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Aliphatic Diether Analogs of Glyceride-Derived Lipids. IV. The Occurrence of Di-*O*-dihydrophytylglycerol Ether Containing Lipids in Extremely Halophilic Bacteria*

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ABSTRACT: The lipids of seven extremely halophilic bacteria, which require 4 M NaCl for optimal growth, were compared with those of moderately halophilic bacteria (requiring 1 M NaCl) and related nonhalophilic bacteria. Infrared spectra of the total lipids showed that the extreme halophiles were almost entirely devoid of fatty acid ester groups, in contrast to the moderate and nonhalophiles which had normal fatty acid ester-containing lipids. After saponification, the lipids of the extreme halophiles gave high yields of unsaponifiable material, identified as largely 2,3-di-*O*-dihydrophytylglycerol, and only traces of fatty acids which were probably derived from lipids in the medium; in contrast, the lipids of moderate and nonhalophiles gave no glycerol diethers and large amounts of fatty acids.

The lipids of all the extremely halophilic bacteria examined by silicic acid paper chromatography were found to contain major amounts of the diether analog of phosphatidylglycerophosphate and minor amounts of the diether analog of phosphatidylglycerol and an unidentified ether phosphatide; a diether glycolipid and its sulfate ester were also present. The moderate halophiles were found to contain major amounts of the diester forms of phosphatidylglycerol and its aminoacyl esters, phosphatidylethanolamine, and several minor unidentified components. Two nonhalophilic *Sarcina* species had major amounts of diester phosphatidylglycerol, and traces of its aminoacyl ester, but no phosphatidylethanolamine.

Previous studies on the lipids of the extremely halophilic bacterium, *Halobacterium cutirubrum*, have established that these lipids consist almost entirely of derivatives of a glycerol diether (Sehgal *et al.*, 1962; Kates *et al.*, 1963; Faure *et al.*, 1963, 1964; Kates *et al.*, 1965a,b), identified as 2,3-di-*O*-(3′,7′,11′,15′-tetramethylhexadecyl)-L-glycerol (di-*O*-dihydrophytylglycerol) (Kates *et al.*, 1963, 1965a,b). Two phosphatide components have so far been identified: a major component, phosphatidylglycerophosphate, diether analog (Kates *et al.*, 1963, 1965b; Faure *et al.*, 1963), and a minor component, phosphatidylglycerol, diether analog (Faure *et al.*, 1964). In addition, a new glycolipid, 1-*O*-[glucosylmannosylgalactosyl]-2,3-di-*O*-dihydrophytyl-L-glycerol, and its sulfate ester, have recently been identified (Kates *et al.*, 1966b).

It was of interest to determine whether these glycerol diether derived lipids were uniquely characteristic of *H. cutirubrum* or whether they were also present in other halophiles and in nonhalophiles. We therefore undertook a survey of the lipids of several species of extremely halophilic, moderately halophilic, and related nonhalophilic bacteria. The present report de-

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TABLE I: Culture Conditions and Yield of Cells.[a]

Sample	Organism	NaCl Concn (%)	Culture Time (hr)	Yield of Cells (g dry weight/l. of culture)	Color of Cells
1	*H. cutirubrum*	25	72	1.2	Red
2	*H. halobium* M	25	80	1.15	Pink
3	*H. halobium* P	25	144	1.73	Red
4	*H. salinarium*	25	168	1.25	Red
5	*S. litoralis*	25	144	1.48	Yellow
6	*Sarcina* sp.	25	168	1.66	Colorless
7	Halophile A_2C	25	80	0.95	Colorless
8	Halophile $A_{31}C$	25	80	0.95	Colorless
9	*M. halodenitrificans* (a)	5.85	48	1.0	Colorless
10	*M. halodenitrificans* (b)	5.85	20	0.72	Colorless
11	*V. costicolus*	5.85	20	1.23	Colorless
12	*S. lutea*	0	20	0.33	Yellow
13	*S. flava*	0	20	0.80	Yellow

[a] All cells were grown in the medium for extreme halophiles (Sehgal and Gibbons, 1960), except for *M. Halodenitrificans* (a), which was grown in a proteose peptone–tryptone medium (Kates *et al.*, 1961).

scribes these studies in detail and presents evidence showing that dihydrophytylglycerol diether derived lipids occur in all of the extremely halophilic bacteria examined, but are absent in the moderately halophilic and nonhalophilic bacteria. A preliminary report of this work has appeared elsewhere (Kates *et al.*, 1966a).

Materials and Methods

Organisms. The extremely halophilic bacteria studied were: *H. cutirubrum*, *Halobacterium halobium* (strain M), *H. halobium* (strain P), *Halobacterium salinarium*, *Sarcina litoralis*, an unidentified halophilic *Sarcina* species, and two uncharacterized colorless halophilic rods, A_2C and $A_{31}C$, which had been found as contaminants in two unidentified cultures of red halophiles. Halophile A_2C grew optimally in 25% NaCl and not at all below 15% NaCl, indicating that it was extremely halophilic. Halophile $A_{31}C$ grew optimally in 7.5–20% NaCl and can be considered moderately halophilic. Other bacteria studied were the moderate halophiles, *Micrococcus halodenitrificans* and *Vibrio costicolus*, and the nonhalophiles, *Sarcina lutea* and *Sarcina flava*.

Culture of Organisms. All organisms studied were grown in 1-l. batches of the standard medium for extreme halophiles (Sehgal and Gibbons, 1960): vitamin-free Casamino acids (Difco), 0.75%; yeast extract (Difco) 1%; trisodium citrate 0.3%; $FeCl_2$, 0.0023%; $MgSO_4 \cdot 7H_2O$, 2%; KCl, 0.2%; sodium chloride was added in the concentrations given in Table I, and the pH was adjusted to 6.2. Incubation was carried out in 4-l. erlenmeyer flasks at 37° with shaking. Cells were harvested by centrifugation at the times indicated (Table I), washed twice in a salt solution of the same composition as in the growth medium, and were finally suspended in 20 ml of the same salt solution used for washing, to give a cell concentration of 20–100 mg dry weight/ml; yields of cells are given in Table I. In all cases the growth phase at the time of harvesting was between the end of the logarithmic phase and the beginning of the stationary phase.

Extraction of Total Lipids. To 20 ml of the above cell suspensions were added 50 ml of methanol and 25 ml of chloroform and the mixture was shaken and left overnight at room temperature. The mixture was centrifuged and the supernatant was diluted with 25 ml each of chloroform and water and transferred to a separatory funnel. The chloroform phase was withdrawn, diluted with an equal volume of benzene, and brought to dryness in a rotary evaporator at 40°. The residual lipid was dissolved in chloroform (25 ml) and the solution (clarified by centrifugation if necessary) was used for subsequent analyses. No effect of salt concentration on the composition of the lipids extracted was observed.

Analytical Methods. Phosphorus was determined by the method of Allen (1940). Salt-free dry weights of cells were determined as described elsewhere (Smithies *et al.*, 1955).

Unsaponifiable material and total fatty acids were determined as follows. Total bacterial lipids (10–20 mg) were heated under reflux in 4.5 ml of 2.5% methanolic-HCl for 3 hr. NaOH (0.5 ml, 7 N) was added and refluxing was continued for 1 hr (Kates, 1964a). After extraction of unsaponifiable material with petroleum ether (bp 30–60°), the hydrolysate was acidified with 6 N H_2SO_4, and the free fatty acids were extracted with petroleum

TABLE II: Yield and Analysis of Total Lipids of Halophilic and Nonhalophilic Bacteria.

Sample	Organism	Lipid Content (% of cell dry weight)	Lipid Analyses (% of total lipids)		
			P	Unsaponifiable Material	Fatty Acids
Extreme halophiles					
1	*H. cutirubrum*	3.5	4.25	70.4	0.6
2	*H. halobium* M	4.1	4.32	73.6	0.4
3	*H. halobium* P	2.6	3.83	68.7	0.3
4	*H. salinarium*	3.6	3.37	66.5	0.7
5	*S. litoralis*	0.7	3.70	64.8	2.3
6	*Sarcina* sp.	3.5	4.23	67.8	0.3
7	Halophile A_2C	3.5	4.41	70.7	0.3
Moderate halophiles					
8	Halophile $A_{31}C$	2.3	3.80	13.8	59.8
9	*M. halodenitrificans* (a)	—	3.33	0.5	62.6
10	*M. halodenitrificans* (b)	6.0	3.74	9.1	68.3
11	*V. costicolus*	9.4	3.76	5.2	60.5
Nonhalophiles					
12	*S. lutea*	7.2	2.89	13.7	68.3
13	*S. flava*	5.9	3.28	13.5	59.6

ether. The extracts of unsaponifiable material and of fatty acids were brought to dryness under a stream of nitrogen and the residues were dried *in vacuo* and weighed.

Since the amounts of fatty acids obtained from the extremely halophilic bacteria were too low for accurate weighing, they were determined as follows. The acids (0.5 mg) were converted to methyl esters by refluxing in 2.5% methanolic–HCl (Kates, 1964a) and a known weight (0.35 mg) of chromatographically pure methyldocosanoate was added. The mixture was then analyzed by gas liquid–partition chromatography on butanediol succinate polyester at 180° as described below. The weight of bacterial fatty acids was then calculated as follows. Weight of acids = (total area of peaks)/(area of 22:0 peak) × weight of 22:0 ester × (average molecular weight of acids)/(average molecular weight of esters).

Chromatography. Fatty acid methyl esters were analyzed by gas–liquid partition chromatography on 4-ft columns (4-mm i.d.) of: (a) 10% Apiezon L on Gas-Chrom P at 195° and 20 psi; and (b) 10% butanediol succinate polyester on Gas-Chrom A at 180° and 16 psi. Peaks were identified by comparison of their relative retentions with those of authentic standards. The chain lengths of unsaturated acids were also verified by analysis of the samples before and after hydrogenation in methanol with platinum catalyst.

Alkyl iodides were analyzed on the butanediol succinate column at 180°. An authentic sample of dihydrophytyl iodide was prepared as described elsewhere (Kates *et al.*, 1965b).

Total lipids were chromatographed on silicic acid impregnated Whatman 3MM paper according to the procedure of Marinetti (1962), using diisobutyl ketone–acetic acid–water (40:25:5, v/v) as solvent. Chromatograms were stained with Rhodamine 6G and viewed under ultraviolet light (360 mμ), with 0.25% ninhydrin in acetone–lutidine to detect amino lipids and with periodate–Schiff reagent (Sastry and Kates, 1964) to detect vicinal glycol-containing lipids.

Thin layer chromatography of unsaponifiable material was carried out on silica gel without binder using chloroform–ethyl ether (9:1) as solvent; lipids were detected by charring with 40% H_2SO_4. An authentic sample of 2,3-di-*O*-dihydrophytylglycerol was synthesized as described previously (Kates *et al.*, 1965a).

Infrared Spectroscopy. Infrared spectra were measured with a Perkin-Elmer Model 21 double-beam spectrograph. Spectra of total lipids were taken in chloroform solution in 0.5-mm NaCl cells; those of the unsaponifiable material were taken in carbon tetrachloride solution.

Cleavage of Diethers. The sample of glycerol diether (5–10 mg) was heated under reflux in 47% hydroiodic acid (3 ml) for 8 hr in a 50-ml standard-tapered erlenmeyer flask with a sealed-on 5-ml side tube (Kates, 1964a). The hydrolysate was diluted with water (2 ml) to fill the side tube and extracted with several 5-ml portions of petroleum ether. The combined extracts were evaporated under a stream of nitrogen and the residual alkyl iodide was subjected to gas–liquid

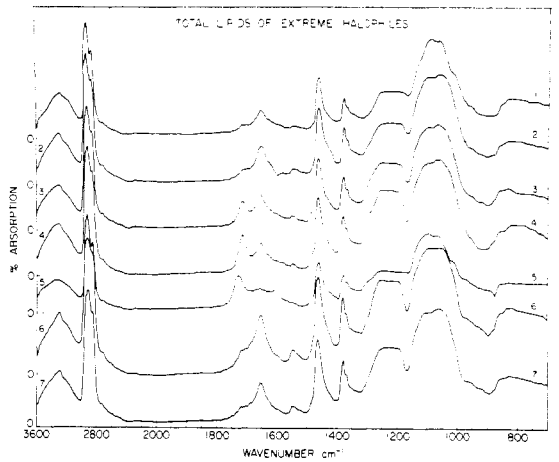

FIGURE 1: Infrared spectra, in chloroform solution, of total lipids of extremely halophilic bacteria: (1) *H. cutirubrum*, (2) *H. halobium* M, (3) *H. halobium* P, (4) *H. salinarium*, (5) *S. litoralis*, (6) halophilic *Sarcina* sp., and (7) halophile A_2C.

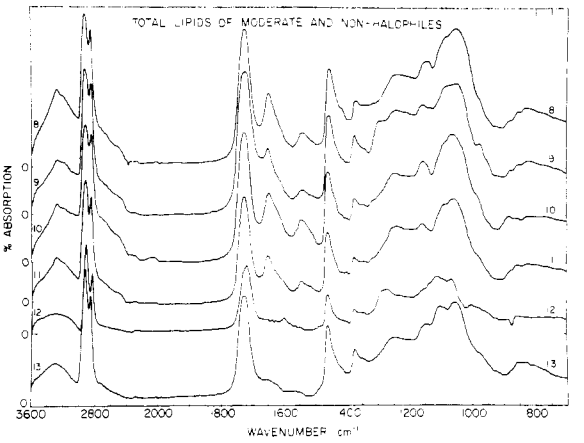

FIGURE 2: Infrared spectra, in chloroform solution, of total lipids of moderately and nonhalophilic bacteria: (8) halophile $A_{31}C$, (9) *M. halodenitrificans* (a), (10) *M. halodenitrificans* (b), (11) *V. costicolus*, (12) *S. lutea*, and (13) *S. flava*.

partition chromatography on a column of butanediol succinate polyester at 180° as described for fatty acid analysis.

Results

Total lipids. The cellular lipid content of the extreme halophiles was found to be in the range 2.6–4.1%, except for *S. litoralis* which had only 0.7% lipids (Table II). The moderate halophiles and nonhalophiles had higher lipid contents, ranging from 6 to 9%, except for halophile $A_{31}C$ which had only 2% lipid.

Most of the extreme halophiles had somewhat higher lipid P contents than the moderate and nonhalophiles (Table II). As will be shown later, this is due to the high proportion of phosphatidylglycerophosphate (%P, 6.6) in the extreme halophiles.

However, the most striking difference between the lipids of the extreme halophiles and the moderate or nonhalophiles became apparent from the data on the contents of unsaponifiable material and fatty acids (Table II). All of the extreme halophiles gave high yields of unsaponifiable material (65–74%) and only traces of fatty acids; in contrast, the moderate and nonhalophiles had high fatty acid contents and gave only small amounts of unsaponifiable material. The presence of traces of fatty acids in the extreme halophile *H. halobium* has been reported recently by Cho and Salton (1966).

The low fatty acid content of the extreme halophiles was also apparent from the infrared spectra of their total lipids (Figure 1), which showed little or no ester carbonyl absorption at 1725 cm^{-1}. This band is very strong in ester-containing lipids, as is evident from the spectra of lipids from moderate and nonhalophiles (Figure 2). Another difference in the spectra of the lipids of extreme halophiles is the presence of a doublet at 1385–1375 cm^{-1}, attributed to $(CH_3)_2C$ groups and characteristic of the dihydrophytyl chain (Figure 1); the spectra of the moderate and nonhalophile lipids lack this doublet and have only a single band at 1387 cm^{-1} owing to terminal CH_3 groups of the fatty acids (Figure 2). The spectra of the extreme halophile lipids also show a strong band at 1100 cm^{-1} which may be due to COC ether groups, but overlapping POC bands, present in the spectra of lipids from all the bacteria studied, makes absorption in this region difficult to interpret.

Identification of Glycerol Diethers. The presence of long-chain glycerol ethers in the extreme halophiles was confirmed by examination of the unsaponifiable fractions. Thin layer chromatography of these fractions revealed a major component (spot b, Figure 3) present only in the extreme halophiles, having R_F 0.4 identical with that of an authentic specimen of 2,3-di-*O*-(3′,7′,11′,15′-tetramethyl)glycerol (dihydrophytyl glyceryl diether). This component was eluted from thin layer plates of unsaponifiable material from each of the extreme halophiles using ethyl ether as eluting solvent. In each case the infrared spectrum (Figure 4) was identical with that of authentic dihydrophytyl glyceryl diether. The characteristic absorption bands were: OH (3580 cm^{-1}), CH_2 and CH_3 (2920, 2850, and 1465 cm^{-1}), $(CH_3)_2C$ (1385–1375 cm^{-1}, doublet), COC ether (1110 cm^{-1}), and primary hydroxyl CO (1040 cm^{-1}).

The nature of the long-chain groups in the eluted glycerol diethers was determined by gas–liquid partition chromatography of the alkyl iodide obtained after cleavage of the ethers with HI (Table III). In each case, the relative retention of the alkyl iodide was identical with that of dihydrophytyl iodide.

Some minor components were also present in the unsaponifiable fraction of the extreme halophiles. Spot d (Figure 3) is probably nonpolar material, chiefly

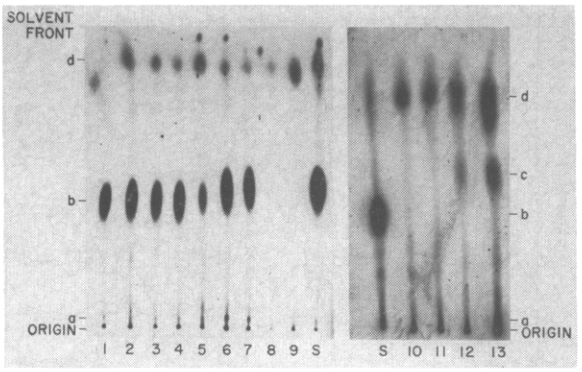

FIGURE 3: Thin layer chromatogram of unsaponifiable material from extremely halophilic (1–7), moderately halophilic (8–11), and nonhalophilic (12, 13) bacteria; identity of bacteria 1–13 given in Figure 1 and 2. Abbreviations: S, synthetic 2,3-di-*O*-dihydrophtylglycerol; a, glycerol monoalkyl ethers (tentative); b, di-*O*-dihydrophtylglycerol; c, unidentified; d, nonpolar components (pigments, hydrocarbons, etc.).

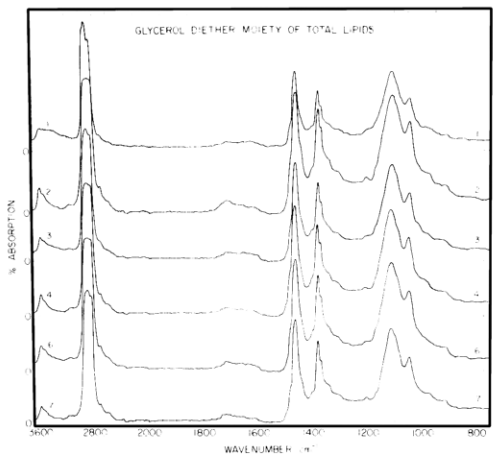

FIGURE 4: Infrared spectra, in carbon tetrachloride solution, of purified glycerol diether moiety of total lipids from: (1) *H. cutirubrum*, (2) *H. halobium* M, (3) *H. halobium* P, (4) *H. salinarium*, (6) *Sarcina* sp., and (7) halophile A_2C. Insufficient material from *S. litoralis* was available for spectral determination.

TABLE III: Gas–Liquid Partition Chromatographic Identification of Alkyl Iodide Derived from the Glycerol Diether Moiety of Lipids of Extremely Halophilic Bacteria.

Sample	Halophile[a]	Rentention Rel to Octadecyl Iodide[b]
1	*H. cutirubrum*	0.80
2	*H. halobium* M	0.80
3	*H. halobium* P	0.81
4	*H. salinarium*	0.81
6	*Sarcina* sp.	0.82
7	Halophile A_2C	0.82
	Authentic dihydrophytyl iodide	0.80

[a] Insufficient material from *S. litoralis* (sample 5, Table II) was available for analysis. [b] On butanediol succinate polyester at 178°. All samples gave only a single peak.

hydrocarbons, pigments, and several unidentified substances which were not further investigated; nonpolar components (spot d) were also present in the moderate and nonhalophiles (Figure 3). Spot a (Figure 3) may represent traces of glyceryl monoethers, and their presence in the extreme halophiles was confirmed by infrared spectroscopy and thin layer chromatography (R_F 0.41 in ethyl ether) on material eluted from spot a. These ethers have also been isolated by silicic acid column fractionation from the unsaponifiable fraction of *H. cutirubrum;* they represented only 2–3% of this fraction and have been identified as an equimolecular mixture of 2-*O*-dihydrophytylglycerol and 3-*O*-dihydrophytylglycerol (M. Kates, C. N. Soo, and T. Shier, unpublished results). Monoethers may also be present in the moderate and nonhalophiles (spot a, Figure 3) but this must be confirmed by isolation and analysis.

Fatty Acid Composition. Because of the very small amounts of fatty acids in the lipids of the extremely halophilic bacteria, accurate determination of their composition was not possible. All samples from these bacteria showed small peaks on gas–liquid partition chromatograms corresponding to lauric, myristic, palmitic, stearic, and C_{16}- and C_{18}-monoenoic acids. To test whether these fatty acids could have been derived from the culture medium, 2 g of silica gel was suspended in 1 l. of culture medium for 3 days. The silica was centrifuged, washed with salt solution, and extracted as described for the halophile cells. A small amount of neutral lipid was obtained which on hydrolysis gave 0.9 mg of fatty acids and 1.2 mg of unsaponifiable material. The latter contained no glycerol diether and consisted of nonpolar lipids with mobility on thin layer chromatography corresponding to spot d (Figure 3). The fatty acids had the same composition as those found in the extreme halophiles, and the amount obtained, if adsorbed on the halophile cells would have accounted for 1–2% of the total lipids. These results thus show that the fatty acids found in the extreme halophiles could have been derived entirely from the culture medium.

Fatty acid analyses of the moderate and nonhalophiles (Table IV) are included here for comparison with compositions reported previously. The values

TABLE IV: Fatty Acid Composition of Moderate and Nonhalophilic Bacteria (in moles per cent).[a]

Fatty Acid[b]	Halophile $A_{31}C$	*M. halodenirificans* a	*M. halodenirificans* b	*V. costicolus*	*S. lutea*	*S. flava*
12:0	—	Trace	Trace	1.1	0.4	0.1
br-13:0	—	—	—	—	—	0.2
br-14:0	—	—	—	—	0.8	1.5
14:0	0.3	0.2	0.2	0.6	Trace	2.9
br-15:0	—	0.3	1.0	1.3	73.7	60.0
br-16:0	—	—	—	—	10.3	9.5
16:0	53.4	24.5	18.5	27.3	3.6	8.8
16:1	—	18.3	13.0	37.0	—	3.0
br-17:0	11.6	—	—	—	11.2	12.0
18:0	3.5	Trace	Trace	2.3	Trace	Trace
18:1	—	56.6	67.3	30.0	—	1.0
Unidentified	3.8					
br-19:0	21.4	—	—	—	—	1.0
Unidentified	6.0	—	—	—	—	—

[a] Data are averages of values obtained by analysis on butanediol succinate and Apiezon L columns. [b] Abbreviations: the first number indicates chain length; the second gives the number of double bonds; br, branched.

found for the two cultures of *M. halodentitrificans* grown in different media (Table I) are similar, and agree fairly well with those reported previously for this bacterium grown in a proteose peptone–tryptone medium in 1 M salt (Kates *et al.*, 1961); the major acids were palmitic and C_{16}- and C_{18}-monoenoic acids. *V. costicolus* had a fatty acid composition qualitatively similar to that of *M. halodenitrificans* but contained more of the C_{16}-monoenoic and less of the C_{18}-monoenoic acids. The two nonhalophilic *Sarcina* species had similar fatty acid compositions, the major acid being a C_{15}-branched chain acid with smaller amounts of C_{16}- and C_{17}-branched acids. Comparison of their relative retentions with those of authentic acids indicated that the C_{15}- and C_{17}-branched acids were the anteiso forms whereas the C_{14} and C_{16} acids were the iso forms. The present results for *S. lutea* agree well with previous analyses, insofar as the predominant acid is the C_{15}-branched chain acid (Cho and Salton, 1966; Huston and Albro, 1964; Akashi and Saito, 1960), but both the anteiso and iso forms of this acid were identified by Huston and Albro (1964).

The halophile $A_{31}C$ was found to have an unusual composition; the major acids were palmitic and C_{17}- and C_{19}-branched acids. However, the position of the branch group in these acids was not determined since their relative retentions did not correspond exactly with those of authentic iso- or anteiso-branched C_{17} or C_{19} acids. Two unidentified homologous acids, differing by two carbons in chain length, were also detected; their retention data suggest that they might be C_{18}- or C_{20}-dienoic acids, but this must be confirmed by chemical analysis.

Lipid Components. Chromatograms of the total lipids of the extremely halophilic bacteria showed that each contained the same major phospholipid, phosphatidylglycerolphosphate diether form (spot 6, Figure 5), and the glycolipid sulfate (spot 2, Figure 5), previously identified in *H. cutirubrum* (Kates *et al.*, 1965b, 1966b). Of the minor components, phosphatidylglycerol, diether form (spot 8, Figure 5) was also detected in all the extreme halophiles except the *Sarcina* sp.; an unidentified phosphatide (spot 4, Figure 5) was present in all except *S. litoralis*, the *Sarcina* sp., and the halophile A_2C; the glycolipid (spot 3, Figure 5) was present in all except *H. halobium* P and *H. salinarium*.

The pattern of lipids in the moderate and nonhalophiles was clearly distinguishable from that of the extreme halophiles (Figures 5 and 6). The moderate halophiles contained chiefly phosphatidylglycerol, diester form (spot 13), its aminoacyl ester (spot 11, Figures 5 and 6), diphosphatidylglycerol (spot 14, Figures 5 and 6), and phosphatidylethanolamine (spot 12, Figure 5 and 6). The nonhalophilic *Sarcina* species had major amounts of phosphatidylglycerol (spot 13, Figure 6) and only traces of its aminoacyl ester (spot 11, Figure 6) and diphosphatidylglycerol (spot 14, Figure 6). Huston *et al.* (1965) reported higher proportions of the aminoacyl ester and diphosphatidylglycerol, but this is not unexpected (see Kates, 1964b) since the culture medium and growth period used by these authors differed from those used here. Comparison of the lipid patterns of halophiles A_2C and $A_{31}C$ shows clearly that the former has components characteristic of the extremely halophilic bacteria whereas the latter has the pattern of the moderate halophiles (Figure 5).

Discussion

The results of the present studies show that glycerol diether derived lipids are not confined to the one

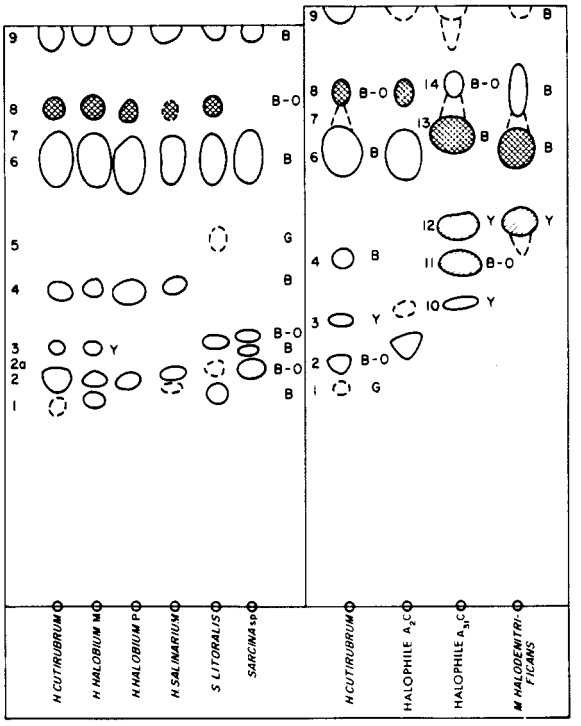

FIGURE 5: Chromatogram of total lipids of extremely halophilic and moderately halophilic bacteria on silicic acid impregnated paper; solvent, diisobutyl ketone–acetic acid–water (40:25:5; v/v). Identity of components: (1, 2a, 4, 5, 10) unidentified, (2) glycolipid sulfate, (3) glycolipid, (6 and 7) phosphatidylglycerophosphate (diether form), (8) phosphatidylglycerol (diether form), (9) pigments and nonphosphatides, (11) phosphatidylaminoacylglycerol (diester form), (12) phosphatidylethanolamine (diester form), (13) phosphatidylglycerol (diester form), and (14) diphosphatidylglycerol (diester form). Chromatograms were stained with Rhodamine 6G (Y, yellow; B, blue; O, orange; G, grey), ninhydrin (singly hatched spots), and periodate–Schiff reagent (doubly hatched spots).

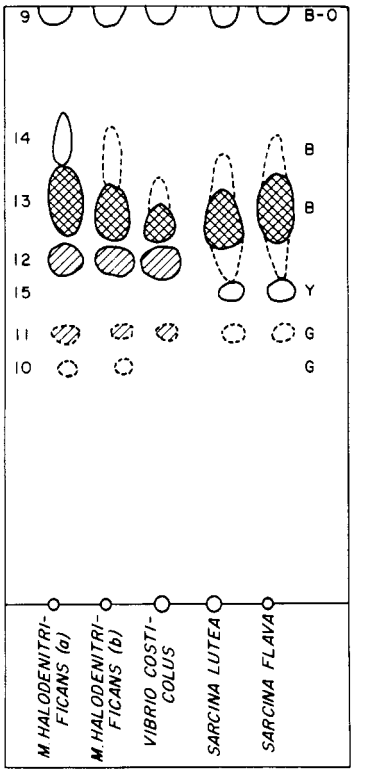

FIGURE 6: Chromatogram of total lipids of moderate and nonhalophilic bacteria, developed and stained as in Figure 5, abbreviations and identity of components as in Figure 5.

species of extremely halophilic bacteria, *H. cutirubrum*, studied previously but are also found in the six other extremely halophilic species examined here (Figure 3). Furthermore, the major lipid components in the latter species are phosphatidylglycerophosphate (diether form), phosphatidylglycerol (diether form), and the glycolipid sulfate [2,3-di-*O*-dihydrophytyl-L-glycerol-1-*O*-(glucosylmannosylgalactosyl)sulfate] (Kates *et al.*, 1966b), as was previously found for *H. cutirubrum* (Figure 5). In contrast, the moderately halophilic bacteria examined completely lacked any glycerol diether derived lipids, and had fatty acid ester-derived components, chiefly phosphatidylglycerol, its amino-acyl ester, diphosphatidylglycerol, and phosphatidylethanolamine (Figures 5 and 6).

Of the two uncharacterized colorless halophiles, one (A_2C) was extremely halophilic, showing no growth below 20% salt, and the other ($A_{31}C$) appeared to be moderately halophilic, growing best in the range 10–15% salt, but being able to survive and grow in up to 25% salt. However, the lipid patterns obtained (Figure 5) are clearly consistent with the classification of the A_2C halophile as an extremely halophilic bacterium, and the $A_{31}C$ halophile as a moderately halophilic organism. Although colorless mutants of extreme halophiles have been obtained by Larsen (1963), it is not certain whether the rods A_2C and $A_{31}C$ are colorless mutants derived from the cultures of red halophiles in which they were found. Despite lack of information on this point, it may at least be concluded that these colorless halophiles were probably derived from or related to genetically different precursors, the A_2C from an extreme halophile, and the $A_{31}C$ from a moderate halophile.

The two nonhalophilic *Sarcina* species, *S. lutea* and *S. flava*, were chosen for comparison with the two morphologically similar extreme halophiles, *S. litoralis* and the unidentified *Sarcina* sp. The major lipid in the two halophilic species is the dihydrophytyl diether form of phosphatidylglycerolphosphate, whereas the branched-chain fatty acid diester form of phosphatidylglycerol predominates in the nonhalophilic *Sarcina* species (Figures 5 and 6). Since different enzyme systems are involved in synthesis of dihydro-

phytyl groups and monomethyl-branched long chains, and of diester and diether forms of phosphatides, some doubt is raised concerning the assignment of the extremely halophilic *Sarcina* species to the same genus as the nonhalophilic *Sarcina* species. Further investigation of this point would be desirable.

The amounts of fatty acids (0.3–2%) found in the lipids of the extreme halophiles (Table II) were just above the level of detection by the procedure used, and their qualitative composition was the same for all the halophilic species. Furthermore, it was shown that the fatty acids detected could have been derived entirely from lipids in the culture medium. Nevertheless, the question remains whether the extreme halophiles are capable of synthesizing fatty acids. In preliminary studies on the biosynthesis of lipids in *H. cutirubrum* (M. Kates, M. K. Wassef, and D. J. Kushner, unpublished results), little or no incorporation of malonate-2-^{14}C into the lipids was observed and none of the incorporated ^{14}C was present in fatty acids. Both acetate-2-^{14}C and mevalonate-2-^{14}C were strongly incorporated and most of the activity appeared in the dihydrophytyl chains but not in fatty acids. It thus appears that *H. cutirubrum* does not contain an active malonyl-CoA[1] fatty acid synthetase system, but instead uses the mevalonate enzyme system for synthesis of the isoprenoid dihydrophytyl groups. In view of these results it seems highly unlikely that fatty acids are synthesized by extremely halophilic bacteria.

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[1] Abbreviation used: CoA., coenzyme A.

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