electrons can be relayed both by tunneling and by motion in and of protein chains. For distances of >8 Å tunneling rates (currents in our electrodes) decrease exponentially with the distance between electron donors ($FADH_2$ centers) and electron acceptors (oxidized relays) and also between relays and electrodes. The tunneling rates increase when the relays are fast redox couples, i.e., when there is little change in the structure of the relay and its solvent environment upon oxidation or reduction. Furthermore, directional or vectorial tunneling, i.e., flow of current, requires that the potentials of the donor and the acceptor differ. Our design of modified enzymes, involving shortened tunneling distances, fast redox couple relays, and potential gradients, is consistent with that required for effective electron-tunneling systems. At the same time, motion of the protein chains to which the relays are attached, i.e., protein dynamics, enhances electron transfer, through transiently reducing the tunneling distances between the $FADH_2$ centers and the relays and between the relays and the metal or carbon electrodes.

Acknowledgment. We thank Pierre Wiltzius for measuring the diffusion coefficients of the native and the chemically modified enzymes, Anthony M. Williams for assays of iron and ruthenium, and Tetsuo Yamane for valuable discussions, and Barry Miller for improving the manuscript.

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Biosynthesis of Isoprenoid Membranes in the Methanogenic Archaebacterium *Methanospirillum hungatei*

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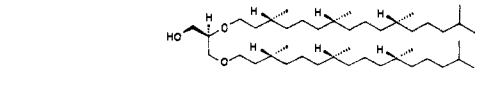
Abstract: The biosynthesis of 2,3-di-*O*-phytanyl-*sn*-glycerol (**1**) and 2,3-di-*O*-biphytanyl-*sn*-diglycerol (**2**), which are primary lipid components of the cell membrane of *Methanospirillum hungatei*, was studied by incorporation of ^{14}C-labeled diether **1** and its monophosphate **1**-P, ^{14}C-labeled tetraether **2** and its monophosphate **2**-P, [1-^{3}H]phytanol (**3**), [1-^{3}H]-(*E*)-phytol (**4**), and [1-^{3}H]-(*E*,*E*,*E*)-geranylgeraniol (**5**). No interconversion was found between the di- and tetraethers. Geranylgeraniol (**5**) was readily incorporated into ethers **1** and **2**; phytol (**4**) was incorporated poorly; and phytanol (**3**) was not incorporated. The results suggest that the ether linkages in archaebacterial lipids are established before the double bonds in the geranylgeranyl moiety are reduced and that diether **1** is not the immediate precursor of tetraether **2**.

Archaebacteria have recently been recognized as a distinct taxonomic unit that diverged from eubacteria and eukaryotes during the very early stages of evolution.[1] These unusual organisms inhabit hostile environments characterized by high salt (halophiles), high temperatures (thermophiles), low pH (acidophiles), or lack of oxygen (methanogens). Phenotypically, archaebacteria display distinct traits at the molecular level. Sequence analysis of 5S and 16S ribosomal RNA shows a distinct grouping within the archaebacterial kingdom that is far closer to other archaebacterial rRNAs than to eubacterial or eukaryotic rRNAs.[2-4] In addition, archaebacteria utilize metabolic cofactors not found in other organisms,[5-8] and the molecular architecture of lipids in their cell membranes is unique.[9]

In contrast to the fatty acid ester motif found in eubacteria and eukaryotes, archaebacterial membrane lipids are alkyl ether glycerolipids where the alkyl moieties are branched isoprenoid chains. The most commoly encountered structures are 2,3-di-*O*-[3*R*,7*R*,11*R*]-phytanyl-*sn*-glycerol (**1**)[10] and 2,3-di-*O*-[3*R*,7*R*,11*R*,15*S*,18*S*,22*R*,26*R*,30*R*]-biphytanyl-*sn*-diglycerol (**2**),[11] although several related variants have been reported, especially in the C_{40} units of tetraethers from thermophiles.[12] Labeling patterns clearly indicate that the C_{20} and C_{40} isoprenoid chains are derived from acetate via mevalonate by the same general process utilized by eubacteria and eukaryotes.[12] Little is known, however, about the sequence of steps beyond the geranylgeranyl stage of chain construction, especially with respect to the timing of condensation and hydrogenation reactions. We now report incorporation studies with advanced precursors that help clarify the intermediate stages of ether lipid biosynthesis in the strict anaerobe *Methanospirillum hungatei*.



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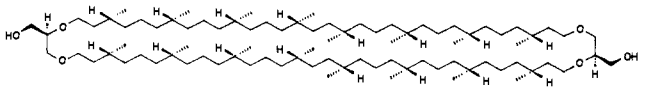

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Experimental Section

General Procedures. All solvents were of reagent grade or higher and distilled. Tetrahydrofuran was heated at reflux over lithium aluminum hydride, and methylene chloride and acetonitrile were heated at reflux over phosphorus pentoxide for several hours before distillation. Silica gel used for flash chromatography was from Baker (230–400 mesh). Analytical and preparative TLC of nonphosphorylated materials was carried out on Merck 60 GF 254 (0.25-mm) and Analtech silica gel GF (0.5-mm) plates, respectively. Compounds were visualized as colored spots by spraying with vanillin/H_2SO_4 (1:134, w/w) and then by heating on a hot plate. Analytical and preparative TLC of phosphorylated compounds were carried out on Merck cellulose plates (0.25 mm). Compounds were visualized as white spots on a pink background by spraying with a solution of 0.2% $FeCl_3$ in 80% EtOH and then a solution of 1% sulfosalicyclic acid in 60% EtOH. Dowex AG 50W-X8 cation-exchange resin (hydrogen form) was purchased from Bio-Rad Laboratories.

4-(*N,N*-Dimethylamino)pyridine, pyridinium dichromate, and dimethyl sulfide were purchased from Aldrich Chemical Co. Phytol was purchashed from Accurate Chemical and Scientific Corp. and used without further purification. Phytanol was prepared according to a previously described procedure.[13] (*E,E,E*)-Geranylgeraniol was provided by Dr. Desiree Bartlett. Ribonuclease and deoxyribonuclease were purchased from Sigma. Radiochemicals were from New England Nuclear.

IR spectra were recorded on a Perkin-Elmer Model 299 infrared spectrophotometer and calibrated to the 1602-cm^{-1} band in polystyrene. ^1H and ^{13}C NMR spectra were obtained on a Varian SC-300 spectrometer, using tetramethylsilane as an internal standard. ^{31}P NMR spectra were taken on a Varian FT-80A spectrometer, using 85% phosphoric acid as an external standard. Radioactivity was measured in a Packard Tri-carb 4530 liquid scintillation counter, and the samples were analyzed in 10 mL of Insta-fluor or Insta-gel (Packard) liquid scintillation cocktails. Mass spectra were obtained on a Varian MAT 731 mass spectrometer.

Cultures of *M. hungatei* GP1 and *Methanobacterium thermoautotrophicum* Δ*H* were maintained under anaerobic conditions as described by Wolfe and co-workers.[14,15] Cultures (100 mL) were grown from a 5% innoculum at 37 and 62 °C in 500-mL Wheaton bottles equipped with rubber stoppers and sealed with aluminum caps under an atmosphere of 4:1 (v/v) hydrogen/carbon dioxide according to the Balch and Wolfe technique.[15] Large cultures were prepared in a New Brunswick Microgen 10-L fermentor gassed with approximately 0.5 L/min of H_2/CO_2. Medium for *M. hungatei* consisted of the following ingredients diluted to 1 L with deionized water: K_2HPO_4 (0.82 g), $MgCl_2·6H_2O$ (1.6 mL of a 4% w/v solution), NH_4Cl (0.40 g), $CaCl_2·2H_2O$ (0.8 mL of an 8% w/v solution), NaCl (0.30 g), sodium acetate (1.64 g), sodium formate (1.36 g), Na_2SO_4 (0.70 g), resazurin (4 mL of a 0.025% solution), vitamin solution (10 mL), and mineral solution (10 mL). The vitamin solution contained the following materials in a final volume of 1 L of distilled water: biotin (2 mg), folic acid (2 mg), pyridoxime hydrochloride (10 mg), riboflavin (5 mg), thiamine (5 mg), nicotinic acid (5 mg), pantothenic acid (5 mg), vitamin B_{12} (0.1 mg), *p*-aminobenzoic acid (5 mg), and thioacetic acid (5 mg). The mineral solution contained nitrilotriacetic acid (1.5 g), $MnCl_2·4H_2O$ (0.10 g), $FeCl_2·4H_2O$ (0.15 g), $CaCl_2·2H_2O$ (0.05 g), $CoCl_2·6H_2O$ (0.20 g), $ZnCl_2$ (0.10 g), $CuSO_4$ (0.05 g), $Na_2MoO_4·2H_2O$ (0.05 g), and $NiCl_2·6H_2O$ (0.50 g) in a final volume of 1 L of distilled water. Medium for *M. thermoautotrophicum* was prepared as previously described.[16] For culture bottles the solution was adjusted to pH 6.0 with carbon dioxide, followed by addition of 1 M Na_2CO_3 to a final pH of 7.2. After the medium was placed under an anaerobic atmosphere of H_2/CO_2 (4:1, v/v), $Na_2S·9H_2O$ (final concentration of 1 mM) and L-cysteine hydrochloride (final concentration of 1.3 mM) were added, and the resulting solution was autoclaved. Fermentor media was mixed in the vessel, autoclaved, and then made anaerobic with H_2/CO_2. At this time Na_2S was added and pH was adjusted.

Isolation and Characterization of 1 and 2. a. From *M. Thermoautotrophicum* Δ*H*. Wet cells (180 g) of *M. thermoautotrophicum* Δ*H* were lyophilized for 3 days to give 36.2 g of a dry powder. The material was divided into 12 portions. Each was suspended in 30 mL of water and disrupted by two passes through a French press at 15000 psi. Lipids were extracted from the combined samples by a modification of the Bligh-Dyer procedure.[17] The aqueous suspension was diluted with 10 mL of water and sonicated for 3 min. Methanol (100 mL) and chloroform (50 mL) were added, followed by sonication for 2 min. Additional chloroform (50 mL) was added, followed by sonication for 45 s, and water (50 mL), followed by sonication for 1 min. The phases were separated. A mixture of methanol/chloroform/water (1:1:0.9) was added to the aqueous layer, followed by sonication for 3 min. The organic layer was removed, and the extraction was repeated two more times. The organic layers were combined, and solvent was removed at reduced pressure to give 633 mg of a reddish oil. The residue was washed three times with 30 mL of hexane to yield 570 mg of insoluble material.

The aqueous layer, which contained suspended cell debris, was filtered through filter paper by suction. The residue and the filter paper were extracted with 600 mL of a methanol/chloroform (2:1) for 2 days in a Soxhlet extractor. Solvent was removed at reduced pressure, and the residue was extracted with hexane, as described above, to yield 760 mg of hexane-insoluble material. The combined hexane-insoluble residues were treated with 175 mL of 6% methanolic HCl for 6 h. Water (175 mL) was added. The hydrolysate was extracted with three 100-mL portions of hexane and two 100-mL portions of chloroform. The extracts were combined and dried over magnesium sulfate. Solvent was removed at reduced pressure to give 605 mg of a light brown oil. The material was purified by flash chromatography on silica gel (hexane/ethyl acetate, 4:1) to yield **1** and **2** as colorless oils.

2,3-Di-*O*-phytanyl-*sn*-glycerol (1): 63 mg; R_f 0.65 (hexane/ethyl acetate, 4:1); $[\alpha]^{25}_D$ +7.8° (*c* 6.3, $CHCl_3$); IR (neat) 3450, 2960, 2930, 2870, 1463, 1378, 1365, 1115, 1048 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.83–0.90 (30 H, CH_3), 1.20 and 1.24 (48 H, CH_2, CH), 2.18 (1 H, exchanges in D_2O, OH), 3.30–3.93 (9 H, OCH_2, OCH); ^{13}C NMR ($CDCl_3$) δ 78.30 (d), 70.93 (t), 70.13 (t), 68.62 (t), 63.06 (t), 39.25 (t), 37.38 (t), 37.33 (t), 37.28 (t), 37.24 (t), 37.16 (t), 36.95 (t), 36.46 (t), 32.67 (d), 29.71 (d), 27.82 (d), 24.64 (d), 24.32 (d), 24.20 (d), 22.55 (q), 22.46 (q), 19.59 (q), 19.52 (q); FABMS, *m/z* at 653 $(M + H)^+$.

2,3-Di-*O*-biphytanyl-*sn*-diglycerol (2): 226 mg; R_f 0.33 (hexane/ethyl acetate, 4:1); $[\alpha]_D^{25}$ +8.6° (*c* 3.5, $CHCl_3$); IR (neat) 3450, 2950, 2925, 2860, 1460, 1375, 1115, 1050 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.83–0.89 (48 H, CH_3), 1.2 (104 H, CH_2, CH), 2.18 (2 H, exchange with D_2O, OH), 3.33–3.77 (18 H, OCH_2, OCH); ^{13}C NMR ($CDCl_3$) 78.60 (d), 71.27 (t), 70.30 (t), 68.81 (t), 63.26 (t), 37.63 (t), 37.48 (t), 37.17 (t), 36.70 (t), 34.42 (t), 33.16 (t), 32.89 (d), 29.93 (d), 29.88 (d), 24.54 (t), 19.91 (q); FABMS, *m/z* at 1301 $(M + H)^+$.

b. From *M. hungatei* GP1. A 2-g aliquot of lyophilized cells was processed by scaling down the above procedure 15-fold. After chromatography on silica gel, compounds identical with diether **1** (13 mg) and tetraether **2** (18 mg) were obtained.

[^{14}C]-2,3-Di-*O*-phytanyl-*sn*-glycerol ([^{14}C]1) and [^{14}C]-2,3-Di-*O*-biphytanyl-*sn*-diglycerol ([^{14}C]2). Wet cells (2.44 g) of *M. hungatei* GP1 grown on media supplemented with [2-^{14}C]acetate were treated by a scaled-down procedure identical with that used for unlabeled cells. After purification by chromatography on silica gel, 0.6 mg of [^{14}C]**1** (2.1 μCi/μmol) and 0.9 mg of [^{14}C]**2** (3.6 μCi/μmol) were obtained.

2,3-Di-*O*-phytanyl-*sn*-glyceryl Phosphate (1-P). (1-Phenyl-1,2-dibromoethyl)phosphonic acid (26 mg, 76 μmol) was added to a solution of 25 mg (38 μmol) of diether **1** in 1 mL of methylene chloride. The flask was covered with aluminum foil, and the mixture was stirred under nitrogen at room temperature for 10 min. Diisopropylethylamine (20 mg, 155 μmol) was added by syringe, and stirring was continued for 24 h. Solvent was removed at reduced pressure, and the residue was extracted three times with 5 mL of diethyl ether. Solvent was again removed at reduced pressure, and the residue was purified by TLC on cellulose to give 18 mg (53%) of a colorless gum, R_f 0.41 (acetonitrile/water/diisopropylamine, 84:15:1). The residue was dissolved in 2.5 mL of tetrahydrofuran/water (3:2) and passed through a 0.5-mL column of Dowex AG 50W-X8 cation-exchange resin (Na^+ form). The eluent was lyophilized to yield 15 mg (50% overall) of a colorless gum: ^1H NMR ($CDCl_3$) δ 3.55 (7 H, OCH_2, OCH), 3.86 (2 H, CH_2OPO_3); ^{31}P NMR ($CDCl_3$) δ 3.10.

2,3-Di-*O*-biphytanyl-*sn*-diglyceryl Phosphate (2-P). Following the procedure described for **1**-P, 34 mg (26 μmol) of tetraether **2** was phosphorylated with 18 mg (52 μmol) of (1-phenyl-1,2-dibromoethyl)-phosphonic acid and 13 mg (104 μmol) of diisopropylethylamine to yield 14 mg (36%) of a colorless oil, R_f 0.79 (2-propanol/chloroform/acetonitrile/0.1 M ammonium bicarbonate, 5:2.5:1:1.5). After ion exchange on Dowex AG 50W-X8 (Na^+ form), 11 mg (30% overall) of a colorless gum was obtained: ^1H NMR δ 3.47 (14 H, OCH_2, OCH), 3.85 (4 H, CH_2OPO_3); ^{31}P NMR ($CHCl_3$) δ 3.70.

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[^{14}C]-**2,3-Di-*O*-phytanyl-*sn*-glyceryl Phosphate** ([^{14}C]**1-P**). Following the procedure described for **1**-P, 0.27 mg (0.4 μmol, 2.1 μCi/μmol) of [^{14}C]**1** and 6 mg (9.2 μmol) of **1** were phosphorylated to yield 2.4 mg (22%) of a colorless gum (0.09 μCi/μmol).

[^{14}C]-**2,3-Di-*O*-biphytanyl-*sn*-diglyceryl Phosphate** ([^{14}C]**2-P**). Following the procedure for **2**-P, 0.36 mg (0.3 μmol, 3.6 μCi/μmol) and 6 mg (4.6 μmol) of unlabeled **2** were phosphorylated to yield 0.7 mg (4%) of a colorless gum (0.18 μCi/μmol).

(3*R*,7*R*,11*R*)-Phytanol (3). 2,3-Di-*O*-phytanyl-*sn*-glycerol (**1**; 16 mg, 24.5 μmol) from *M. thermoautotrophicum* was heated at reflux in 2 mL of 47% HI for 24 h. The mixture was extracted with 20 mL of ether, and the ether extract was washed in succession with water, saturated potassium carbonate, and 50% sodium thiosulfate. The ether layer was dried over magnesium sulfate, and solvent was removed at reduced pressure. The residue was dissolved in 1.6 mL of glacial acetic acid, 42 mg of powdered silver acetate was added, and the suspension was stirred at reflux for 24 h. Ether (20 mL) was added, the suspension was filtered, and the filtrate was washed in succession with water, brine, saturated sodium bicarbonate, and 40% sodium thiosulfate. The ether layer was dried over potassium carbonate, and solvent was removed at reduced pressure. Sodium hydroxide in methanol (1.5 mL, 3:97, w/v) was added, and the solution was heated at reflux for 1 h. Water (1.5 mL) was added, and the mixture was extracted three times with 4 mL of ether. The combined ether layers were washed with water and dried over magnesium sulfate. Solvent was removed at reduced pressure, and the residue was chromatographed on silica gel to yield 2.0 mg (14%) of a colorless oil, R_f 0.17 (hexane/ethyl acetate, 9:1). The material gave an identical R_f on TLC and an IR spectrum and a ^{1}H NMR spectrum similar to those of a sample obtained by hydrogenation of phytol (Aldrich).

(3*R*,7*R*,11*R*)[1-^{3}H]Phytanol ([1-^{3}H]3). Pyridinium dichromate (8 mg)[18] was added to a solution of 2.0 mg (6.7 μmol) of (3*R*,7*R*,11*R*)-phytanol (**3**) in 1 mL of methylene chloride. The mixture was stirred at room temperature for 24 h, diluted with 2 mL of ether, and filtered in sequence through small plugs of anhydrous magnesium sulfate and silica gel. Solvent was removed at reduced pressure, and the residue was purified by preparative TLC on silica gel to give 1.8 mg (91%) of a colorless oil, R_f 0.48 (hexane/ethyl acetate, 19:1). The oil was dissolved in 0.5 mL of methanol, and 0.2 mL of a solution containing 5.0 μequiv of sodium [^{3}H]borohydride (New England Nuclear) in methanol was added.[19] The material was allowed to stand for 1 h at room temperature. Cold **3** (7.0 mg) was added, solvent was removed with a gentle stream of nitrogen, and the residue was purified by TLC on silica gel to yield 8.1 mg (90%, 11.5 μCi/μmol) of a colorless oil, R_f 0.17 (hexane/ethyl acetate, 9:1), that comigrated with authentic **3**.

(7*R*,11*R*)[1-^{3}H]Phytol ([1-^{3}H]4). A suspension of 390 mg of active manganese dioxide and 20 mg (68 μmol) of (7*R*,11*R*)-phytol (**4**) in 3 mL of hexane was stirred for 1.5 h at room temperature.[19] The material was filtered, and solvent was removed at reduced pressure. The residue was purified by preparative TLC on silica gel to give 13 mg (65%) of a colorless oil: R_f 0.67 (hexane/ethyl acetate, 5:1); ^{1}H NMR ($CDCl_3$) δ 5.73 (1 H, d, J = 6 Hz, H at C2), 9.79 (1 H, d, J = 6 Hz, H at C1). A 9-mg (31-μmol) portion of the oil was reduced with sodium [^{3}H]-borohydride as described for [1-^{3}H]**3** to yield 5.7 mg (62%, 54 μCi/μmol) of a colorless oil, R_f 0.33 (hexane/ethyl acetate, 5:1), that comigrated with authentic **4**.

(*E*,*E*,*E*)[1-^{3}H]Geranylgeraniol ([1-^{3}H]5). Following the procedure described for [1-^{3}H]**4**, 36 mg (124 μmol) of **5** was treated with 389 mg of manganese dioxide to yield 26 mg (73%) of a colorless oil, R_f 0.59 (hexane/ethyl acetate, 85:15). A 3.6-mg (12.5-μmol) portion of the sample was treated with sodium [^{3}H]borohydride to yield 3.0 mg (83%, 64 μCi/μmol) of a colorless oil, R_f 0.38 (hexane/ethyl acetate, 85:15), that comigrated with authentic **5**.

Feeding Experiments. Wheaton bottles (500 mL) containing 100 mL of medium were prepared as described under general procedures, given a 5% innoculum of *M. huntagei* GP1, and incubated with shaking for 3 days at 37 °C. Labeled precursors were dissolved in 20 μL of ethanol and injected into the bottles at day 3. At day 10, cells were harvested by centrifugation (17000 rpm, 45 min, 2 °C). The pellet was repeatedly resuspended in 30 mL of water and centrifuged until the total radioactivity in the supernatant dropped below 200 dpm. The cells were washed with 30 mL of 0.1% Tween 80, centrifuged, and lyophilized. Typical yields were 20 mg of lyophilized material/100 mL of 10-day-old culture. Membranes were obtained by suspending 40 mg of labeled cells in 30 mL of 50 mM $NaHCO_3$. The suspension was adjusted to pH 10.0 with 1 M NH_4OH, and 3 mL of 1 M sucrose and 8 mg of dithiothreitol were added. The suspension was gently stirred at room temperature for 45 min and spun at 27100g for 30 min at 4 °C. The pellet was resuspended in 25 mL of ice-cold water containing 0.1 mg each of ribonuclease and deoxyribonuclease. After standing for 30 min at 4 °C, the mixture was sonicated for 1 min and centrifuged at 176600g for 2 h at 4 °C. The pellet was resuspended in 15 mL of water and centrifuged again. The pellet was lyophilized to give 14 mg of a lightly colored residue. Diether **1** and tetraether **2** were isolated from the membranes by scaled-down versions of the procedures described above. Materials were purified by TLC and gave single radioactive spots that comigrated with authentic samples.

Table I. Distribution of Radioactivity in Diether **1** and Tetraether **2** from Membranes of *M. hungatei* Labeled with [^{14}C]**1** and [^{14}C]**2**

		radioactivity, dpm			
		hexane soluble		hexane insoluble[a]	
substr	substr	**1**	**2**	**1**	**2**
[^{14}C]**1**	1.7×10^5	1.4×10^4			
[^{14}C]**2**	2.3×10^5		7.7×10^4		1.6×10^3
[^{14}C]**1**-P	3.2×10^4			8.6×10^2	
[^{14}C]**2**-P	1.5×10^4		2.0×10^3		2.6×10^2

[a] **1** and **2** obtained after acidic hydrolysis.

Results

Incorporation Studies with [^{14}C]1 and [^{14}C]2. ^{14}C-Labeled **1** and **2** were obtained by growing *M. hungatei* on [^{14}C]acetate. The cells were ruptured in a French press, and lipids were extracted with a mixture of methanol and chloroform. The extract was subjected to acidic hydrolysis, and labeled di- and tetraethers were purified by chromatography on silica gel. The corresponding monophosphates **1**-P and **2**-P were prepared from the free alcohols by the procedure of Rameriz, et al.[20] The diisopropylethylammonium salts of **1**-P and **2**-P were purified by preparative TLC and converted to the corresponding sodium salts by ion-exchange chromatography.

Rabiolabeled ethers **1** and **2** and the corresponding phosphates **1**-P and **2**-P were injected into 3-day-old cultures of *M. hungatei*. After 7 additional days the bacteria were harvested by centrifugation. The cells were repeatedly washed until the total radioactivity in the wash dropped below 200 dpm and were then washed once with 0.1% Tween 80 to remove any loosely associated radioactivity from the cell surface. The cells were disrupted by osmotic shock, and the membrane fraction was isolated according to the procedure of Sprott and co-workers.[21] Lipids were extracted from the membranes with a mixture of methanol/chloroform/water, and the lipid fraction was extracted with hexane. Cold **1** and **2** were added to the extract, and the hexane-soluble material was purified by thin-layer chromatography on silica gel. The hexane-insoluble lipids were hydrolyzed at reflux for 6 h in 6% methanolic HCl. Cold carriers were added, and ethers **1** and **2** were purified by thin-layer chromatography. All of the radioactivity from the bacterial isolates comigrated with **1** or **2**. Chromatography of control samples showed that diether **1** (R_f 0.65) was cleanly separated from tetraether **2** (R_f 0.33) in our experiments.

The results for diether **1**, tetraether **2**, and their respective phosphates are summarized in Table I. All of the radioactivity in membranes labeled with [^{14}C]**1** or **1**-P was recovered in the diether fractions. Specifically, radioactivity introduced in **1** was located exclusively in the hexane-soluble diether fraction, while radioactivity introduced in **1**-P was found exclusively in the hexane-insoluble diether fraction. Radioactivity from tetraethers **2** and **2**-P was located exclusively in the tetraether fractions. In both cases, higher amounts, 48- and 8-fold, respectively, were recovered from hexane-soluble than from hexane-insoluble fractions.

Incorporation Studies with [1-^{3}H]3–5. Radiolabeled (3*R*,7*R*,11*R*)-phytanol (**3**), (7*R*,11*R*)-phytol (**4**), and geranyl-

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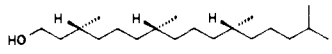
Table II. Distribution of Radioactivity in Diether **1** and Tetraether **2** from Membranes of *M. hungatei* Labeled with [1-^{3}H]**3**–**5**

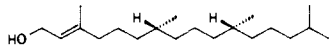
		radioactivity, dpm					
		hexane soluble			hexane insoluble[a]		
substr	substr	**1**	**2**	C_{20} alcohol	**1**	**2**	C_{20} alcohol
[1-^{3}H]**3**	7.7×10^6			3.3×10^6			3.6×10^4
[1-^{3}H]**4**	6.3×10^7	1.0×10^4	1.8×10^4	1.4×10^7	2.2×10^4	1.3×10^4	3.0×10^4
[1-^{3}H]**5**	7.2×10^6	7.4×10^4	5.3×10^4	1.4×10^6	6.5×10^5	5.3×10^5	3.8×10^4

[a] After acidic hydrolysis.

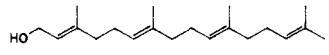
geraniol (**5**) were prepared by reduction of the corresponding aldehydes with sodium [^{3}H]borohydride according to procedures commonly used for isoprenoids.[19] The compounds were ad-



3



4



5

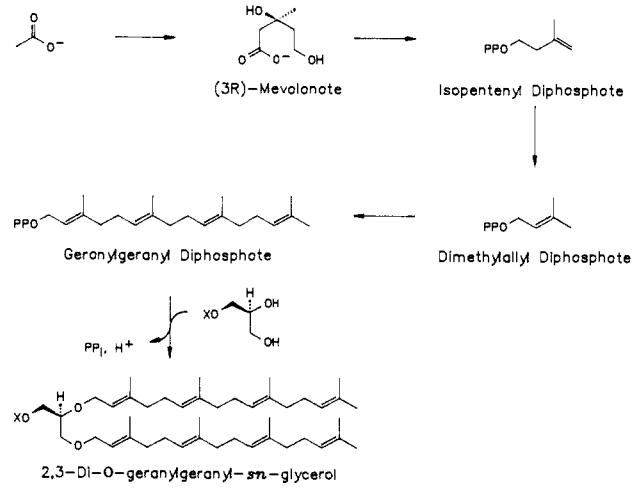
ministered to 3-day-old cultures of *M. hungatei*, which were incubated for 7 additional days. Membranes from labeled cells were treated as previously described to yield mixtures of diethers and tetraethers from hexane-soluble and hexane-insoluble extracts. The hexane-soluble fractions were heavily contaminated with unconsumed **3**, **4**, or **5**. In all instances the C_{20} alcohols migrated between diether **1** and tetraether **2** during the thin-layer chromatography. From control experiments we established that there was very little spillover (<0.005%) from the alcohols into the diether fractions. However, the C_{20} alcohols tailed substantially into the tetraether fractions. TLC separations were optimized for each C_{20} alcohol on 20-cm plates as follows: for **3**, R_f(**1**) 0.56, R_f(**3**) 0.46, R_f(**2**) 0.18 with 4:1 hexane/ethyl acetate; for **4**, R_f(**1**) 0.56, R_f(**4**) 0.39, R_f(**2**) 0.17 with 4:1 hexane/ethyl acetate; and for **5**, R_f(**1**) 0.65, R_f(**5**) 0.49, R_f(**2**) 0.28 with 9:1 chloroform/ether. The most difficult separation was for geranylgeraniol. In the chloroform/ether system approximately 1% of the radioactivity in **5** was recovered in the tetraether fraction. However, residual contamination dropped to less than 0.03% with greater than 95% recovery of radioactivity in the geranylgeraniol fraction when the tetraether was rechromatographed two additional times. Rechromatography of the tetraethers contamined with phytanol or phytol gave residual radioactivities of 0.001% and 0.002%, respectively, with excellent recoveries of counts in the C_{20} alcohol fractions.

The distributions of radioactivity in diether **1** and tetraether **2** from cells labeled with the three C_{20} alcohols are given in Table II. All of the entries for **1** and **2** are for thrice chromatographed material. Phytanol (**3**) did not label **1** or **2** above the background (0.001%) established in control experiments. Phytol (**4**) gave low, but significant (0.016–0.035%), incorporations, while geranylgeraniol (**5**) efficiently labeled **1** and **2**. Diether **1** and tetraether **2** contained 9% and 7.4%, respectively, of the radioactivity originally introduced in [1-^{3}H]**5**. The recoveries of total radioactivity from the incubations were 43% for **3**, 22% for **4**, and 38% for **5**.

Discussion

Archaebacteria have an unusually high demand for isoprenoids because of the unique nature of their membrane lipids. Several studies indicate that the early stages of the pathway in archaebacteria are identical with those found in eubacteria and eukayrotes. The isoprenoid side chains of archaebacterial ether lipids of *Thermoplasma*,[22] *Halobacterium*,[23] and *Sulfolobus*[24,25] were

Scheme I. Proposed Biosynthetic Pathway for C_{20} Diethers



selectively labeled by acetate or mevalonate in the manner expected. Activity for 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase, a key regulatory enzyme in the isoprenoid pathway of higher animals, was recently discovered in *Halobacterium halobium*.[26] Growth of the bacterium was blocked by mevinolin, a known potent inhibitor of HMGCoA reductase, and a concomitant rapid accumulation of intracellular HMGCoA was noted. All of these observations are suggestive of a normal mevalonate-based pathway.

Later stages of diether and tetraether biosynthesis have not been extensively explored. Presumably, the hydrocarbon chains are assembled from dimethylallyl diphosphate and isopentenyl diphosphate in the standard manner, as shown in Scheme I.[27,28] Kates[29] originally suggested that the double bonds in the isoprenoid chains were removed before they were attached to the glycerol moiety, while DeRosa and co-workers[12] favored attachment before hydrogenation. From a mechanistic viewpoint the latter scenario is more reasonable. Most of the carbon–carbon and carbon–heteroatom bonds in the early stages of the isoprenoid pathway are constructed during prenyl-transfer reactions.[27,28] The mechanisms of these reactions are typically electrophilic alkylations of prenyl acceptors (most often the double bond in isopenteyl diphosphate) by allylic carbocations. The ether linkages in **1** and **2** could easily be formed in a similar manner by a prenyltransferase that utilizes glycerol, or a related derivative, as the prenyl acceptor. The allylic double bond in the prenyl diphosphate donor is essential

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for the substrate to be reactive in the reaction.[27,28,30,31]

Our studies with [1-^{3}H]**3**–**5** clearly support a prenyl-transfer mechanism for attachment of the isoprene residues to the glyceryl moiety. [1-^{3}H]Phytanol (**3**) did not significantly label diether **1** or tetraether **2** in the hexane-soluble or hexane-insoluble membrane fractions from *M. hungatei*. A small, but significant, amount of radioactivity was incorporated from (7*R*,11*R*)-phytol (**4**). (*E*,-*E*,*E*)[1-^{3}H]Geranylgeraniol (**5**) was, however, very efficiently incorporated into **1** and **2** in the hexane-soluble and hexane-insoluble components of the membrane. Diether **1** and tetraether **2** from the hexane-soluble fraction contained 1.03% and 0.74%, respectively, of the total radioactivity originally adminstered in **5**, while **1** and **2** from the hexane-insoluble fraction contained 9.0% and 7.4% of the total radioactivity, respectively. Clearly, the presence of an allylic double bond is necessary, and unsaturation in the tail is strongly preferred. At the present time the form of glycerol that serves as the acceptor (Scheme I) is not knonw. Since prenyl-transfer reactions utilize allylic diphosphates as substrates, *M. hungatei* must posses kinases capable of phosphorylating isoprenoid alcohols. The preference for incorporation of **5** rather than **4** could occur because of differences in transport properties or differences in substrate specificities by the relevant kinases and prenyltransferases. We favor the latter explanation. The C_{20} alcohols should have similar solubility characteristics, and analysis of the distribution of radioactivity in the hexane-soluble fractions indicates that all three alcohols are efficiently taken into the membranes of the bacterium. It is also well documented that prenyltransferases have strong preferences for the nonallylic double bonds in their substrates.[28] The high levels of incorporation of geranylgeraniol into **1** and **2** strongly suggest that the prenyl-transfer step precedes hydrogenation of the nonallylic double bonds. Cell-free studies will ultimately be needed to resolve many of the uncertainties raised above.

Feeding experiments with ^{14}C-labeled **1**, **2**, and their corresponding phosphates gave no significant levels of incorporation into other metabolites. Substantial amounts of radioactivity remained in the medium or were recovered in the washes. All of the radioactivity in diether **1** remained in the hexane-soluble membrane fraction, and radioactivity from **1**-P was located in the insoluble fraction, as expected if neither were metabolized. Clearly **1** and **1**-P are not substrates for the tetraethers. Radioactivity from **2** and **2**-P was located in both hexane fractions, perhaps as the result of phosphorylation/dephosphorylation reactions.

In summary, the results of our labeling experiments with phytanol (**3**), phytol (**4**), and geranylgeraniol (**5**) can be readily explained by a simple prenyl-transfer reaction mechanism for alkylation of glycerol as outlined in Scheme I. The ether-forming step in *Sulfolobus sulfataricus*, a thermoacidophile, occurs without loss of hydrogen from carbons in the glyceryl moiety and without any intervening oxidized forms of glycerol.[32] Given the very high efficiency for incorporation of **5** relative to **4**, and the lack of conversion of **1** to **2**, it is likely that the prenyl transfers that generate the ether linkages and the unique 4–4′ condensation that joins the termini of the C_{20} hydrocarbon moieties both occur before saturation of the isoprene chains.

Acknowledgment. This work was supported by Grant GM 21328 from the National Institutes of Health.

Registry No. 1, 23315-10-8; [^{14}C]**1**, 113303-36-9; **1**-P, 31979-09-6; [^{14}C]**1**-P, 113303-39-2; **2**, 99529-31-4; [^{14}C]**2**, 113303-37-0; **2**-P, 113303-38-1; [^{14}C]**2**-P, 113303-40-5; **3**, 18654-63-2; [1-^{3}H]**3**, 113303-41-6; [1-^{3}H]**4**, 55497-16-0; **5**, 24034-73-9; [1-^{3}H]**5**, 74730-48-6.

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