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Long-Chain Glycerol Diether and Polyol Dialkyl Glycerol Triether Lipids of *Sulfolobus acidocaldarius*

THOMAS A. LANGWORTHY, WILLIAM R. MAYBERRY, AND PAUL F. SMITH
Department of Microbiology, University of South Dakota, Vermillion, South Dakota 57069

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Cells of *Sulfolobus acidocaldarius* contain about 2.5% total lipid on a dry-weight basis. Total lipid was found to contain 10.5% neutral lipid, 67.6% glycolipid, and 21.7% polar lipid. The lipids contained $C_{40}H_{80}$ isopranol glycerol diethers. Almost no fatty acids were present. The glycolipids were composed of about equal amounts of the glycerol diether analogue of glucosyl galactosyl diglyceride and a glucosyl polyol glycerol diether. The latter compound contained an unidentified polyol attached by an ether bond to the glycerol diether. The polar lipids contained a small amount of sulfolipid, which appeared to be the monosulfate derivative of glucosyl polyol glycerol diether. About 40% of the lipid phosphorus was found in the diether analogue of phosphatidyl inositol. The remaining lipid phosphorus was accounted for by approximately equal amounts of two inositol monophosphate-containing phosphoglycolipids, inositolphosphoryl glucosyl galactosyl glycerol diether and inositolphosphoryl glucosyl polyol glycerol diether.

Brock et al. (6) reported the isolation of a new genus of acidophilic, thermophilic, sulfur-oxidizing bacterium, *Sulfolobus acidocaldarius*. The organism requires a pH of 3.0 or less and a temperature of 65 to 90 C for growth. It grows autotrophically on elemental sulfur and heterotrophically on yeast extract. The requirements of high temperature and low pH of *Sulfolobus* are similar to those of *Thermoplasma acidophilum*, which requires pH 2.0 and 59 C for growth. Previous reports (16, 26) have shown the unusual nature of *Thermoplasma* membranes and lipids, which contain very long-chain isopranol glycerol diethers. A characterization of the lipids was undertaken to determine whether similar lipid structures exist in *Sulfolobus*.

MATERIALS AND METHODS

Growth of cells. *Sulfolobus acidocaldarius* (isolate, 98-3) was provided by Thomas D. Brock, University of Wisconsin, Madison, Wis. Cultivation of the organism was carried out heterotrophically in a liquid medium (6, 23) of the following composition: $(NH_4)SO_4$ (1.3 g), KH_2PO_4 (0.28 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), $CaCl_2 \cdot H_2O$ (0.07 g), yeast extract (1 g) (Difco), in a total volume of 1 liter. After adjustment to pH 3.0 with 10 N H_2SO_4, the medium was sterilized by autoclaving. The inoculum consisted of a 10% volume of a 48-h culture. Incubation was carried out with shaking at 70 C in 2-liter volumes contained in 3-liter flasks. After 48 h, organisms were chilled and harvested by concentration in a Sharples centrifuge followed by sedimentation at 10,000 × *g* in a Sorvall RC-2B centrifuge and finally lyophilized.

When isotopically labeled lipids were desired, 50-ml cultures were grown in the above medium supplemented with 50 μCi of [2-^{14}C]acetate, 5 μCi of [2-^{14}C]mevalonic acid, dibenzylethylenediamine salt, 1 mCi of [^{35}S]sodium sulfate, or 1 mCi of [^{32}P]orthophosphate. In some instances, the medium was modified to avoid isotope dilution. Medium containing ^{35}S was modified to contain chloride in place of sulfate salts, and the pH was adjusted to 3.0 with HCl. For ^{32}P, the KH_2PO_4 was omitted from the medium. Radioisotopes were obtained from New England Nuclear Corp. The following methods are essentially those described previously (16).

Lipid extraction. Lipids were routinely extracted from 15 g of lyophilized cells or from washed, isotopically labeled wet sediments by stirring with 50 volumes of chloroform-methanol (2:1, vol/vol) for 3 h at room temperature. The suspension was filtered, and the cell residue was extracted an additional hour with fresh solvent. The combined filtrates were evaporated to dryness, and the residue was dissolved in chloroform-methanol-water (60:30:4.5, vol/vol/vol) and passed through Sephadex G-25 to remove nonlipid contaminants (29). Several batches of cells were extracted with chloroform-methanol-water (1:2:0.8, vol/vol/vol) according to the method of Bligh and Dyer (4).

Fractionation of lipids. Lipids were fractionated on silicic acid columns (2 by 8 cm) prepared from Unisil (100 to 200 mesh; Clarkson Chemical Co., Inc., Williamsport, Pa.). Neutral lipids were eluted with 500 ml of chloroform, glycolipids were eluted with 500 ml of acetone, and polar lipids were eluted with 500

ml of methanol. Isotopically labeled lipids were fractionated on silicic acid columns (1 by 2 cm) by using 20 ml of each solvent. Polar lipids were purified by fractionation on diethylaminoethyl (DEAE)-cellulose columns (2.5 by 22 cm) in the acetate form (20, 21). Polar lipids were applied in chloroform-methanol-water (60:30:4.5, vol/vol/vol). Any glycolipid was eluted with 10 column volumes of chloroform-methanol (7:3, vol/vol). Polar lipids were eluted with 10 column volumes of chloroform-methanol-ammonia (70:30:2, vol/vol/vol) containing 0.4% ammonium acetate. Lipids were further fractionated by thin-layer chromatography (TLC).

Thin-layer chromatography. TLC was carried out on 0.25-mm layers of either Silica Gel H or Silica Gel H made with 0.1 N boric acid in place of water. Plates were activated by heating at 100 C for 1 h. Solvents for glycolipids and polar lipids included chloroform-methanol-water (65:25:4 or 60:10:1, vol/vol/vol) and chloroform-methanol (9:1, vol/vol). Neutral lipids were separated with isopropyl ether-acetic acid (96:4, vol/vol) followed by n-hexane-diethyl ether-acetic acid (90:10:1, vol/vol) (24). Chloroform-diethyl ether (9:1, vol/vol) was used for glycerol diethers (14), and chloroform-methanol-water (60:10:1, vol/vol) was used for polyol dialkyl glycerol triethers. Alditols and polyols were separated with chloroform-methanol-formic acid (65:25:10, vol/vol/vol). Isolated bands on preparative TLC were scraped and eluted through sintered-glass filters with chloroform-methanol (2:1, vol/vol) for ethers and intact lipids, chloroform-methanol-water (60:30:4.5, vol/vol/vol) for phosphoglycolipids, and methanol-water (1:1, vol/vol) for polyols.

Lipids were detected by charring with 50% methanolic-sulfuric acid or iodine vapor or by spraying plates to transparency with water. Diphenylamine (24) or phenol-sulfuric acid (10) was used for glycolipids, periodate-Schiff reagent (3) was used for vicinal glycols, the reagent of Vaskovsky and Kostetsky (28) was used for phospholipids, and 0.2% ninhydrin in water-saturated butanol was used for amines.

Degradative procedures. Hydrolyses were done in screw-cap tubes (13 by 100 mm) with Teflon-lined closures. Acid methanolysis of lipids was carried out in anhydrous N methanolic hydrochloride at 100 C for 3 h. After cooling, equal volumes of chloroform and water were added, the contents were mixed well, and the phases were separated by centrifugation. The chloroform- and methanol-water-soluble products were analyzed after being dried under a stream of nitrogen or in vacuo. Partial degradation of lipids by mild acid methanolysis was performed as above by using 0.05 N methanolic hydrochloride at 60 C for 15 h. Strong alkaline hydrolysis was done in N NaOH at 100 C for 3 h followed by partition with equal volumes of methanol and chloroform. The methanol-water phase was neutralized with Dowex 50 (H^+) before examination of hydrolysis products. Mild alkaline hydrolysis was carried out by the method of Dittmer and Wells (8) at 17 C for 15 min or with sodium methoxide at room temperature for 1 h (19). Phosphate was released from inositol phosphate esters by hydrolysis in 6 N HCl at 100 C for 72 h.

Glycerol ethers were hydrolyzed in 57% hydroiodic acid at 110 C for 5 h to release alkyl iodides (15, 17). The hydrolysate was extracted with n-hexane, and the n-hexane was washed with 10% NaCl, saturated Na_2CO_3, and 50% $Na_2S_2O_3$. The alkyl iodides were reduced to the alkanes by refluxing with zinc in acetic acid (17) or converted to alkyl acetates by refluxing with silver acetate in acetic acid for 24 h (15). Alkyl acetates were converted to the alcohols by hydrolysis in 0.2 N methanolic sodium hydroxide at 100 C for 2 h.

Liquefied boron trichloride in chloroform (1:1, vol/vol) was used to release alkyl chlorides, glycerol, polyols, or carbohydrates from glycerol ethers or intact lipids (5, 9, 15, 16). To samples in 1 ml of chloroform was added 1 ml of boron trichloride, liquefied at −70 C, the tubes being capped tightly and mixed well. After standing for 24 h at room temperature, samples were evaporated under nitrogen, 1 ml of methanol was added, and the samples were again evaporated. The residues were partitioned between n-hexane and water, and the two phases were examined. Under these conditions ether, ester and glycosidic bonds were broken while free polyols and carbohydrates remained unaltered.

Gas-liquid chromatography. A Hewlett-Packard model 402 biomedical gas chromatograph, equipped with flame ionization detectors, and a model 3370A digital electronic integrator were used for gas-liquid chromatography (GLC). Stainless-steel columns (215 by 0.6 cm, outer diameter) were packed with 5.5% SE-30 (Applied Science Laboratories, State College, Pa.) and 7% OV-17 (Ohio Valley Specialty Chemical Co., Marietta, Ohio) on Gas Chrom Q; glass columns (183 by 0.6 cm) were packed with 10% EGSS-X (Applied Science Laboratories, State College, Pa.) on Chromosorb W. Oven temperatures were 325 C for long-chain alkanes, alcohols, and alkyl chlorides, 195 C for carbohydrates, and 215 C for polyols. Flow rates were as follows: helium, 60 ml/min; hydrogen, 80 ml/min; and oxygen, 200 ml/min. Detector temperatures were 50 C higher than oven temperature. Trimethylsilyl (TMS) derivatives were prepared by using a mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane-bis-trimethysilyl trifluoroacetamide (2:2:1:1, by volume). Acetate derivatives were prepared by using pyridine-acetic anhydride (3:1, vol/vol) (21). Relative response factors were determined experimentally.

Analyses. Carbohydrates were estimated by the anthrone (7) or phenol-sulfuric acid (2) procedure. Phosphorus was determined by the method of Ames (1). Glycerol was assayed enzymatically (30). Sulfate was estimated with barium chloranilate (8). Reducing sugar was determined by the Park-Johnson ferricyanide method (18), and acetate esters were determined by the ferric hydroxamate procedure (8). Specific compounds were quantitatively assayed by GLC with internal standards as follows: n-dotriacontane for alkanes and alcohols; arabinitol for inositol, glucose, and galactose; and inositol for the polyol. Lipid content, glycerol diether, and alkyl chlorides were determined gravimetrically after drying to constant weight. Infrared spectra were obtained on a Beckman model IR 18A instrument with samples as thin films between

sodium chloride crystals. Ultraviolet and visible spectra were obtained on a Beckman DK-2A scanning spectrophotometer. A Packard Tri-Carb liquid scintillation spectrometer was used for counting radioactive samples.

Materials. *Thermoplasma* diethers, alkanes, and glycolipids were obtained as previously described (16). All materials and standards were the best grades commercially available. Solvents were freshly distilled before use.

RESULTS

The normal yield of organisms from 48-h cultures was approximately 150 mg (dry cell weight) per liter. Total lipid accounted for 2.6% of the cellular dry weight obtained either by extraction with chloroform-methanol (2:1, vol/vol) or by extraction with chloroform-methanol-water (1:2:0.8, vol/vol/vol). Re-extraction of cell residues with chloroform-methanol (2:1, vol/vol), adjusted to 0.1 N with respect to HCl, did not release any further significant amount of lipid. Fractionation of total lipids on silicic acid columns gave 13.9% neutral lipid (chloroform fraction), 53.6% glycolipid (acetone fraction), and 32.4% polar lipid (methanol fraction). Considerable amounts of glycolipid usually contaminated the polar lipid fraction. In addition, significant amounts of extremely polar lipid remained in the original flask even after rinsing with acetone and methanol elution solvents. This remaining polar lipid and the polar lipid fraction obtained from silicic acid fractionation were combined in chloroform-methanol-water (60:30:4.5, vol/vol/vol), applied to a DEAE-cellulose column, and eluted with chloroform-methanol (70:30, vol/vol) for glycolipids followed by chloroform-methanol-ammonia (70:30:2, vol/vol/vol) containing 0.4% ammonium acetate for polar lipids. Salts were removed from the polar lipid eluate (21), and the glycolipids were combined with the glycolipid fraction obtained by silicic acid fractionation. For analytical work, polar lipids were routinely purified by DEAE-cellulose column chromatography. The lipid content of a typical batch of cells is shown in Table 1.

TABLE 1. *Lipid composition of S. acidocaldarius*[a]

Fraction	Percent of cell dry weight	Percent of total lipid
Total lipid	2.6	100
Neutral lipid	0.27	10.5
Glycolipid	1.74	67.6
Polar lipid	0.56	21.8

[a] Lipid percentages were determined gravimetrically after fractionation by silicic acid and DEAE column chromatography.

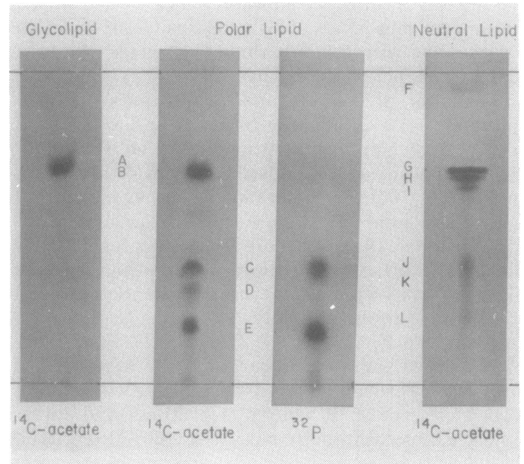

FIG. 1. *Autoradiographs of glycolipid, polar lipid, and neutral lipid fractions from S. acidocaldarius grown with [^{32}P]orthophosphate or [2-^{14}C]acetate. All components incorporated [2-^{14}C]mevalonate. Thin-layer chromatograms developed in chloroform-methanol-water (65:25:4, vol/vol/vol) for glycolipids and polar lipids, and isopropyl ether-acetic acid (96:4, vol/vol) followed by n-hexane-diethyl ether-acetic acid (90:10:1, vol/vol/vol) for neutral lipids.*

Autoradiographs of thin-layer chromatograms of each lipid class are shown in Fig. 1. All of the lipids became labeled with [2-^{14}C]acetate as well as [2-^{14}C]mevalonate, which indicated the presence of isoprenoid-like structures. Polar lipids C and E incorporated ^{32}P. None of the lipids incorporated ^{35}S as [^{35}S]sodium sulfate. Distribution of the labeled precursors between lipid classes is shown in Table 2. Treatment of each lipid class with sodium methoxide at room temperature for 1 h or sodium hydroxide at 37 C for 30 min did not release any water-soluble products or detectable fatty acids. All components remained unaltered after saponification when examined by TLC. Resistance to saponification suggested the presence of ether linkages. The staining characteristics and distribution of individual glycolipids and polar lipids are illustrated in Table 3.

Glycerol diethers. To determine the presence of glycerol ethers, a portion of the glycolipid fraction was subjected to acid methanolysis in N methanolic hydrochloride at 100 C for 3 h. After partition by addition of equal volumes of chloroform and water, the chloroform phase was examined by TLC developed in chloroform-diethyl ether (9:1, vol/vol). Two major spots were present, one remaining at the origin and the other (R_f 0.35) migrating faster than the

glycerol monoethers batyl (R_f 0.19), chimyl (R_f 0.15), or selachyl (R_f 0.22) alcohol, and slower than the long-chain glycerol diether from *Thermoplasma* (R_f 0.49). The migrating material was periodate-Schiff negative, indicating the absence of vicinal hydroxyl groups. The infrared spectrum showed OH absorption at 3,450 cm^{-1}; akyl absorption at 2,960, 2,929, 2,860, 1,460, and 1,375 cm^{-1}; ether C—O—C absorption at 1,115 cm^{-1}; and primary alcohol C—O absorption at 1,045 cm^{-1}. No double bond absorption was apparent. The infrared spectrum was essentially identical to that of the long-chain glycerol diether from *Thermoplasma*.

The O-alkyl side chains of the diether were examined after hydrolysis with 57% hydriodic acid and conversion of the alkyl iodides to the corresponding alkane or alcohol. The alkanes and TMS derivatives of the alcohols were examined by GLC. Both derivatives showed only one major component with an equivalent chain length of 37.9 on 5.5% SE-30 and 38.1 on 7% OV-17 columns. The two alkanes from the *Thermoplasma* glycerol diether had equivalent chain lengths of 35.2 and 36.7, respectively, on 5.5% SE-30 and 34.6 and 36.6, respectively, on 7% OV-17.

Analysis of the mass spectrum of the alkane from the *Sulfolobus* diether showed the molecular ion *m/e* 560, corresponding to the molecular formula $C_{40}H_{80}$, which indicates a possible ring in the structure. The mass spectrum of the alcohol as the acetate derivative showed the molecular ion *m/e* 619, an increase of *m/e* 59, accounted for by one acetate group, indicating the presence of one hydroxyl group in the long-chain alcohol. Both the *Sulfolobus* alkane derivative (molecular weight 560) and *Thermoplasma* alkane derivatives (molecular weights 560, 562) have similar molecular weights (16). However, the longer retention time of the *Sulfolobus* alkane derivative may be indicative of less methyl branching than in the *Thermoplasma* alkane derivatives.

A portion of the intact diether was treated with boron trichloride in chloroform (1:1, vol/vol) at room temperature for 24 h to release alkyl chlorides and glycerol. After evaporation, the residue was partitioned between *n*-hexane and water. The *n*-hexane phase containing alkyl chloride (molecular weight 595) was dried and weighed. Glycerol in the water phase was estimated enzymatically. Examination of the water phase by TLC developed in chloroform-methanol-formic acid (65:25L10, vol/vol/vol) gave only one spot (R_f 0.65), which was rapid periodate-Schiff positive and migrated identically to glycerol. Alkyl content was also estimated on an equal portion of the intact diether by hydrolysis with 57% hydriodic acid, conversion of the alkyl iodide to the alkane, and analysis by GLC with *n*-dotriacontane as inter-

TABLE 2. *Distribution of labeled precursors into lipid classes of S. acidocaldarius*

Precursor supplied	Percent distribution[a]		
	Neutral lipids	Glyco-lipids	Polar lipids
[2-^{14}C]acetate	19.5	19.2	61.2
[2-^{14}C]mevalonate	30.1	25.3	44.5
[^{32}P]orthophosphate[b]	0	0	100
[^{35}S]sodium sulfate[b]	0	0	0

[a] Cultures (50 ml) containing 50 μCi of [2-^{14}C]acetate, 5 μCi of [2-^{14}C]mevalonate, 1 mCi [^{32}P]orthophosphate, or 1 mCi of [^{35}S]sodium sulfate were extracted after 24 h, and lipids were fractionated on silicic acid columns (1 by 2 cm).

[b] Medium was modified to avoid isotope dilution as described in Materials and Methods.

TABLE 3. *Staining reactions and distribution of individual lipids*[a]

Component	Staining reaction			Percent of total lipid phosphorus	Percent of lipid class	Percent of total lipids
	Diphenylamine	Rapid periodate-Schiff	Phosphate			
Glycolipid						
A	+	−	−	0	43.6	29.5
B	+	+	−	0	56.3	38.0
Polar lipid						
C	−	−	+	40.5	38.0	8.3
D	+	+	−	0	6.0	1.3
E	+	+	+	59.5	55.9	12.2

[a] Thin-layer chromatography on Silica Gel H, or Silica Gel H containing 0.1 N boric acid, developed in chloroform-methanol-water (65:25:4, vol/vol/vol). Lipids were determined both gravimetrically and colorimetrically as carohydrate or phosphate.

nal standard. The diether had a ratio of 2.1 alkyl : 1.0 glycerol.

A sample of the diether was acetylated in pyridine-acetic anhydride (3:1, vol/vol) at 100 C for 1 h. The acetylated diether was examined for acetate ester by the ferric hydroxamate procedure, and glycerol and alkyl chloride were determined after boron trichloride degradation. The diether had a ratio of 2.1 alkyl : 1.0 glycerol : 1.01 acetate ester, indicating the presence of one free hydroxyl group. The diether derived from *Sulfolobus* glycolipid is a 1,2-substituted glycerol diether containing two identical $C_{40}H_{80}$ hydrocarbon chains.

Polyol dialkyl glycerol triether. The second component released by acid methanolysis from *Sulfolobus* glycolipids was examined by TLC in chloroform-methanol-water (60:10:1, vol/vol/vol). The material migrated (R_f 0.70) faster than diglucosyl diglyceride (R_f 0.27), monoglucosyl diglyceride (R_f 0.50), and the *Sulfolobus* glycolipids (R_f 0.26, 0.23), but had a mobility similar to that of the monoglycosyl diether from *Thermoplasma* (R_f 0.76). Long-chain glycerol diether migrated with the solvent front. The material was rapid periodate-Schiff positive, indicative of vicinal hydroxyl groups, diphenylamine negative, and nonreducing, and did not react in the phenol-sulfuric acid assay for carbohydrates. The infrared spectrum was identical to that of the glycerol diether except for a rather broad absorption from 1,045 cm^{-1} to 1,100 cm^{-1} characteristic of secondary hydroxyl groups and a much stronger OH absorption at 3,450 cm^{-1}. Hydrolysis of the material in 57% hydriodic acid followed by conversion of alkyl iodides to the alkanes yielded one alkane which was identical to the $C_{40}H_{80}$ alkane from the glycerol diether when examined by GLC. The *n*-hexane and water-soluble products of the ether were examined after treatment with boron trichloride-chloroform (1:1, vol/vol) at room temperature for 24 h. TLC of the water phase in chloroform-methanol-formic acid (65:25:10, vol/vol/vol) revealed two rapid periodate-Schiff-reactive polyols in about equal proportion. One co-chromatographed with glycerol (R_f 0.65), whereas the other (R_f 0.22) migrated more slowly than glucitol (R_f 0.36) and slightly slower than glucoheptitol (R_f 0.25). The initial pink color of the periodate-Schiff reaction on the straight-chain alditols disappeared after 24 h, whereas the polyol developed a dark blue color, which may suggest the presence of a cyclic structure (22). Determined by GLC on a column of 5.5% SE-30 or 10% EGSS-X as the TMS derivative, the unknown polyol had a retention time of 2.06 relative to inositol. The polyol had an estimated carbon number of 7.8 by GLC when compared with a curve constructed by plotting the carbon number of C_4 through C_7 straight-chain alditols against log retention times relative to inositol. The polyol was unaltered by treatment with sodium borohydride at room temperature for 24 h and did not give a reaction for reducing sugar or react with the phenol-sulfuric acid assay for carbohydrates. Mild periodate oxidation of the polyol, with a 10-μmol excess of $NaIO_4$, at room temperature for 10 min followed by neutralization with excess glycerol and reduction with sodium borohydride gave an unidentified product with a retention time of 0.55 relative to inositol by GLC. Under these conditions, inositol remained unaltered and glucitol was completely destroyed. The structure of the polyol remains undetermined. Preliminary evidence suggests that it behaves as a heptitol or octitol and may be cyclic or have a greater number of carbons than hydroxyl groups.

To determine the composition of the intact ether and the number of free hydroxyl groups present, a portion was acetylated in pyridine-acetic anhydride (3:1, vol/vol) at 100 C for 1 h and purified by TLC in *n*-hexane-diethyl ether (1:1, vol/vol). Two products were obtained. The fully acetylated product (R_f 0.37) showed complete absence of hydroxyl absorption in the infrared spectrum. The incompletely acetylated product had an R_f of 0.18. The fully acetylated product was assayed for acetate ester. Polyol was estimated by GLC after treatment of the fully acetylated product with the boron trichloride-chloroform mixture. The ether had a ratio of 2.1 alkyl : 1.0 glycerol : 1.11 polyol : 6.1 acetate, indicating six free hydroxyl groups in the structure and the polyol linked via an ether linkage to glycerol. To determine whether the alkyl groups are linked to the glycerol or to the polyol, a portion of the ether was dissolved in 3 ml of chloroform-methanol (1:2, vol/vol), and 0.5 ml of water containing 50 μmol of $NaIO_4$ was added. After 1 h, 1 ml of chloroform and 1 ml of 10% sodium bisulfite were added to stop the reaction, and the organic phase was removed. The products of the organic phase were taken up in 3 ml of chloroform-ethanol (1:2, vol/vol), and 0.5 ml of 10% sodium borohydride was added. After 15 h, the contents were partitioned by addition of 1 ml of chloroform and 1 ml of water, and the organic phase was examined by TLC in chloroform-methanol-water (60:10:1, vol/vol/vol). A major product was present (R_f 0.8) that was rapid periodate-Schiff negative and migrated faster than the original ether (R_f 0.7). After treatment of the material with the

boron trichloride-chloroform mixture, only glycerol was present in the aqueous phase as indicated by TLC in chloroform-methanol-formic acid (65:25:10, vol/vol/vol) (R_f 0.65). No intact polyol was present as determined by GLC. The material had a ratio of 2.3 alkyl:1.0 glycerol. Glycerol was not destroyed by periodate oxidation. The alkyl groups, therefore, are most likely linked to glycerol.

This second ether obtained from *Sulfolobus* lipids has properties very similar to those of a monoglycosyl glycerol diether except that it has an as yet unidentified polyol in an ether linkage to the glycerol diether. It is not known whether the polyol is linked through the 1 or 2 position of the glycerol diether. The material appeared to be a polyol dialkyl glycerol triether or could be considered a polyol-substituted glycerol diether.

Glycolipids. The glycolipids comprise two major components, as judged by TLC (Fig. 1). The two components could be separated (R_f 0.70 and 0.80) for analytical work by TLC on Silica Gel H layers made with 0.1 N boric acid and developed in chloroform-methanol-water (65:25:4, vol/vol/vol). When the total glycolipid fraction was subjected to acid methanolysis to release glycerol diether or polyol dialkyl glycerol triether, a small amount of fatty acid methyl ester was consistently detected by TLC in chloroform-diethyl ether (9:1, vol/vol) (R_f 0.77). When examined by GLC, these fatty acids were found to consist of 14:0 (4.7%), 15br (2.6%), 15:0 (3.2%), 16:0 (58.2%), 18:1 (21.5%), and 18:0 (9.8%). To determine whether these fatty acids are associated with the two major glycolipids, the glycolipid fraction was further purified by chromatography on a DEAE-cellulose column eluted with 10 column volumes of chloroform, followed by 10 column volumes of chloroform-methanol (98:2, vol/vol). The eluate of the second solvent contained the two glycolipids. They were chromatographed on a silicic acid column, eluted in the acetone fraction, and finally separated by TLC on boric acid plates developed in chloroform-methanol-water (65:25:4, vol/vol/vol). No fatty acids were released from glycolipid A or B upon saponification, nor was any ester absorption in the 1,730 cm^{-1} region apparent in the infrared spectrum. The fatty acids, therefore, are not part of the two major glycolipids.

Glycolipid A. Glycolipid A was diphenylamine positive and rapid periodate-Schiff negative. Acid methanolysis in N methanolic hydrochloride at 100 C for 3 h released glycerol diether and two carbohydrates, galactose and glucose, determined as the TMS derivatives of the methyl glycosides by GLC, in a proportion of 0.98:1. Analysis of intact glycolipid A after treatment with boron trichloride-chloroform (1:1, vol/vol) at room temperature for 24 h gave 2.2 alkyl:1.0 glycerol:0.95 galactose:1.07 glucose. To determine the order of carbohydrate linkage, the glycolipid was subjected to mild acid methanolysis in 0.05 N methanolic hydrochloride at 60 C for 15 h. The sample was partitioned by addition of equal volumes of chloroform and water, and the organic phase was examined by TLC in chloroform-methanol-water (60:10:1, vol/vol/vol). Three components were present. One (R_f 0.26) was unreacted glycolipid. The second, the monoglycosyl product (R_f 0.72), migrated similarly to the polyol dialkyl glycerol triether (R_f 0.70) and was diphenylamine positive. Glycerol diether migrated with the solvent front. Acid methanolysis of the monoglycosyl glycerol diether released glycerol diether and galactose, indicating that galactose is internally linked to glycerol diether. Attempts to use alpha- or beta-galactosidases or glucosidases in various buffers or detergents to determine linkages were unsuccessful, probably because of the extreme hydrophobicity of these lipids in aqueous systems. A weak absorption band was present in the infrared spectrum of both glycolipids A and B at 897 cm^{-1}, which may indicate the presence of beta-linkages (27). Glycolipid A is tentatively identified as the diether analogue of glucosyl galactosyl diglyceride.

Glycolipid B. Glycolipid B was diphenylamine and rapid periodate-Schiff positive. Acid methanolysis released polyol dialkyl glycerol triether and only glucose, determined as the TMS derivative of the methyl glycoside by GLC. Analysis after degradation of intact glycolipid B with boron trichloride gave 2.06 alkyl:1.03 glycerol:1.0 polyol:1.12 glucose, which is consistent with a glucosyl polyol glycerol diether structure of glycolipid B.

Polar lipids. Three polar lipids, C, D, and E, were present in *Sulfolobus* as shown by TLC developed in chloroform-methanol-water (65:25:4, vol/vol/vol) (R_f 0.38, 0.32, and 0.18, respectively) (Fig. 1). All three components were present in the polar lipid fraction eluted from DEAE columns with chloroform-methanol-ammonia (70:30:2, vol/vol/vol) containing 0.4% ammonium acetate. Components C and E incorporated ^{32}P. Component D did not incorporate ^{32}P and appears to be a polar glycolipid. None of the components incorporated ^{35}S from [^{35}S]sodium sulfate under the conditions used in this study. The infrared spectrum of each component showed the complete absence of any

ester absorption in the 1,730 cm^{-1} region. Prolonged acid hydrolysis in 6 N HCl at 100 C for 96 h released all of the phosphorus as inorganic phosphate, indicating the absence of any phosphonate bonds. Under these conditions, 2-aminoethyl phosphonate remained intact. No ninhydrin-positive components were detected in the polar lipid fraction.

Polar lipid C. Polar lipid C was phosphate positive and diphenylamine and rapid periodate-Schiff negative. Acid methanolysis in N methanolic hydrochloride at 100 C for 3 h released chloroform-soluble glycerol diether and a methanol-water-soluble phosphate ester. The phosphate ester was further hydrolyzed in 6 N HCl at 100 C for 72 h. No glycerol was detected enzymatically or by TLC in chloroform-methanol-formic acid (65:25:10, vol/vol/vol). Instead, determined by GLC on columns of 5.5% SE-30 or 10% EGSS-X as the TMS derivative, myo-inositol was identified as the sole organic product. Inositol and phosphate were present in a ratio of 1.0:0.93. Strong base hydrolysis of intact polar lipid C released methanol-water-soluble inositol and the monophosphate ester. Examination of the chloroform-soluble products by TLC in chloroform-methanol-water (65:24:4, vol/vol/vol) showed intact polar lipid C (R_f 0.38), a phosphate-positive streak running up the plate from polar lipid C, typical of phosphatidic acid, and glycerol diether which migrated with the solvent front. A portion of polar lipid C was treated with boron trichloride-chloroform (1:1, vol/vol) at room temperature for 24 h. The products of degradation were assayed after partition of the residue between *n*-hexane and water. A sample of the water phase was hydrolyzed in 6 N HCl for 72 h to release phosphate and inositol. Analysis showed a content of 1.87 alkyl:1.02 glycerol:1.0 phosphorus:1.03 inositol. Polar lipid C may be identified as the diether analogue of phosphatidyl inositol.

Polar lipid D. Polar lipid D was diphenylamine and periodate-Schiff positive and phosphorus negative. It was always present in small amounts and eluted with the polar lipid fraction from DEAE-cellulose columns. Acid methanolysis released polyol dialkyl glycerol triether and glucose. No sugar acids were detected by GLC. Strong alkaline hydrolysis in N NaOH at 100 C for 3 h did not affect the chromatographic mobility of component D. The products of mild acid methanolysis in 0.05 N methanolic hydrochloride at 60 C for 15 h were examined by TLC in chloroform-methanol-water (65:25:4, vol/vol/vol). Three products were present. One (R_f 0.32) migrated as intact component D. A diphenylamine-positive spot co-chromatographed with glycolipid B and released glucose and polyol dialkyl glycerol triether after acid methanolysis. The third component (R_f 0.85) was diphenylamine negative and co-chromatographed with polyol dialkyl glycerol triether when examined by TLC in chloroform-methanol-water (60:10:1, vol/vol/vol). After boron trichloride degradation, analysis of component D gave 1.88 alkyl:1.06 glycerol:1.0 polyol:0.98 glucose. Glucose and polyol were estimated by GLC with inositol as internal standard. The polar glycolipid was examined by the barium chloranilate procedure (8) after acid methanolysis in N methanolic hydrochloride at 100 C for 3 h or after oxidation in boiling nitric acid. In both instances, sulfate was detected in a proportion of 0.85 sulfate:1.0 glucose. The infrared spectrum was very similar to the spectrum of glycolipid B except for an absorption band at 1,220 cm^{-1}, characteristic of S—O bonds (11). The sulfur appeared to be present in a sulfate linkage.

No ^{35}S supplied as [^{35}S]sodium sulfate was detectable in component D even though a sulfate salt-free medium was used. A different source of sulfate may be required. Apparently component D is the monosulfate derivative of glycolipid B. From preliminary evidence, it may be tentatively identified as the monosulfate derivative of glucosyl polyol glycerol diether.

Polar lipid E. Component E was phosphorus, diphenylamine, and rapid periodate-Schiff positive. The material was extremely polar, requiring chloroform-methanol-water (60:30:4.5, vol/vol/vol) for solution or elution from silica gel. Acid methanolysis in N methanolic hydrochloride released approximately equal proportions of glycerol diether and polyol dialkyl glycerol triether. Examination of the methanol-water phase showed the presence of carbohydrate, phosphorus, and inositol in the proportions 1.59:1.0:0.93. The carbohydrate consisted of galactose and glucose in a ratio of 1.05:2.0, determined as the TMS derivatives by GLC. Strong alkaline hydrolysis in N NaOH at 100 C for 3 h released two glycolipids which co-chromatographed with glycolipids A and B when examined by TLC in chloroform-methanol-water (65:25:4, vol/vol/vol). Analysis of the two glycolipids showed them to be identical to glucosyl galactosyl glycerol diether and glucosyl polyol glycerol diether. Examination of the water-soluble product after strong alkaline hydrolysis revealed the sole presence of inositol and phosphorus in a ratio of 1.06:1.0. Analysis of polar lipid E after degradation with boron trichloride-chloroform (1:1, vol/vol) at

room temperature for 24 h gave 2.1 alkyl:0.92 glycerol:0.51 polyol:0.52 galactose:0.99 glucose:1.0 phosphorus:1.02 inositol. Polar lipid E appeared to be a mixture of two phosphoglycolipids. Exhaustive attempts to resolve the mixture by TLC in acidic, basic, or neutral solvents, either as the intact lipid or acetylated derivative, proved unsuccessful. A partial separation was obtained by TLC developed in butanol-isopropanol-water (5:3:1, vol/vol/vol). A spot was obtained which streaked about 3 cm out of the origin. Acid methanolysis of the origin released about 80% of the polyol dialkyl glycerol triether, and the migrating streak released approximately 90% of the glycerol diether. A second approach was attempted to alter the polyol-containing phospholipid by periodate oxidation while leaving the other intact. Polar lipid E was dissolved in chloroform-methanol-water (1:2:0.5, vol/vol/vol) containing 50 μmol of $NaIO_4$. After 1 h, excess periodate was destroyed by addition of glycerol, and the contents, were partitioned by addition of chloroform and water. The organic phase was examined by TLC in chloroform-methanol-water (65:25:4, vol/vol/vol). Several phosphorus-positive spots were present. The one migrating as intact polar lipid E (R_f 0.18) was eluted and subjected to acid methanolysis. This phosphorus-containing material released only glycerol diether. Polyol dialkyl glycerol triether was absent. Galactose and glucose were present in a proportion of 1:1.38. Polar lipid E appear to consist of a mixture of approximately equal amounts of two phosphoglycolipids, designated E_1 and E_2, which are the inositol monophosphate derivatives of the two glycolipids found in the organism. It is not known whether the inositol monophosphate is linked to the external glucose or to the internal polyol or galactose in each structure. Phosphoglycolipid E_1 may be tentatively identified as inositolphosphoryl glucosyl galactosyl glycerol diether, and phosphoglycolipid E_2 may be tentatively identified as inositolphosphoryl glucosyl polyol glycerol diether. The analytical composition of *Sulfolobus* glycolipids and polar lipids is shown in Table 4.

Neutral lipids. The neutral lipids of *Sulfolobus* have not been examined in detail. Thin-layer chromatograms developed in the two-step solvent system, isopropyl ether-acetic acid (96:4, vol/vol) followed by *n*-hexane-diethyl ether-acetic acid (90:10:1, vol/vol/vol), revealed seven components, F through L (Fig. 1). Component F consisted of a series of hydrocarbons, although no long-chain $C_{40}H_{80}$ hydrocarbons were detected by GLC. Component G was yellow-orange in color and exhibited absorption peaks at 460, 438, 415, and 334 nm similar to those of carotenoids. Component J migrated as glycerol diether. The remaining components, H, I, K, and L, have not been examined.

DISCUSSION

The lipids of *S. acidocaldarius* contain ether linkages and are almost completely devoid of ester-bound fatty acids. Acid methanolysis releases two unsaponifiable ethers. One is a 1,2-substituted long-chain diether of glycerol. The second ether is unique. It contains two long-chain O-alkyl groups and glycerol linked to a polyol through an ether bond. Subjecting the ether to periodate oxidation did not destroy glycerol in the structure, as would be expected if glycerol were unsubstituted. The glycerol must,

TABLE 4. *Analytical composition of S. acidocaldarius glycolipids and polar lipids*

Component	Composition[a]	Molar ratio	Tentative identity
Glycolipid			Diether analogues of:
A	O-alkyl[b]-glycerol-galactose-glucose	2.20:1.00:0.95:1.07	Glucosyl galactosyl diglyceride
B	O-alkyl-glycerol-polyol[c]-glucose	2.20:1.03:1.00:1.12	Glucosyl polyol diglyceride
Polar lipid			
C	O-alkyl-glycerol-P-inositol	1.87:1.02:1.00:1.03	Phosphatidyl inositol
D	O-alkyl-glycerol-polyol-glucose/ SO_4^{-2}	1.88:1.06:1.00:0.98:0.85	Glucosyl polyol diglyceride mono sulfate
			Mixture of:
E	O-alkyl-glycerol-polyol-galactose-glucose-P-inositol	2.1:0.92:0.51:0.52:0.99:1.00: 1.2	Inositolphosphoryl glucosyl galactosyl diglyceride and inositolphosphoryl glucosyl polyol diglyceride

[a] Analysis after degradation in boron trichloride-chloroform (1:1, vol/vol).
[b] Determined as the alkyl chloride, molecular weight 595, or alkane, molecular weight 560.
[c] As yet unidentified polyol, ether linked to 1,2-dialkyl glycerol ether.

therefore, be substituted with at least one and most likely both long-chain alkyl groups. The intact ether contains six free acetylatable hydroxyl groups. The structure of the polyol remains unidentified, although it behaves as a heptitol or octitol and may be cyclic. We believe this ether to be a polyol dialkyl glycerol triether which may be the first natural occurrence of a substituted glycerol triether. It may be considered as the diether analogue of a monoglycosyl diglyceride, except that a polyol is ether linked rather than a sugar glycosidically linked to diglyceride.

The O-alkyl side chains of the ethers contained only one species of alkyl group when the alcohol or alkane derivative was examined by GLC. No double-bond absorption was apparent in the infrared spectrum of the intact ether or alkyl derivatives. [2-^{14}C]mevalonate was incorporated into all of the lipid components, suggesting isoprenoid-like branching. Analysis of the alkane by mass spectrometry showed a hydrocarbon of molecular weight 560, accounted for by the formula $C_{40}H_{80}$, and requiring the presence of a ring in the structure. The acetate derivative of the long-chain alcohol had a molecular weight of 619, indicating the presence of one hydroxyl group in the structure. The O-alkyl side chains of the ethers appear to consist of some type of isopranol derivative.

The lipids of *Sulfolobus* contain several new and unique lipid species. The glycolipids, making up nearly 70% of the total lipids, are composed of two major components, A and B, tentatively identified as the diether analogue of glucosyl galactosyl diglyceride, and glucosyl po-

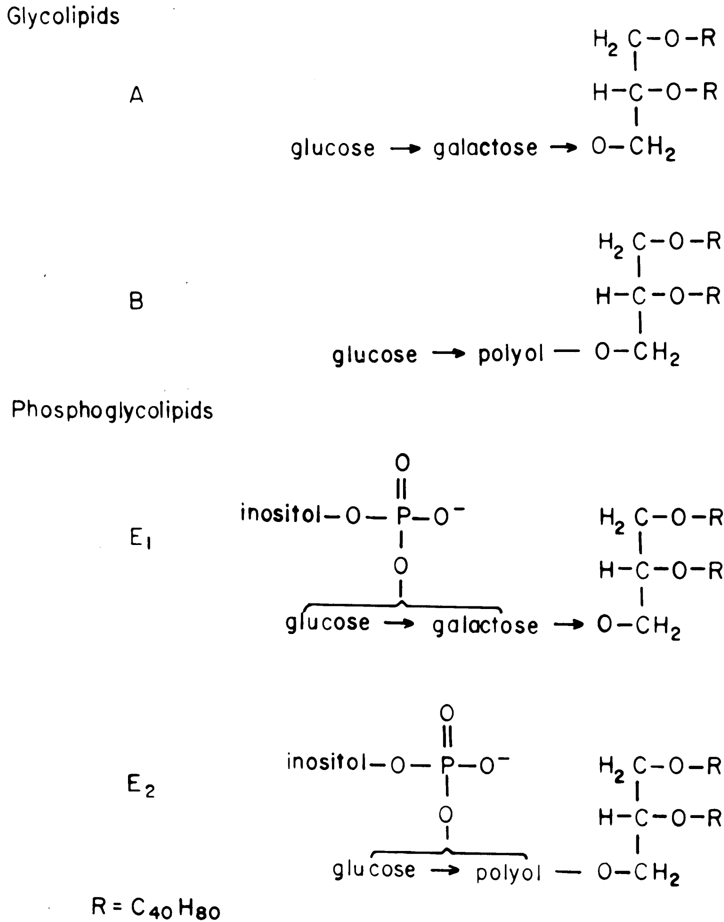

FIG 2. *Tentative structures of S. acidocaldarius glycolipids and phosphoglycolipids: (A) glucosyl galactosyl glycerol diether; (B) glucosyl polyol glycerol diether; (E_1) inositolphosphoryl glucosyl galactosyl glycerol diether; (E_2) inositolphosphoryl glucosyl polyol glycerol diether. The unidentified polyol exists in an ether linkage to glycerol diether as a polyol dialkyl glycerol triether.*

lyol glycerol diether, respectively. A small amount of a polar glycolipid, component D, appeared to be the monosulfate derivative of glycolipid B, although in this study it did not incorporate ^{35}S. Release of sulfur by acid methanolysis suggests the absence of a sulfonate linkage. It is tentatively identified as the monosulfate derivative of glucosyl polyol glycerol diether. The glycolipids appeared similar to the dihydrophytanyl glycerol diether glycolipids found in *Halobacter cutirubrum* except for the presence of polyol-substituted glycerol diethers in several of the *Sulfolobus* glycolipids (13).

The phospholipids of the organism appeared to be unique. Phospholipid C, accounting for about 40% of the lipid phosphorus, is the diether analogue of phosphatidyl inositol. Phospholipid E contains a mixture of two phosphoglycolipids, E_1 and E_2, which we believe to be the inositol monophosphate derivatives of the two glycolipids found in the organism. E_1 is tentatively identified as inositolphosphoryl glucosyl galactosyl glycerol diether, and E_2 is tentatively identified as inositolphosphoryl glucosyl polyol glycerol diether. The presence of only inositol-containing phospholipids is quite unusual, since phosphoinositides are generally not present to a great extent in bacteria (12). The tentative structures of glycolipids A and B and of the two phosphoglycolipids, E_1 and E_2, are illustrated in Fig. 2.

The lipids of *S. acidocaldarius* are of the long-chain isopranol glycerol ether type, which seems to be a common denominator of the low-pH-, high-temperature-requiring life forms. Both *Sulfolobus* and *Thermoplasma* glycerol diethers contain 40 carbon O-alkyl side chains, although they appear to differ slightly in their structures (16). The occurrence of long-chain glycerol diethers in *Sulfolobus* lends support to the suggestion (16) that thermophily can be related to the long isopranol chains of the lipids and acidophily can be related to the sole presence of ether lipids in these obligatory thermophilic, acidophilic organisms. The glycolipids and polar lipids of both organisms are composed exclusively of carbohydrate- or carbohydrate derivative-containing structures (16). Their presence may be related to the nature of the deficient wall of *Sulfolobus* (6) and the complete absence of a wall in *Thermoplasma*. It has been suggested that the accumulation of phosphoglycolipids in certain mycoplasmas may be related to the inability of these organisms to synthesize a normal cell wall structure (25). Surprisingly, only a small amount of sulfolipid exists in *Sulfolobus* considering that the organism is a facultative autotroph capable of elemental sulfur oxidation. The organism used in the study was grown heterotrophically in the absence of elemental sulfur. Possibly an altered lipid composition may exist when the organism is actively oxidizing elemental sulfur under autotrophic conditions.

A detailed study to characterize the structure of the polyol in the lipids of *S. acidocaldarius* is in progress.

ADDENDUM IN PROOF

Cells grown on a sulfate salt-free medium containing 200 μCi of $H_2{}^{35}SO_4$ per ml incorporated ^{35}S into polar lipid D.

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