after addition of TCA and no decrease of phosphorus or sugar content in this lipid extract (TCA-acid extract or total lipid = neutral solvent-extractable lipid + residual lipid, see below) was observed. The level of lipid-sugar was almost the same as that of the HCl-acid extract and the level of lipid-phosphorus was slightly higher than the maximum level of the HCl-acid extract. The extrapolation of the phospholipid degradation curve after 5 h of the HCl-acid extraction crossed the ordinate at the level of the maximum yield of lipid-phosphorus obtained by the TCA-acid extraction. Lipid extracted by this method comprised 5.58% of the dry cell weight, a reasonable value compared to the lipid contents of other bacteria (*24*). When the residue of the first neutral extraction was extracted again with the TCA-acid solvent, the extract (residual lipid) contained lipids corresponding to the difference between the

neutral and TCA-acid extractions. This indicated that the effect of the acidified solvent on the recovery was really to extract more lipid, not merely to re-partition highly polar lipids from the aqueous upper layer to the lower chloroform layer. A 2 M KCl solution, which is sometimes used instead of water in lipid extraction, had little effect on the recovery of lipid in the case of *M. thermoautotrophicum* cells (Table I). When lipid was extracted from cell homogenate prepared by passage through a French pressure cell with the usual Bligh-Dyer solvent, the recovery was almost the same as that obtained by the TCA-acid extraction of the intact cells. No difference in the lipid pattern on TLC between the TCA-acid extract and the neutral extract from the disrupted cells was observed.

The aqueous methanol phase was subjected to strong acid hydrolysis followed by extraction with chloroform. Only a trace amount (0.1% of the cell dry weight) of C_{40} tetraether was detected by TLC. That is, there was only a trace amount of extremely polar lipid which had been partitioned in the upper phase even after the acidic extraction.

Polar Lipid Composition—The total lipid was composed of 15% neutral lipid and 85% polar lipid. Figure 2 shows a two-dimensional TLC pattern of the total lipids. At least 23 spots of polar lipids were detected by acid charring. The lipids were tentatively designated as shown in Fig. 2, for example PNL1a, *etc.* They were classified by the use of specific staining tests into five groups; PL, PNL, PNGL, PGL, and GL mean phospholipids (10 spots), aminophospholipids (6 spots), aminophosphoglycolipids (3 spots), phosphoglycolipids (2 spots), and glycolipids (2 spots), respectively. The last letter "a" or "b" on some designations was given to pair lipids that were located close to each other on the chromatogram ("a" refers to the spot with higher R_f value) and showed the same responses to the spray reagents, implying that these were C_{40} tetraether type and C_{20} diether type of lipids, respectively, with the same polar head groups. This implication was partly based on the report of Kushwaha *et al.* (*22*) that the lipid with a C_{40} tetraether residue had a higher R_f value on TLC than that with a C_{20} residue if they had the same polar head group. Furthermore, the accompanying paper (*14*) shows that two lipids with "a"-designation (PNL1a, GL1a) had C_{40} tetraether as a core residue.

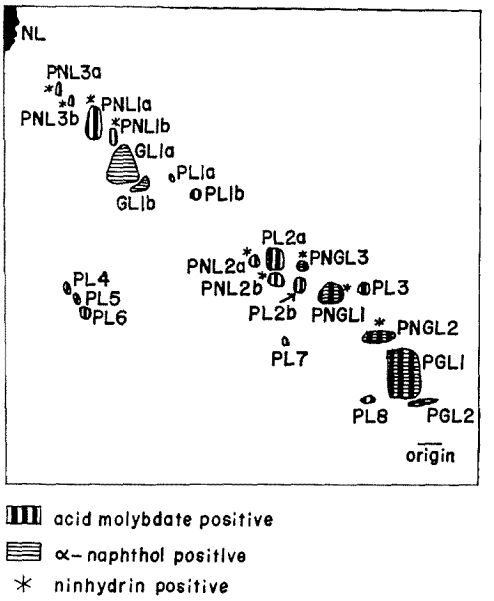

Fig. 2. TLC chromatogram of total lipid of *M. thermoautophicum.* Details of the development of lipids and the detection of each lipid are given in " MATERIALS AND METHODS."

Table II shows the composition of these polar lipids. The predominant polar lipids were a highly polar phosphoglycolipid designated as PGL1 (30%) and a glycolipid which had a glucosylglucose residue (GL1a, 16%). The other major lipids included an aminophospholipid containing phosphoethanolamine residue (PNL1a, 9%), a phospholipid (PL2a, 7%), and an aminophosphoglycolipid possessing phosphoethanolamine and sugar residues identical with those of GL1a (PNGL1, 7%). PNGL1 was structurally characterized as well as PNL1a and GL1a (*14*). The composition of polar lipids in the neutral extract was somewhat different from the composition in the acid extract. The contents of GL1a, PGL1, and other lipids with "a" designation were relatively low in the neutral extract. In contrast, high proportions of these lipids were found in the residual lipids. In the HCl-acid extract obtained after a 48 h extraction the amount of PGL1 greatly decreased, accompanied with a corresponding increase of GL1a. Thus, prolonged extraction with the HCl-acid solvent caused the specific degradation of PGL1 into GL1a. The conversion of PGL1 to GL1a and a water-soluble

TABLE II. Polar lipid composition of *M. thermoautotrophicum*.

Lipid	P(%)	Sugar(%)	Mol%[a]
Phospholipid			
PL1a	<0.5		<0.5
PL1b	<0.5		<0.5
PL2a	9		7
PL2b	4		3
PL3	4		3
PL4	2		2
PL5	3		2
PL6	1		1
PL7	<0.5		<0.5
PL8	<0.5		<0.5
Aminophospholipid			
PNL1a	12		9
PNL1b	2		2
PNL2a	2		2
PNL2b	3		2
PNL3a	2		2
PNL3b	<0.5		<0.5
Aminophosphoglycolipid			
PNGL1	9	12	7
PNGL2	7	8	6
PNGL3	2	2	2
Phosphoglycolipid			
PGL1	36	45	30
PGL2	1	1	1
Glycolipid			
GL1a		27	16
GL1b		5	3

[a] mol% was calculated assuming that each lipid contained 1 phosphate moiety and/or 2 glucose moieties as established for the major lipids, PNGL1, PNL1a, GL1a, PGL1, PNL2b, and PL2b (Refs. *13*, *11*, and Morii, Nishihara, and Koga, unpublished results).

organic phosphate compound was confirmed by a model experiment in which purified PGL1 was incubated in the HCl-acid solvent for 48 h under conditions similar to those of the HCl-acid extraction (data not shown). Since 7 sugar-containing lipids were detected, sugar in the lipid fraction was analyzed to determine how many kinds of sugar were present. Only D-glucose was detected as a sugar constituent of lipids of this organism (see the accompanying paper (*14*)).

Composition of Glyceryl Ether Core Residues— Only 52% of phosphodiester bonds were cleaved by acid methanolysis of the total lipid while sugar moieties were completely released. PL4, PL5, PL6, PNL1a, PNL1b, PNL2a, PNL2b, PNL3a, and PNL3b remained intact even after acid methanolysis. On the other hand, acetolysis without H_2SO_4 was suitable to remove all of the phosphate moiety, while the glycosidic bond was not broken. Thus, acetolysis followed by acid methanolysis resulted in the complete cleavage of all polar head groups in the total lipid, and this is prerequisite for the correct analysis of core ether lipid composition. A densitometric tracing of a TLC plate of chloroform-soluble materials obtained by this method from the total lipid showed that tetraether was predominant (82% of the total material on the TLC). The levels of C_{20} diether and other unidentified materials were 6 and 12%, respectively. That is, the ratio of C_{20} diether and C_{40} tetraether was 1 : 14. A small part (less than 7% of total lipid) of C_{20} diether and C_{40} tetraether was originally present as the neutral lipids in the total lipid (the latter was again predominant).

Although the content of tetraether lipid reported here was clearly higher than those described by earlier workers (26 : 74 by Makula and Singer (*8*) and 45 : 55 by Tornabene and Langworthy (*10*)), a densitometric determination is accompanied with an inherent error when one spot is extremely large compared to the other, as in the present case. To determine the composition of diether and tetraether more precisely, alkyl chlorides prepared with BCl_3 from the total lipid were analyzed by GLC (Table III). Biphytanyl (C_{40}) chain consistently predominated (83 mol%) in the alkyl groups of the total lipid obtained by TCA-acid extraction from the intact cells or neutral extraction from the disrupted cells. The apparent high proportion of phytanyl (C_{20}) chain in the neutral-solvent-extractable lipid reflects preferential extraction of polar lipids with the C_{20} diether core. On the other hand, C_{40} tetraether polar lipids were relatively hard to extract with the neutral solvent but were readily extractable with the TCA-acid solvent. Almost all the residual lipids consisted of C_{40} chain. There were non-negligible amounts of

TABLE III. Composition of alkyl chains in the lipid extracts. Alkyl chains were analyzed by GLC as the alkyl chlorides.

Lipid extract	Alkyl chain		
	Phytanyl	Biphytanyl (mol%)	Others[a]
Neutral extract	35.3	36.0	28.7
Residual lipid	1.6	97.4	1.0
Total lipid	10.0	82.6	7.4
Disrupted cell lipid	9.2	83.2	7.6

[a] Four species of alkyl chlorides other than phytanyl chloride and biphytanyl dichloride were present on the gas chromatogram. The retention times of these alkyl chlorides relative to that of phytanyl chloride were 0.65, 1.20, 1.45, and 1.71, respectively. For the calculation of mol%, the molecular weights of these unidentified peaks were assumed to be the same as that of phytanyl chloride based on the average retention time of the two major peaks (the former two).

alkyl chains other than C_{20} and C_{40} chains detected on a gas-liquid chromatogram. These may be the C_{15} and C_{25} chains reported by Mancuso *et al.* (*25*). Fatty acids or acyl glycerides were not detected in appreciable quantities in total lipids or polar lipids.

Chloroform-Unextractable C_{40} Tetraether-Containing Material—The fluffy residue obtained after TCA-acid extraction was washed twice with the acidified solvent and then subjected to strong acid hydrolysis followed by extraction with chloroform. While the washings of the residue contained little lipids, an appreciable amount of chloroform-soluble substance (2.04% of the dry cell weight) was obtained. TLC analysis with solvent C showed that almost all of this substance was C_{40} tetraether. More than 98% of the alkyl chains was biphytane. This result suggested that C_{40} tetraether-containing material, which was not extracted with the TCA-acid solvent and precipitated by TCA or chloroform, was present in the cells.

DISCUSSION

The most effective method for extraction of lipid from *M. thermoautotrophicum* cells was established. Although the disruption of cells gave quantitatively and qualitatively the same results as the use of the TCA-acid solvent, the latter method was more convenient, especially in experiments requiring a quantitative processing of a small amount of cells. TCA was readily removed by washing with methanol-water. Because the lipid contents per unit weight of cells obtained by three kinds of extractions (the TCA-acid extract, the HCl-acid extract and the extract from disrupted cells) coincided well with each other, the value could be considered as the maximum extractable lipid amount. That is, all the extractable lipid was actually extracted by either method. The requirement of an acidic solvent and a fairly long extraction time for the complete extraction of lipids might suggest that the residual lipids were bound to macromolecular components *via* covalent bonds which were cleaved by the acid solvent. This possibility was, however, excluded because the breakage of cells had the same effect on the lipid extraction without the use of an acid solvent. It is not known why large amounts of lipids are tightly bound to the cellular constituents. Considering the facts that almost all the residual lipids are C_{40} tetraether type which may form monolayer membranes (*26*) and that TCA, a strong protein-denaturing agent, or cell wall disruption was required, one can suppose either that the interactions of membrane proteins with monolayer membrane lipids (C_{40} tetraether lipids) are stronger than those with the usual bilayer lipids or that the rigid pseudomurein cell walls interfere with the usual extraction of tetraether monolayer lipids.

By this method 23 kinds of polar lipids were detected. The complexity of the lipid pattern was striking compared to the eubacterial lipid pattern, *e.g.*, only 3 to 5 lipids in *Escherichia coli* (*24*).

Makula and Singer (*8*) and Tornabene and Langworthy (*10*) reported the approximate composition of diether and tetraether core lipids of several kinds of methanogens. The present paper describes the presence of phospholipids resistant to acid methanolysis. The polar head group lacking free hydroxyl groups could not be split off from the core residue in the case of glycerol diether or tetraether lipids because ether bonds are not readily hydrolyzed or methanolyzed. The cyclic phosphate is able to be formed only on the polar residues with a free hydroxyl group adjacent to the original phosphodiester. Thus, the entity of the unmethanolyzable lipids is di- or tetraether

lipid with polar residues such as phosphate, phosphoethanolamine, or phosphoserine. Because these were found to be cleaved by the combination of acetolysis and acid methanolysis, core residue compositions obtained by the simple methanolysis of various methanogens should be revised if the total lipid contained unmethanolyzable components. The tetraether content (83 mol% or 90% in weight) of *M. thermoautotrophicum* reported here is much higher than that (55%) reported by Tornabene and Langworthy (*10*). Makula and Singer (*8*) described the presence of acid-stable phospholipids and Tornabene and Langworthy (*10*) stated that some materials remained at the origin of the thin-layer chromatogram of methanolyzates of total lipid from 7 of 9 methanogens. These reports indicate the general occurrence of unmethanolyzable phospholipids. The identities of some of them are, for example, the diether analog of phosphatidylserine which was detected in 6 species of *Methanobacteriaceae* (*12*), and PNL1a and PNGL1 as described in the accompanying paper (*14*). The proportion of unmethanolyzable lipid-phosphorus (48%) almost corresponded to the sum of the percentage of 6 aminophospholipids, 3 phospholipids (PL4, PL5, PL6), and 3 aminophosphoglycolipids.

Glucose, galactose, and mannose are the sugars commonly found in glycolipids and phosphoglycolipids of bacteria including archaebacteria (*22*, *27–29*). On the other hand, glucose was the sole sugar found in lipids of *M. thermoautotrophicum* ΔH. Galactose and galactosamine contents in the pseudomurein of this strain are also particularly low compared with those in other members of *Methanobacteriaceae* (*30*).

Strong acid hydrolysis of the fluffy residue after TCA-acid extraction showed the presence of an appreciable amount of chloroform-unextractable C_{40} tetraether-containing material, while only a trace amount of C_{40} tetraether was obtained on similar treatment of the aqueous methanol phase of the extraction into which highly polar lipids would be partitioned. There is, therefore, little possibility that the chloroform-unextractable C_{40} tetraether-containing material is extremely polar "lipid." Perhaps the original material is C_{40} tetraether bound covalently to other TCA-precipitable cell component(s) of high molecular weight such as polysaccharide, proteins or teichoic acid-like cell-wall components. Such kinds of materials would not be extracted with chloroform and would be precipitated by TCA or chloroform. It should be noted that such a compound would not be classified as lipid even if it contained C_{40} tetraether. An analogous lipoglycan found on the cell surface of *Thermoplasma acidophilum* (*31*, *32*) resembled lipopolysaccharides of Gram-negative bacteria. It was extracted with hot aqueous phenol and had an extended 25-sugar chain which was attached to one side of the tetraether molecule. It is not extractable with the Bligh and Dyer solvent and yields the C_{40} tetraether core upon acid hydrolysis. It is not known whether the chloroform-unextractable C_{40} tetraether-containing material in *M. thermoautotrophicum* is extractable with phenol or contains a long sugar chain. Further experiments are necessary to elucidate the original structure of this material in the cells.

The features found in *M. thermoautotrophicum* lipids, such as the requirement of an acidified solvent for complete extraction, the presence of a number of aminophospholipids and PGL1 or the occurrence of glucose as the sole sugar constituent of glycolipids, are common to *M. arboliphilus* A2 (Nishihara and Koga, unpublished results) and probably to all methanogens of the same family.

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