CDP-2,3-di-O-geranylgeranyl-sn-glycerol synthase (CDP-archaeol synthase) activity was discovered in the membrane fraction of the methanoarchaeon *Methanothermobacter thermoautotrophicus* cells. It catalyzed the formation of CDP-2,3-di-O-geranylgeranyl-sn-glycerol from CTP and 2,3-di-O-geranylgeranyl-sn-glycerol-1-phosphate (unsaturated archaeic acid). The identity of the reaction product was confirmed by thin layer chromatography, fast atom bombardment-mass spectrometry, chemical analysis, and by UV spectroscopy. One mole of the product was formed from approximately 1 mol of each of the reactants. The enzyme showed maximal activity at pH 8.5 and 55 °C in the presence of Mg²⁺ and K⁺ ions. By in vivo pulse labeling of phospholipids with [³²P]₀, CDP-archaeol was found to be an intracellular intermediate. A cell-free homogenate of *M. thermoautotrophicus*, when incubated with L-serine, converted the product of CDP-archaeol synthase reaction to a product with the same chromatographic mobility as archaetidylserine. It was concluded from these results that both CDP-archaeol and CDP-archaeol synthase were involved in cellular phospholipid biosynthesis. Among various synthetic substrate analogs, both enantiomers of unsaturated archaeic acid possessing geranylgeranyl chains showed similar levels of activity, while archaeic acid with saturated or monounsaturated isoprenoid or straight chains was a poor substrate, despite having the same stereostructure as the fully active substrate. The ester analogs with geranylgeraniol chains showed significant activities. These results suggest that the enzyme does not recognize ether or ester bonds between glycerophosphate and hydrocarbon chains nor the stereostructure of the glycerophosphate backbone but mainly targets substrates with geranylgeranyl chains.

To our knowledge, 100 or more polar lipids have been identified from 25 species of Archaea (LipidBank for Web, available via the World Wide Web). Because all the archaen phospholipids consist of ether linkages between glycerophosphate (GP), and isoprenoid alcohols, they are usually referred to as “ether lipids.” Another remarkable feature of archaenal phospholipids is the stereocconfiguration of the GP backbone. The GP backbone of phospholipid in Archaea is sn-glycerol-1-phosphate (G-1-P), which is the enantiomer of its bacterial and eucaryal counterparts (1). So far, no exception to this arrangement in the stereostructure of GP has been found, and remains the most fundamental distinguishing characteristic of the organisms in each of these domains (2). In *vitro* studies of polar lipid biosynthesis in Archaea have been carried out since 1990, and the enzymes catalyzing the first three steps have been identified (Refs. 3–6, Fig. 1). The last intermediate (2,3-di-O-geranylglycerol-G-1-P = 2,3-GG-GP ether = unsaturated archaeic acid) corresponds to phosphatidic acid (the fatty acyl ester form of the phospholipid intermediate) in phospholipid biosynthesis of *Escherichia coli*, which is then activated by CTP to become diacylglycerol by the action of CTP-phosphatidate cytidyltransferase (EC 2.7.7.41) (7). CDP-diacylglycerol plays a central role in the biosynthesis of a number of phospholipids in bacteria (8). Although 2,3-GG-GP ether is the analogous intermediate of polar lipid biosynthesis in Archaea, its hydrocarbon chains are still highly unsaturated unlike the final products in which the chains are completely saturated. Therefore, one may reasonably expect to encounter hydrogention, nucleotide activation, and polar group attachment in the archaenal polar lipid biosynthetic pathway, but their exact sequence of events is not known. In order to elucidate this pathway of polar lipid biosynthesis in Archaea, we began by looking for an enzyme capable of activating the unsaturated archaeic acids by CTP. We found such activity in the methanogenic archaenan *Methanothermobacter thermoautotrophicus* (formerly *Methanobacterium thermoautotrophicum*, Ref. 9), which catalyzed the formation of CDP-di-O-geranylglycerol (CDP-unsaturated archaenal) from CTP and 2,3-GG-GP ether (CTP:2,3-GG-GP ether cytidyltransferase, e.g., CDP-di-O-geranylglycerol synthase or CDP-archaeol synthase) according to Reaction 4, shown in Fig. 1.

The enzymes catalyzing steps 1–3 in Fig. 1 appear to be specific for G-1-P and derivatives of the same stereoconfigura-
The present paper reports, for the first time, the characterization of CDP-archaeol synthase in the Methanoarchaeon *M. thermoautotrophicus*. The nomenclature of archaeal lipids proposed by Nishi-hara and comments upon the biosynthetic significance of the enzyme. The identities of two of the final products, 2,3-di-phyto-glycerol (10), are described in the present paper.

**EXPERIMENTAL PROCEDURES**

**Growth of Microorganisms—** *M. thermoautotrophicus* 
*ΔH* (DSM 1053) was grown at 65 °C in a 15-liter fermentor (Mituwa, Japan) containing 5 liters of medium 2 (11) or in a 50-liter fermentor (Marubishi Model MSJ-N2) containing 33 liters of the same medium. Cells were harvested at log phase. *Halobacterium salinarum* (JCM 8981) was grown in 1 liter of JCM medium 12 (JCM Catalogue of Strains 1999) with shaking at 37 °C for 2 days. *E. coli* (JCM 1649) was grown in 1 liter of JCM medium 12 (JCM Catalogue of Strains 1999) with shaking at 37 °C for 6 h.

**Thin Layer Chromatography (TLC)**—TLC was carried out on a Silica Gel 60 plate (Merck) with the following solvents: solvent A, chloroform, methanol, and 7 % ammonia (60:35:8); solvent B, chloroform, methanol, acetic acid, and water (80:30:15:5). Phospholipid spots were visualized by spraying acid molybdate reagent. The radioactive spots were recorded by a Fujifilm FLA-2000 fluor image analyzer with an imaging plate (Fujifilm type BAS-TR for 3H-labeled lipids and type BAS-MS for 14C- and 32P-labeled lipids).

**Chemical Synthesis of Archaeidic Acids**—The chemical syntheses of various archaeidic acids are summarized in Fig. 2. Unsaturated and saturated archaetidic acids were synthesized from geranylgeraniol (1, 5, 5', 8, 8', 11, 11', 14, 14'), phytol (2, 6, 9, 12, 15), oleyl (3, 7, 10, 13, 16), or phytanyl (17, 18, 19) group. 4 and 5–16, 1-O-benzyl-sn-glycerol and its derivatives, respectively; 4', 5', 8', 11', and 14', 2-O-benzyl-sn-glycerol and its derivatives, respectively. 17, 18, 19, 2,3-di-O-phytanyl-sn-glycerol and its derivatives, respectively. DMSO, dimethyl sulfoxide; DMAP, 4-(dimethylamino)pyridine.

**Fig. 1.** Enzymatically identified reactions of biosynthesis of polar lipids in Archaea. Reactions 1–3 have already been reported (3–6), and Reactions 4 and 5 are described in the present paper. PPI, pyrophosphate; DHAP, dihydroxyacetone phosphate.

**Fig. 2.** Synthesis of unsaturated and saturated archaeidic acids. R represents a geranylgeranyl (1, 5, 5', 8, 8', 11, 11', 14, 14'), phytol (2, 6, 9, 12, 15), oleyl (3, 7, 10, 13, 16), or phytanyl (17, 18, 19) group. 4 and 5–16, 1-O-benzyl-sn-glycerol and its derivatives, respectively; 4', 5', 8', 11', and 14', 2-O-benzyl-sn-glycerol and its derivatives, respectively. 17, 18, 19, 2,3-di-O-phytanyl-sn-glycerol and its derivatives, respectively. DMSO, dimethyl sulfoxide; DMAP, 4-(dimethylamino)pyridine.
RESULTS

CDP-archaeol Synthase Activity—A cell-free homogenate of M. thermoautotrophicus catalyzed the conversion of \(^{3}H\)CTP to chloroform-extractable material. Under assay conditions, incorporation of radioactivity into a lipid form continued almost linearly for at least 10 h, and then gradually slowed down (Fig. 3). In routine assays, the incubation time was 2 h. The activity was entirely dependent on the lipid substrate (2,3-GG-GP ether) revealed \(m/z\) 752 (M + Na + H\(^+\)) and \(m/z\) 695 (M + Na\(^+\)), respectively.

Saturated archaeatic acid with phytanyl chains, 2,3-di-O-phytanyl-sn-glycero-1-phosphate (19, 2,3-phytta-GE ether) was prepared by phosphorylation of 2,3-di-O-phytanyl-sn-glycerol (17, archaee), with diphenyl chloro (Tokyo Kasei, Japan) prepared from total lipid extract of \(H. salinarum\) cells (14). The phenyl groups were removed by catalytic hydrogenolysis (15). Authentic archaeatic acid (16), obtained from M. thermoautotrophicus, was co-chromatographed with product 19 on one-dimensional TLC plates using solvents A (R\(_F\) 0.41) and B (R\(_F\) 0.73), and product 19 was also confirmed by \(m/z\) 733 (M + H\(^+\)) of FAB-MS.

Chemical Synthesis of Phosphatidic Acids—Several kinds of phosphatidic acids (diacyl glycerocephosphate) were synthesized by acylation of G-1-P as described (14). sn-Glycerol-phosphate (G-3-P, Nacalai Tesque, Japan), or rac-glycerophosphate (rac-GP, Nacalai Tesque) with geranylgeranoyl anhydride or oleic anhydride prepared with \(N, N\)‘-dicyclohexylcarbodiimide from the corresponding carboxylic acids as described (17). Geranylgeranoyl acid was prepared from geran-

Enzyme Assay—(a) For CDP-archaeol synthase, the complete assay mixture (final volume, 0.2 ml) contained the membrane fraction of M. thermoautotrophicus (200 \(\mu\)g of protein), 0.16 mM Bicine buffer (Dojin Laboratories, Kumamoto, Japan), pH 8.5, 0.5 mM KCl, 1.5 mM MgCl\(_2\), 2 mM lipid substrate, and 2 mM [\(^{3}H\)]CTP (PerkinElmer Life Sciences, 6.25 Ci/mmol). After incubation at 55 °C for 1 h, the activity was measured at room temperature at 589 nm with an automated spectrophotometer (Shimadzu UV-2200D spectrophotometer).

Preparative Enzymatic Synthesis of CDP-archaeol—To obtain a fairly large amount of the enzyme reaction product for structural analysis, CDP-archaeol was enzymatically synthesized with 7.5 mg of 2,3-GG-GP ether, 4.8 mg of unlabeled CTP, and M. thermoautotrophicus membrane fraction (4.4 mg of protein). These reactants were incubated in the same buffer as the CDP-archaeol synthase assay (final volume, 5 ml) for 22 h. Since CDP-archaeol is soluble in an aqueous alkaline phase but soluble in the chloroform phase only under neutral or acidic conditions, the product was first extracted using the Bligh and Dyer solvent (23) under acidic condition followed by extraction into an alkaline aqueous layer. After removal of the lower organic layer, the aqueous solution was acidified slightly with dilute HCl and re-extracted by the Bligh and Dyer method (acidic-alkaline partitioning). Most cellular phospholipids remained in the chloroform fraction while highly acidic lipids, such as CDP-archaeol and archaetidylserine, were extracted into the aqueous phase under alkaline conditions. The product was finally purified by TLC with solvent B.

Analytical Methods and Degradation Procedure of CDP-archaeol—Phosphate (24) and protein (25) were determined as described previously. CDP-archaeol was hydrolyzed with 5% HCl-methanol at 80 °C for 1 h. Equal volumes of chloroform and water were added to the reaction mixture, and the two layers were separated. A portion of the aqueous product was dried under a stream of nitrogen. The resulting residue was dissolved in 0.1 M HCl. UV spectra (220–320 nm) of this solution were recorded by a Shimadzu UV-2200A spectrophotometer at pH 2 and 13. Cytidine was determined by the measurement of A\(_{260}\) at pH 2 (e = 12800).

Physical Measurements—FAB-MS was recorded with a mass spectrometer (JEOL JMS DX-303) with a matrix of \(m\)-nitrobenzyl alcohol in a positive and/or a negative mode. In the case of CDP-unsatuated archaeol, the matrix contained a small amount of NaI. Optical rotation was measured at room temperature at 589 nm with an automatic digital polarimeter (PM-201, Photon, Japan). Radioactivity was counted using a liquid scintillation spectrometer (Alkova LSC-3500E, Japan) with Aquaso-2 (Packard) as scintillator.

Detection of CDP-archaeol in M. thermoautotrophicus Cells—At the midlog phase of growth of M. thermoautotrophicus grown in 50 ml of the low phosphate medium (26), 1 ml of \([^{32}P]P\) (Amersham Pharmac Biotech, 50 Ci/mol) was added and the culture was allowed to continue to grow. After 60 min the cells were harvested, and unlabeled CDP-unsatuated archaeol was added (60 \(\mu\)g/10 mg cells). Total lipid was extracted, and the CDP-archaeol-containing fraction was roughly purified by the acid-alkaline partitioning method described above. A quarter of the final acidic chloroform fraction was developed on two-dimen-

Time (h)

nmol/assay

0
5
10
15
20
25

FIG. 3. Conversion of \(^{3}H\)CTP to lipid catalyzed by M. thermoautotrophicus membrane fraction incubated with 2,3-GG-GP ether in the presence of 0.5 M K\(^+\) and 2 mM Mg\(^{2+}\) at 55 °C. Data are the average of duplicate assays ± S.E.
CDP-archaeol Synthase in the Methanoarchaeon

TABLE I

| Incorporation of [3H]CTP into lipid by the membrane fraction of *M. thermoautotrophicus* |
|---------------------------------------------------------------|
| Reaction mixture | [3H]CTP incorporated into lipid |
| Complete | 3.6 |
| − 2,3-GG-GP ether | 0.01 |
| − Membrane fraction | 0.08 |
| − Membrane fraction + heated membrane | 0.09 |
| − MgCl2 | 0.00 |
| − KCl | 0.14 |

| a | The membrane fraction was heated in a boiling water bath for 30 min. |

The complete reaction mixture contained the membrane fraction, 2 mM 2,3-GG-GP ether, and 2 mM [5-3H]CTP as described under "Experimental Procedures." After incubation at 55 °C for 2 h, the reaction was stopped and 3H in chloroform-extractable material was counted.

Substrate Specificity of CDP-archaeol Synthase—Archaeal glycerolipids are fundamentally characterized by the G-1-P stereoskeleton of their backbone. Substrate stereospecificity of the enzymes involved in polar lipid biosynthesis is therefore of special interest. To examine this issue, we synthesized a variety of substrate analogs with ether or ester bonds between GP and both saturated and unsaturated hydrocarbons, and with both stereoisomers of the GP backbone. Table III shows relative activities of the enzyme in the presence of 2 mM of each of the substrate analogs. Both enantiomers of archaetidic acid possessing geranylgeranyl chains (2,3-GG-GP ether and 1,2-GG-GP ether) showed similar activities as a substrate for the enzyme. In contrast to the stereostructure of GP backbone, unsaturation of the hydrocarbon chains of these substrates was critical; archaetidic acid with saturated (2,3-phyta-GP ether) or monounsaturated (2,3-phyto-GP ether) isoprenoid chains of the same carbon number were poor substrates as were analogs with monounsaturated straight chains (2,3-ole-GP ether), even though they had the same stereostructure as the fully active natural substrate (2,3-GG-GP ether). As to the ester analogs of the substrate, the GP esters with geranylgeranyloxy chains again showed high activities. In this case, the 1,2-GG analog showed a half-maximal activity. The activities to the ester analogs of the substrate with oleyl chains were also in low levels. These results suggest that the enzyme does not recognize ether or ester bonds between GP and hydrocarbon chains nor the stereostructure of the GP backbone but mainly targets substrates with geranylgeranyl chains.

Substrate specificity of *E. coli* CDP-diacylglycerol synthase for the stereostructure and ether or ester linkage has not been reported previously. The substrate specificity of *E. coli* CDP-diacylglycerol synthase was investigated in order to form a baseline against which the substrate specificity of CDP-archaeol synthase could be compared. The synthetic enantiomeric phosphatidic acid (2,3-ole-ester) was a poor substrate for *E. coli* CDP-diacylglycerol synthase. Both enantiomers of archaetidic acid (ether type) with oleyl chains showed similarly low activities when introduced as substrates. These results indicate that *E. coli* CDP-diacylglycerol synthase recognizes ester bonds between GP and the hydrocarbon chain as well as the stereostructure of the GP backbone.

Conversion of CDP-archaeol to Archaeoylserine—In order to confirm that CDP-archaeol could be an actual intermediate metabolite of phospholipid biosynthesis in this Archaeon, we investigated the ability of a cell free extract of *M. thermoautotrophicus* to catalyze the conversion of CDP-archaeol to its likely next state, archaeoylserine. Cell-free homogenate of *M. thermoautotrophicus* was found to catalyze the incorporation of [14C]serine into the chloroform-extractable fraction in the presence of chemically synthesized CDP-archaeol. The incorporation of [14C]serine continued linearly for 30 min. The activity was completely dependent on the CDP-archaeol and the homo-
генерован от M. thermoautotrophicus (Таблица IV). Когда разработаны на одномерном TLC с растворителем B, этиллипидная фракция мигрировала как сингл спот, хроматографически идентичный с археатидилсерин. При этих условиях 30% CDP-археола было окислено в археатидилсерин. Энзиматически синтезированный CDP-археол также был окислен в растворимую в хлороформ фракцию при значительному, но несколько более низком, темпе, по сравнению с химически синтезированным CDP-насыщенным археолом.

**Presence of CDP-archaeol in M. thermoautotrophicus Cells—**

**Table II**

| CDP-archaeol                  | Cytidine | Phosphate |
|-------------------------------|----------|-----------|
|                               | Water-soluble | Chloroform-soluble |
| Chemically synthesized saturated | 1.20     | 1.17      |
| Chemically synthesized unsaturated | 1.96     | ND        |
| Enzymatically synthesized     | 2.10     | ND        |

*Not determined because no phosphate-positive material was detected on TLC.*

**FIG. 4. Effects of pH (A), temperature (B), K⁺ concentration (C), and Mg²⁺ concentration (D) on CDP-archaeol synthase activity.** The conditions of the experiments were the same as described in Table I except that Mg²⁺ concentration was 4 mM in A and C and at pH 8 in C. In panel A, imidazole (pH 6.5–8.0, ○) and Bicine (pH 8.0–9.0, □) buffers were used. Data in A and B are the average of duplicate assays ± S.E.

**Total lipid extract of M. thermoautotrophicus** was chromatographed using a two-dimensional TLC in an attempt to detect an acid-labile phospholipid species co-migrating with synthetic CDP-archaeol. However the effort was unsuccessful, as only trace amounts would be present, if at all, and several large spots were distributed around the location of the reference spot on the TLC plate. [³²P]CDP-archaeol was concentrated from a total lipid extract of M. thermoautotrophicus cells pulse-labeled in [³²P]containing medium using the acidic-alkaline partitioning method. This treatment removed significant amounts, but not all, of phospholipids such as diphytanoylarchaeol, which interferes with the detection of CDP-archaeol. To improve resolution of the pertinent spots on the two-dimensional TLC, the TLC was developed twice in the first direction using the same solvent. The solvent was allowed to dry between the first and second developments. In this way we detected a tiny [³²P]-spot superimposed on the spot of carrier CDP-unsaturated archaeol detected by means of acid molybdate spray (Fig. 5).

The [³²P]-phospholipid (8202 cpm) corresponding to CDP-archaeol spot detected by I₂ vapor was scraped off from the TLC plate and extracted from the silica gel with Bligh-Dyer solvent. The extracted [³²P]-lipid (and carrier CDP-archaeol) was incubated at 55 °C for 30 min with L-serine, cell-free homogenate of M. thermoautotrophicus, and Bicine buffer with the composition as described for the archeatidylserine synthase assay. After the incubation, a chloroform-soluble fraction was obtained and Triton X-100 was removed by acetone precipitation of phospholipid. A significant amount of radioactivity, most of which were impurities, was removed during this process. The reaction product was developed on TLC with solvent B. A tiny [³²P]-spot (144 cpm) cochromatographed with archeatidylserine was detected. As CDP-archaeol and archeatidylserine contain two and one phosphate groups in their molecules, respectively, and it is assumed that 30% of CDP-archaeol is converted to archeatidylserine under the conditions, roughly 10% of the [³²P]-phospholipid recovered from the TLC plate should be [³²P]CDP-archaeol.
able labeled material was counted. Reaction at 55 °C for 30 min, reaction was stopped, and chloroform-extract
mogenate as described under “Experimental Procedures.” After incuba-(CDP-2,3-di-
(a) The molecular weight of the product coincided
backbone and the molecular weight of cytidine plus two phosphate

**Table III**

| Compound                    | Hydrocarbon          | Double bond | GP backbone | Lipid substrate Relative activitya |
|-----------------------------|----------------------|-------------|-------------|-----------------------------------|
| 2,3-GG-GP ether             | Geranylgeranylb      | 4           | G-1-P       | 100                               |
| 1,2-GG-GP ether             | Geranylgeranyl       | 4           | G-3-P       | 90.0 ± 0.8                         |
| 2,3-Phyto-GP ether          | Phytanyl             | 0           | G-1-P       | 3.1 ± 0.2                          |
| 2,3-Phyt-GP ether           | Phytanyl             | 1           | G-1-P       | 0.0 ± 0.0                          |
| 2,3-Ole-GP ether            | Oleyl                | 1           | G-1-P       | 0.0 ± 0.0                          |
| 1,2-Ole-GP ether            | Oleyl                | 1           | G-3-P       | 7.8 ± 0.3                          |
| 2,3-GG-GP ester             | Geranylgeraniolyf    | 4           | G-1-P       | 122.0 ± 13                         |
| 1,2-GG-GP ester             | Geranylgeraniol      | 4           | G-3-P       | 49.8 ± 3.8                         |
| rac-GG-GP ester             | Geranylgeraniolyf    | 4           | rac-GP      | 81.4 ± 0.7                         |
| 2,3-Ole-GP ester            | Oleyl                | 1           | G-1-P       | 12.9 ± 0.7                         |
| rac-Ole-GP ester            | Oleyl                | 1           | G-3-P       | 100                                |

Data are the average of duplicate assays ± S.E.

b 3,7,11,15-Tetramethylhexadec-2E,6E,10E,14E-tetraenyl.

c 3,7R,7R,11R,15-Tetramethylhexadec-2E-enyl.

d 3,7R,11R,15-Tetramethylhexadec-2E-ethyl.

f (Z)-Octadec-9-ethyl.

TABLE IV

| Reaction mixture | [14C]Serine incorporated into lipid | nmol |
|------------------|-----------------------------------|------|
| Complete         |                                   | 7.4  |
| Homogenate       |                                   | 0.0  |
| CDP-unsaturated archaeol |                   | 0.0  |
| CDP-unsaturated archaeol + enzymatically synthesized CDP-archaeol | | 5.7  |
| CDP-unsaturated archaeol + CDP-2,3-di-O-phytanyl glycerol | | 4.9  |

**DISCUSSION**

The enzyme activity of CDP-archaeol synthase was found in the membrane fraction of M. thermoautotrophicus. The enzyme catalyzes the reaction analogous to CDP-diacylglycerol that plays the central role in phospholipid biosynthesis in bacteria. Because it is a newly described enzyme activity, the details of the reaction should be established unambiguously. The product of CDP-archaeol synthase reaction was identified as CDP-di-O-geranylgeranylglycerol (CDP-unsaturated archaeol) based on the following observations. (a) Most of the radioactivity in the chloroform-soluble extract of the reaction products was recovered in a single spot on TLC, which co-migrated with chemically synthesized CDP-archaeol. (b) The product contained cytidine and phosphate in a molar ratio of 1:2. (c) The mild acid hydrolysis of the product yielded two water-soluble phosphate groups, but the same treatment of CDP-saturated archaeol yielded one water-soluble phosphate group and one chloroform-soluble arachidonic acid, suggesting that the core lipid portion of CDP-archaeol is composed of acid-labile allyl ether bonds. (d) The molecular weight of the product coincided with that of CDP-di-O-geranylgeranylglycerol. The difference between the total molecular weight of CDP-unsaturated archaeol and the molecular weight of cytidine plus two phosphate groups is consistent with the molecular weight of digeranylgeranylglycerol. These results strongly support the theory that this enzyme activity catalyzes the conversion of unsaturated arachidonic acid and CTP to CDP-archaeol. The stoichiometry of this reaction was also established. Since this is the first report of CDP-archaeol synthase activity, it was necessary to address a concern that the putative substrates added to the reaction might simply stimulate incorporation of CTP into an endogenous lipid. When 7.5 mg of 2,3-GG-GP ether was incubated with the membrane fraction (equivalent to 4.4 mg of protein) of M. thermoautotrophicus cells, 1.2 mg of CDP-unsaturated archaeol was synthesized. Although the membrane fraction contained 2 mg of endogenous lipid, up to 0.47% of it was allyl ether type phospholipids determined by a newly developed method of reductive cleavage of ether bonds and GLC analysis of the resultant hydrocarbon (data not shown), i.e. the unsaturated lipid in this membrane fraction amounted to no more than 0.0094 mg. Since the hydrocarbon groups of the product were composed of geranylgeranyl chains, far more unsaturated product was produced from the substrate than from the endog-
ensous lipid present in the membrane fraction. This means that CTP was actually incorporated into the added 2,3-GG-GP ether.

It has been shown that it is possible to convert enzymatically synthesized CDP-archaeol to archaeidylserine in vitro. In addition, the fact that CDP-archaeol, the product of the CDP-archaeol synthase reaction, was detected in trace amounts in the phospholipid biosynthetic pathway in Archaea have not been definitively reported, CDP-archaeol could be one of the important precursors for phospholipid biosynthesis in Archaea.

Although intermediate steps other than Reactions 1–5 (Fig. 1) in the phospholipid biosynthetic pathway in Archaea have not been definitively reported, CDP-archaeol could be one of the most important precursors for phospholipid biosynthesis in Archaea based on analogy with the bacterial pathway. After CDP-archaeol is synthesized in this pathway, two major issues continue to pose problems; saturation of the hydrocarbon chains must occur before or after the attachment of polar groups, and the formation of tetra-ether polar lipids. With regard to the former, three possibilities may be considered. Hydrogenation could occur (a) at GG-GP ether (archaeidic acid), (b) at CDP-archaeol, or (c) after attachment of a polar group (Fig. 6). Although the hydrogenation step has not been identified, it could occur at step b or c since CDP-archaeol synthase was found to be specific for unsaturated lipid.

One of the most remarkable features of archaeal phospholipids is the stereoconfiguration of G-1-P backbone. This work, therefore, focused on stereospecificity of the lipid substrate for CDP-archaeol synthase. Surprisingly, the CDP-archaeol synthase recognized neither the stereochemical structure of a glycerophosphate backbone nor the linkage between glycerol and hydrocarbon groups (ester or ether linkage). It can be concluded that the stereostructure of archaeal polar lipids is established prior to or at the generation of unsaturated arachidonic acid (just before the CDP-archaeol synthase step). It should be noted that a geranylgeranyl chain contains four double bonds in the alkyl group. It is most likely that CDP-archaeol synthase is specific for arachidonic acid or phosphatidic acid with geranylgeranyl chains. However, the possibility has not been excluded that the substrate specificity might indicate the presence of two or more enzymes with different specificities since the enzyme has not yet been purified. Another possibility worth considering is that the preference of this enzyme for polyunsaturated hydrocarbon chains may be due to difficulty in dispersing the saturated compound in the reaction mixture as described by Sparrow et al. (27). If this is the sole reason of the low activity of CDP-archaeol synthase with the saturated substrate, the activity, although low, ought to be detected in significant amounts. Table III shows, however, that the activity with the saturated substrate was negligible. This suggests that problems associated with the dispersion are unlikely to be the reason for the low activity of CDP archaeol with saturated substrate.

By contrast, CDP-diacylglycerol synthase from E. coli recognized both G-3-P backbone and ester linkages. The complete genomic sequence of *M. thermoautotrophicus* has been reported (28) and functional annotation assigned. However, these do not reveal the presence of the gene encoding CDP-diacylglycerol synthase. Archaeal CDP-archaeol synthase might be quite different in its primary structure from bacterial or eucaryotic CDP-diacylglycerol synthase.

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**Fig. 6. Three possibilities (a, b, and c) of hydrogenation of phospholipid hydrocarbon chains during phospholipid biosynthesis in Archaea.**