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Formation of Unilamellar Liposomes from Total Polar Lipid Extracts of Methanogens†

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Unilamellar liposomes were formed by controlled detergent dialysis of mixed micelles consisting of acetone-insoluble total polar lipids extracted from various methanogens and the detergent *n*-octyl-β-D-glucopyranoside. The final liposome populations were studied by dynamic light scattering and electron microscopy. Unilamellar liposomes with mean diameters smaller than 100 nm were obtained with lipid extracts of *Methanococcus voltae*, *Methanosarcina mazei*, *Methanosaeta concilii*, and *Methanococcus jannaschii* (grown at 50°C), whereas larger (>100-nm) unilamellar liposomes were obtained with lipid extracts of *M. jannaschii* grown at 65°C. These liposomes were shown to be closed intact vesicles capable of retaining entrapped [^{14}C]sucrose for extended periods of time. With the exception of *Methanospirillum hungatei* liposomes, all size distributions of the different liposome populations were fairly homogeneous.

Artificial lipid vesicles have become an important tool in numerous basic and applied research areas. They have been used extensively as biological membrane systems for the study of processes such as transmembrane transport, lipid bilayer permeability, membrane fusion, and lipid-protein interaction. They may also serve as immunological adjuvants or as carriers of drugs, insecticides, genetic material, and enzymes. Indeed, it is easy to recognize their increasing use and importance by the number of books, reviews, and articles that have recently been written about their formation and application (23).

Archaeobacteria possess membrane lipid structures very different from those of eubacteria and eucaryotes. Instead of fatty acyl chains, which are often unsaturated and are esterified to *sn*-1,2 carbons of glycerol, archaeobacterial lipids are composed of saturated phytanyl chains in ether linkage to glycerol carbons with an *sn*-2,3 configuration (15). Moreover, various methanogens, in addition to having the ubiquitous diether $C_{20,20}$-lipid, can also have phytanyl chains modified to give rise to tetraether, hydroxydiether, and macrocyclic diether lipids (30, 31) (Fig. 1).

The novelty of these structural differences has encouraged us to compare liposomes composed of different combinations of methanogenic lipids. We report our results on liposome formation with total polar lipid extracts of *Methanococcus voltae*, *Methanococcus jannaschii*, *Methanosarcina mazei*, *Methanospirillum hungatei*, and *Methanosaeta concilii*. These were chosen because their polar lipid compositions (11) are very different from one another and because they encompass the known spectrum of unusual core lipid structures found in methanogens (Table 1).

MATERIALS AND METHODS

Growth of bacteria. *M. hungatei* GP1 (DSM 1101) was grown at 35°C under an atmosphere of H_2-CO_2 (80:20, vol/vol) in mineral salts medium SA (2) supplemented with 5 μM $NiCl_2$. The aceticlastic methanogen *M. concilii* GP6 (DSM 3671) was cultured under N_2 at 35°C in acetate medium as described by Ferrante et al. (9). *M. jannaschii* JAL-1 (DSM 2661) was grown in defined medium under an atmosphere of H_2-CO_2 (80:20, vol/vol) at 65 or 50°C as described before (11). *M. voltae* PS (DSM 1587) was grown under H_2-CO_2 (80:20, vol/vol) in Balch medium 3 (1) at 35°C. *M. mazei* S6 (DSM 2053) was grown on methanol (0.6%, vol/vol) under N_2 in defined Balch medium 3, that is, Balch medium 3 in which yeast extract and tryptone were replaced by 0.1 g of L-isoleucine per liter and 0.05 g of L-leucine per liter, the NH_4Cl concentration was raised to 0.54 g/liter, and Na_2CO_3 was replaced by $NaHCO_3$.

All microorganisms were grown at their optimum pHs in a 75-liter fermentor (Chemap AG fermentor) containing 55 liters of medium. The dissolved sulfide was maintained at 0.1 mM with the addition of aqueous Na_2S.

Lipid extraction. Total polar lipid extracts were obtained by the method of Bligh and Dyer (11).

The polar lipids were recovered by dissolving the total lipid fraction in $CHCl_3$-CH_3OH (2:1, vol/vol) and precipitating the polar lipids with 20 volumes of cold acetone. The precipitates were collected by centrifugation, dissolved in $CHCl_3$-CH_3OH (2:1, vol/vol), and precipitated again with acetone. This step was repeated a third time. Total polar lipids were quantitated by weighing.

Lipid-detergent mixed-micelle formation. Polar lipids (40 mg) and *n*-octyl-β-D-glucopyranoside were dissolved in $CHCl_3$ (2 ml). The detergent was added in a 20:1 molar ratio (*n*-octyl-β-D-glucopyranoside to lipid), assuming an average molecular weight of 1,000 for the polar lipid extracts (11). The lipid-detergent solution was evaporated to dryness under N_2 and placed in vacuo overnight to remove all traces of $CHCl_3$. The mixed micelles were formed by dissolving the lipid-detergent material in 3 ml of dialysis buffer (10 mM K-phosphate buffer [pH 7.14] containing 160 mM NaCl). Trace amounts of undissolved material were removed by filtration through a 0.22-μm-pore-size nylon filter.

Liposome formation. The liposomes were formed by controlled dialysis of the lipid-detergent mixed micelles at room

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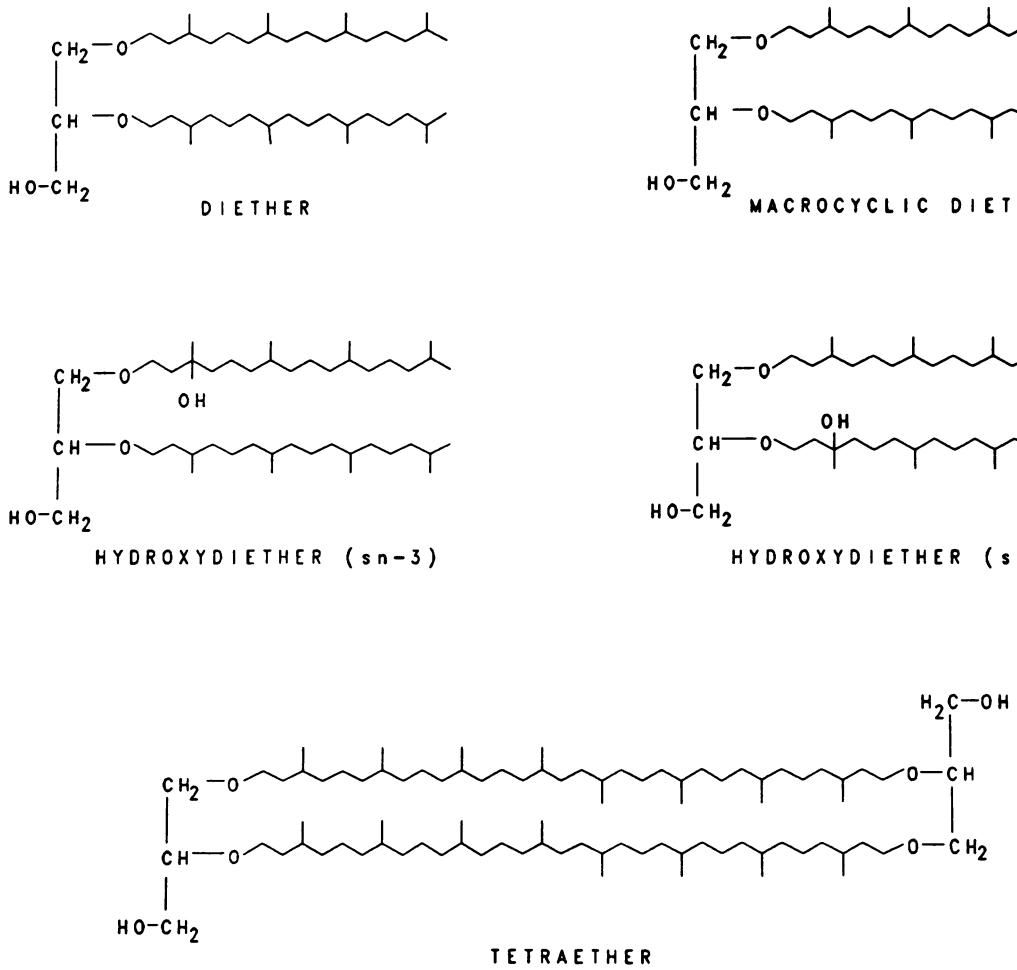

FIG. 1. Structures of the different core lipids found in methanogens.

temperature with a Liposomat (Avestin Inc., Ottawa, Ontario, Canada) operating for 4 h at a flow rate of 0.5 ml/min for the mixed micelles and 2.5 ml/min for the dialysis buffer.

Determination of the internal volume. The internal volume of liposomes was determined with the marker [U-^{14}C]sucrose (NEN Research Products, Mississauga, Ontario, Canada). Mixed micelles and liposomes were formed with dialysis buffer containing 0.3 mCi of [^{14}C]sucrose per liter. Thus, the concentration of [^{14}C]sucrose trapped inside the liposomes was the same as that outside the vesicles. The specific activity of the [^{14}C]sucrose (4.2 mCi/mmol) was used to calculate the intravesicular aqueous compartment volumes, and these were expressed as microliters of aqueous trapped volume per milligram of total polar lipid following removal of free [^{14}C]sucrose.

The separation of free from entrapped sucrose was done on Sephadex G-50 (medium) columns by the microcolumn centrifugation method described by New (24).

Thin-layer chromatography. Polar lipids were separated in chloroform-methanol-acetic acid-water (85:22.5:10:4, vol/vol) on Silica Gel G plates (0.25 mm) and visualized as described by Ross et al. (28). Polar lipid and micellar extracts, both in $CHCl_3$-CH_3OH (2:1, vol/vol), were applied directly to the thin-layer chromatography plates, while samples (5 mg) of the liposome suspensions were dried under N_2 and dissolved in $CHCl_3$-CH_3OH (2:1, vol/vol) prior to their application to the plates.

Size determination. The mean diameter and the number-weighted size distribution of the vesicle preparations were determined by dynamic light scattering (DLS) using a NICOMP submicron particle sizer, model 370 (Nicomp, Santa Barbara, Calif.), and by direct measurements from electron micrographs of negatively stained preparations (24).

TABLE 1. Known distribution of the different ether core lipids present in the methanogens used in this study

Methanogen	Distribution (%) of:				Reference(s)
	Diether	Hydroxydiether	Tetraether	Macrocyclic diether	
M. voltae	>90	<10			4, 30
M. concilii	70	30 (*sn*-3)			10
M. mazei	43	57 (*sn*-2)			30
M. hungatei	50		50		21
M. jannaschii					
65°C[a]	15		42	43	31
50°C[a]	60		21	19	31

[a] Growth temperature of *M. jannaschii*; other methanogens were grown at 35°C.

Electron microscopy. Negative stains were prepared by using Formvar carbon-coated copper grids (200 mesh) and a 1% solution of sodium phosphotungstate (pH 7.2). For a better dispersion of the liposomes, the grids were treated with bacitracin (0.1 mg/ml) (24) for 2 min prior to sample application. A drop of the proper liposome dilution (10^{-1} to 10^{-2}), with phosphotungstate as the diluent, was placed on the grid for 5 min, and the excess stain was drawn off with filter paper. The grids were observed with a Siemens 101 transmission electron microscope at 60 kV. To account for the flattening of the liposomes, the diameters of the measured disks were multiplied by 0.71 to approximate the diameter of the original liposomes (24).

For freeze fracturing, the liposomes were centrifuged at 200,000 × g_{max} for 5 h and the pellet was distributed into gold freeze-etching planchets. These were frozen by plunging them into propane held at liquid nitrogen temperature. The frozen material was fractured and etched (etching time of 30 s) in a Balzers BA 360 freeze-etcher equipped with electron guns as evaporation sources. The platinum-carbon replicas were cleaned of liposomal debris by treatment in concentrated sulfuric acid, 5% (wt/vol) sodium hypochlorite, and distilled water. The replicas were mounted on 400-mesh copper grids and viewed with a Philips EM 300 microscope operating at 60 kV under standard conditions with the cold finger in place.

RESULTS

Mean sizes and size distributions. Controlled detergent dialysis was successfully used to form liposomes with good size homogeneity with total polar lipid extracts of four of the five methanogens tested (Fig. 2), the exception being *M. hungatei* liposomes (see below). DLS analysis of the different liposome preparations yielded coefficients of variation of the size distribution between 0.2 and 0.5 (Table 2); the narrowest size distribution was obtained with *M. jannaschii* liposomes, followed in decreasing order by those of *M. voltae*, *M. mazei*, and *M. concilii*. DLS analysis was done in the vesicle-particle mode as number-weighted diameter distributions because the data agreed well with sizes and standard deviations calculated from microscopic examination (Table 2). Volume-weighted distributions always gave much larger diameter estimates (20 to 80%). The differences in homogeneity observed by DLS were confirmed by electron microscopy of negatively stained liposomes (Fig. 2 and Table 2). Importantly, the size distribution was very reproducible for different liposome preparations made from the same lipid extract (i.e., within 10%).

Large particles (>1 μm), easily observed with the light microscope, were present in the liposome suspension of *M. concilii* after dialysis. By electron microscopy, they appeared to be aggregates of various-sized liposomes. Number-weighted DLS data revealed that removing these particles by filtration through a 0.22-μm-pore-size nylon filter did not affect the mean diameter and size distribution of the population, as expected, because they accounted for only a very small fraction of the liposome population. In fact, in comparison with the smaller, more numerous liposomes, they were judged to be few and rare by electron microscopy. Once this small proportion of large vesicles was removed, a fairly homogeneous suspension of small liposomes remained (Fig. 2D).

Liposomes from *M. hungatei* showed severe size and shape heterogeneity (Fig. 2E and F). Some vesicles were quite large, multiple (freeze fracture revealed these to be multilamellar), and connected by fine tubules (Fig. 2E). Large aggregates were observed much more frequently than with liposome preparations from *M. concilii* lipids. In fact, the size heterogeneity was so great that the submicron particle sizer was unable to properly define this liposome population. Sizing of the liposomes by electron microscopy revealed the following distribution (Fig. 2E): 40% of the liposomes had diameters between 20 and 100 nm, 16% were between 100 and 200 nm, 16% were between 200 and 300 nm, and the remaining 28% were between 300 and 1,000 nm. Some of the electron microscopic fields did show good size distribution (Fig. 2F), although this was the exception.

The average size of the liposomes varied depending on the source of the lipids (Table 2). Unilamellar liposomes with mean diameters smaller than 100 nm were obtained with lipid extracts of *M. voltae*, *M. mazei*, *M. concilii*, and *M. jannaschii* (grown at 50°C), whereas larger (>100-nm) unilamellar liposomes were obtained with lipid extracts of *M. jannaschii* grown at 65°C.

The two extracts containing tetraether lipids, those from *M. jannaschii* grown at 65°C and *M. hungatei*, yielded the larger liposomes. To determine whether the presence of tetraether lipids in the extracts could be responsible for these larger vesicles, liposomes were prepared from the total lipid extracts of *M. jannaschii* grown at 50 and 65°C. Sufficient purified lipids for this purpose were unavailable. Sprott et al. (31) have shown that *M. jannaschii* grown at 50°C contains a higher proportion of diether lipids and less tetraether and macrocyclic diethers than cells grown at 65°C (Table 1). As anticipated, the lipids obtained from *M. jannaschii* grown at 50°C consistently yielded liposomes smaller than the liposomes obtained with lipids of *M. jannaschii* grown at 65°C (Table 2).

Intactness of archaeobacterial liposomes. In order to establish that the polar lipid extracts obtained from the different methanogens did indeed form closed intact vesicles, entrapment experiments with [^{14}C]sucrose were performed. From these experiments, the internal volumes of the different liposomes were obtained (Table 2). There is not a direct relationship between size and internal volume, and this is probably due to the differences in the lipid compositions of the extracts.

After a 2-week incubation period at 4°C, at least 92% of the marker was still present in the liposomes. Lysing the liposomes with 0.2% Triton X-100 released 100% of the label, while the incubation (4 h) of empty liposomes with [^{14}C]sucrose (0.3 mCi/liter) did not show an increase in radioactivity associated with the liposomes. These results confirmed that the [^{14}C]sucrose was entrapped and that the liposomes were sealed vesicles.

Lamellarity of liposomes. Freeze fractures of the liposomes from *M. jannaschii*, *M. voltae*, *M. mazei*, and *M. concilii* (filtered) revealed them to be relatively homogeneous vesicles whose hydrophobic fracture surfaces (both concave and convex) were smooth (Fig. 3 is representative). Multiple fracture planes were never seen, which confirmed the unilamellar nature of these liposomes. Comparison of liposome diameters of freeze fractures and negative stains showed that those from negative stains were slightly larger and suggested them to be somewhat flattened (and artificially expanded) compared with the frozen preparations. A size correction factor of 0.71 (24) was used, therefore, when diameters of negatively stained liposomes were calculated. *M. hungatei* liposomes were a mixture of unilamellar and numerous large multilamellar vesicles.

Lipid composition of liposomes. Comparison by thin-layer

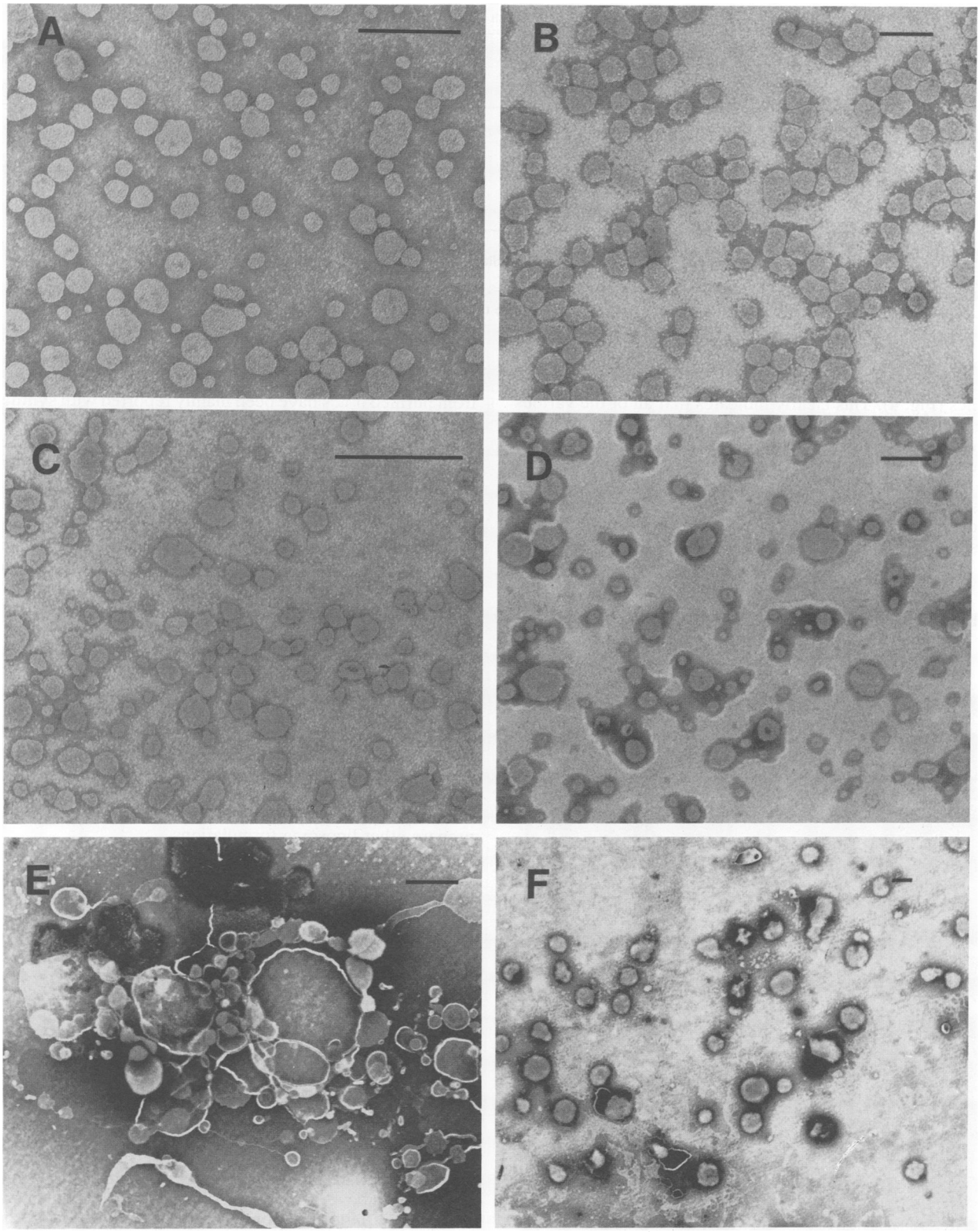

FIG. 2. Transmission electron micrographs of negatively stained liposomes obtained with total polar lipid extracts of *M. voltae* (A), *M. thermolithotrophicus* (grown at 65°C) (B), *M. mazei* (C), *M. concilii* (D), and *M. hungatei* (E and F). Taking into consideration the size factor of 0.71, bars equal 250 nm.

TABLE 2. Size characteristics of liposomes

Origin of total polar lipid extract	Result of:				Trapped vol (μl/mg of lipid[a])
	DLS		Electron microscopy		
	Mean diam ± SD (nm)	Coefficient of variation[b]	Mean diam ± SD (nm)	Coefficient of variation	
M. jannaschii					
65°C[c]	129 ± 31	0.24	113 ± 28 (282)[d]	0.24	4.5
50°C[c]	81 ± 22	0.27	Not determined		4.0
M. voltae	54 ± 19	0.35	56 ± 23 (298)	0.41	4.5
M. mazei	45 ± 19	0.42	49 ± 21 (201)	0.43	1.8
M. concilii[e]	69 ± 30	0.43	70 ± 38 (212)	0.54	3.4

[a] Total polar lipid.
[b] Coefficient of variation of the size distribution = standard deviation/mean diameter.
[c] Growth temperature.
[d] Number of liposomes measured.
[e] Liposomes were filtered through a 0.22-μm-pore-size nylon filter to remove the large aggregates.

chromatography among total polar lipid extracts, mixed micelles, and liposomes revealed essentially identical lipid profiles (Fig. 4). This clearly indicates that all of the various ether lipid species were incorporated into each liposome preparation. Also note the different and characteristic lipid pattern of each methanogen. There was no residual detergent detected by thin-layer chromatography in any of the liposome suspensions (Fig. 4), and therefore, we estimate the detergent concentration to be less than 1 μg in the final liposome populations (40 mg of lipid).

DISCUSSION

The formation of multilamellar ether liposomes from either individual lipid components (3, 25) or polar lipid extracts (3, 20, 25) is well documented. However, the formation and characterization of unilamellar ether liposomes from purified lipids have been mainly reported with synthetic analogs of archaeobacterial lipids (8, 12, 13, 16). With naturally occurring ether lipids, liposome formation has been reported on several occasions but without supporting evidence of their unilamellarity, physical intactness, or size homogeneity (6, 17, 27). However, more recently, Elferink et al. (7) have successfully demonstrated that a lipid fraction of intermediate polarity of the archaeobacterium *Sulfolobus acidocaldarius* could form unilamellar vesicles upon sonication. Concomitantly, we show that unilamellar liposomes can be formed from total polar lipid extracts of methanogens.

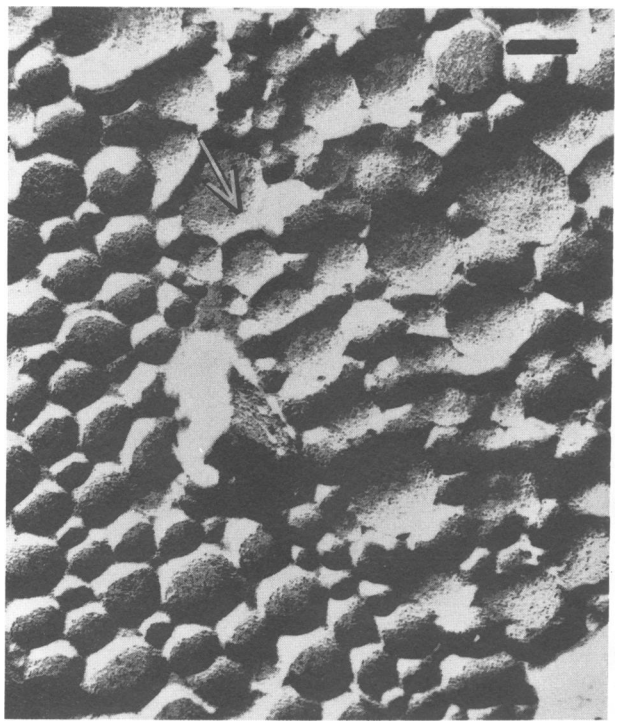

FIG. 3. Transmission electron micrograph of a freeze fracture of *M. voltae* liposomes. It is representative of all liposome preparations shown in Fig. 2 except for *M. hungatei*. Bar = 100 nm. The arrow denotes shadow direction.

Pure methanogenic ether lipids are not available commercially. Isolation of even the most predominant ones in a quantity sufficient to attempt liposome formation is a monumental task, considering that most methanogens synthesize at least 10 different polar lipids (some, such as *M. jannaschii*, have many more). Also, the lipid fraction of most methanogen cells accounts for only about 5% of the cell dry weight. Therefore, we herein explored the feasibility of using natural mixtures of total polar lipids from different methanogens to prepare ether liposome formulations.

We have successfully generated closed unilamellar vesicles with fairly good size homogeneity from total polar lipid extracts of *M. voltae*, *M. jannaschii*, *M. mazei*, and *M. concilii*. In comparison, coefficients of variation for the size distribution of liposomes obtained with ester lipid (egg yolk lecithin) and *n*-alkyl-glucosides as detergents range between 0.15 and 0.35 (22, 29).

There is no strong correlation between the nature of the different core lipid structures in each extract and the size of the liposomes formed. However, the presence of tetraether core lipids does appear to promote the formation of larger vesicles. These observations are in agreement with the findings of Lelkes et al. (18), who have shown that vesicles of egg phosphatidylcholine increased in size with increasing amounts of tetraethers incorporated. The packing constraints imposed on the rigid, membrane-spanning tetraether lipids (18, 27) are probably responsible for the observations mentioned above. These constraints would diminish with increasingly larger liposomes as the curvature of the liposome decreased. The differences in size could also be partly due to the differences in the polar head group compositions of the lipid extracts. It is well established that the nature of the head group partly determines the molecular shape (in-

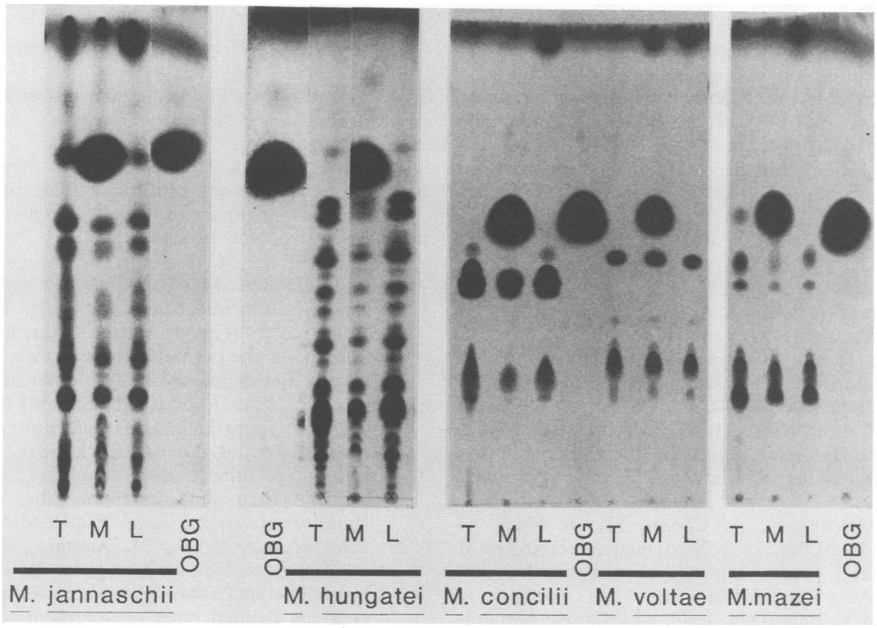

FIG. 4. Thin-layer chromatography of total polar lipid extracts (lanes T), mixed micelles (lanes M), and liposomes (lanes L) obtained for each methanogen. OBG, the detergent *n*-octyl-β-D-glucopyranoside. For *M. jannaschii*, the lipids were obtained from cells grown at 65°C, and for *M. hungatei* liposomes, the final heterogeneous population was used.

verted cone, cylinder, or cone) of the lipid component, which in turn determines its lipid packing order (19). The intravesicular aqueous compartment can also be affected by the head group composition because of its hydration and bulkiness (26).

Because archaeobacterial lipids are generally more stable than eubacterial and eucaryotic membrane lipids, certain advantages in the preparation and use of ether liposomes are anticipated. Not only are alkyl chains fully saturated, imparting resistance to oxidation (14), but also the ether linkage to the phytanyl chains increases resistance to chemical and esterase hydrolysis (5). These characteristics should allow liposomes to be prepared under relatively harsh conditions, such as low pH, or under oxygenic conditions. In comparison, liposomes of eubacterial or eucaryotic lipids often need to be formed under nitrogen. It is further anticipated that archaeobacterial vesicles should have superior stability during storage.

Our success in preparing uniformly sized, unilamellar liposomes from the total polar lipids of certain methanogens will lead to future studies on their chemical and structural stabilities. Ring et al. (27) have shown that tetraether vesicles are structurally more stable than lecithin vesicles in the presence of surface-active agents (alcohols, phenol, and detergents). In addition to potential applications employing entrapment and delivery of substances, these archaeobacterial liposomes may provide the basis for a model membrane system, as was demonstrated with liposomes of *S. acidocaldarius* (7), suitable for studies of methanogenesis and bioenergetic phenomena.

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