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LIPID PROFILE OF THE HALOPHILIC ALGA, DUNALIELLA SALINA

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SUMMARY: The lipid composition comprised more than 50% of the cellular organic material. Greater than 30% of the total lipid composition consisted of hydrocarbons. The aliphatic hydrocarbons consisted of C-17 and C-19 saturated and unsaturated ones, some of them with an internal methyl branch. The remaining lipids consisted of a large quantity of pigmented hydrocarbons together with at least six sterol derivatives, six phospholipids, two glycolipids, one sulfolipid and numerous other components that were not fully identified. This wall-less alga is an immensely rich source of a wide variety of lipids.

INTRODUCTION

The extremely halophilic bacteria and algae are two groups of phenomenal organisms that require saturated or nearly saturated salt solutions for optimum growth and survival (1-5). These organisms occur naturally in salt lakes, concentrated brines and in salt flats. The lipid composition of the halophilic bacteria, which are distinguished by having ether-linked phytanyl groups instead of ester bound fatty acids (6), have been extensively investigated and shown to be important components in the structure and function of the cells. More recently, this same class of lipids was found in methanogens and thermoacidophiles (7-9) and it formed a fundamental basis for taxonomically grouping these bacteria and subsequently dividing them from the vast majority of the remaining bacteria that currently comprise the bacterial world. The lipid composition of halophilic algae, such as those of the genus Dunaliella, has not been reported except for studies that have characterized the cellular fatty acids (10) and the carotenoids (11).

Without the awareness of the nature of the lipid composition of Dunaliella spp., we are lacking basic information for the understanding of the biological relationship between extreme halophiles, the physiology of these algae, and the relationships between the chemical organic markers that exist in the immediate geological environment and those found in ancient sediments (9, 12). In this connection and because of the importance of Dunaliella spp. as potential producers of provitamin A (11, 13, 14) and glycerol (5, 15), this preliminary report describes the general lipid content of Dunaliella salina.

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MATERIALS AND METHODS

An algologically pure strain of D. salina isolated from the Great Salt Lakes of Utah was the object of this investigation. Cells for lipid characterization were grown to late logarithmic phase in a filtered sterilized mineral media previously described (16) but with minor modifications. The NaCl concentration was 10.6% with an additional 1.6% KCl, and the medium adjusted to a pH of 6.7 with KOH. Cells were also prepared in medium containing 1mCi ^{14}C-bicarbonate, 1mCi ^{35}S- sulfate or 1mCi ^{32}P-orthophosphate. The harvested cells were washed three times with a 3.8% saline solution containing a mixture of divalent ions as previously described (17). The ash content was determined on aliquots of dried cells at 425°C. Both wet packed cells and freeze dried preparations were extracted for lipid according to the procedure of Bligh and Dyer (18, 19). Once extracted, cell debris was re-extracted in 0.1 M acetate buffer (pH 5.0) (20). Twice extracted cell debris was then digested in methanol-H_2O (3:7) with 10% NaOH for 2 hours at refluxing temperatures. The lipid material was removed with petroleum ether.

Total lipids were fractionated on heat-activated silicic acid columns (Unisil, 325 mesh). Eluting solvents were hexane, benzene, chloroform, acetone and methanol (21). Total lipids and column fractionated lipids were deacylated by mild alkaline methanolysis (22). For analysis of neutral sugars, the water soluble products were hydrolyzed in 1M HCl at 90°C for 1 hr. The hydrolysates were taken to dryness in a stream of nitrogen and resuspended in methanol-water (10:9, v/v). Unsaponifiable lipids were obtained according to the method of Kates (19). Lipids isolated from thin-layer plates were digested in hydriodic acid to cleave ether linkages (23).

Total and column-fractionated lipids were routinely analyzed on silicic acid coated thin-layer plates in solvent mixtures: (A) diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2) as the first solvent and hexane-diethyl ether (96:4) as the second solvent (24) and (B) chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5) (25). Polar lipids were also separated in (C) chloroform-methanol-28% ammonia (65:35:5) in the first direction followed by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1) in the second (26). Unsaponifiable lipids were run in solvent system (D) petroleum ether-diethyl ether-acetic acid (90:10:2). Lipids were detected by spraying with ninhydrin for amino acids (27), molybdate/H_2SO_4 for phosphatides (28) and 50% H_2SO_4 for general charring. Components were also visualized by exposure to iodine vapors and by autoradiography. Deacylated water-soluble products were separated on cellulose thin-layer plates as previously described (29). Components were detected by dipping the plate in o-tolidine (22) or by autoradiography.

Fatty acid methyl esters were prepared by esterification with 2.5% methanolic-hydrochloride (19). Sugars freed from the lipids by acid hydrolysis were converted to alditol acetates (30). Derivatized lipids and sugars were analyzed on a F&M 5750 or F&M 5840 liquid gas chromatograph (GLC) equipped with dual flame ionization detectors, or LKB-gas liquid chromatograph-mass spectrometer (GLC-MS) combination. Chromatographic analyses of lipids and lipid derivatives were carried out on either a stainless steel capillary column (93 m x 0.075 cm) coated with 5% OV-17, a stainless steel column packed with UC W-982 on 80-100 chromasorb W AW (0.31 cm by 51 cm), or a 10 m x 0.2 mm glass capillary column coated with OV-101. Autoradiograms were made with Kodak no-screen X-ray film. Samples were assayed for radioactivity in a Beckman LS-133 Scintillation system.

RESULTS

The lipid composition of D. salina cultivated under laboratory conditions was 45-55% of the total organic material. In addition, 2% by weight of a yel-

Table I
Fractionation of Lipids of Dunaliella on Silicic Acid Column

	Column Fraction	Rel. % Activity ^{14}C	^{32}P	^{35}S
I	Hexane	7.2	0	0
II	Benzene	41.8	0	0
III	Chloroform	2.5	0	0
IV	Acetone	15.2	10	39.7
V	Methanol	33.3	90	60.3

Radioactive incorporated total lipids were fractionated into non-polar lipids (Fractions I-III); glycolipids (Fraction IV) and phospholipids (Fraction V) on Unisil.

lowish oil was removed from the extracted cell debris by alkaline hydrolysis. The proportions of ^{14}C-, ^{32}P-, and ^{35}S-labeled lipids fractionated on columns of silicic acid are given in Table 1. The ^{35}S-labelled components eluted in both the acetone and methanol eluates; however, further analyses revealed that only one type of sulfur containing component existed representing only a relatively small quantity of the total lipids. Pigments were distributed in all sub-fractions with the exception of the hexane eluate; the largest quantities were eluted in the benzene and methanol eluates. Since the pigments of Dunaliella spp. have been previously reported (11, 31), this preliminary report was limited to the description of the major components comprising the remaining lipids.

The results from the separation of the total lipids by thin-layer chromatography (TLC) with non-polar and polar solvent mixtures are given in Tables II and III. Many of the lipids separated in solvent A (Table II) were visible because of their carotenoid properties. The remaining components were visualized by iodine absorption on TLC, and tentatively identified by comparing their R_f values to those of standards. The lipids separated by unidimensional TLC in solvent system B (Table III) again demonstrated the predominance of the neutral lipids (58.5%) in the total fraction. Thirteen polar lipid components were detected in this system, seven of which contained phosphorus. Components 6 and 7 reacted with ninhydrin indicating the presence of primary amines. Components corresponding to spots 5 and 11 (Table III) eluted in the acetone eluate (Table I). These two components co-chromatographed with digalactolsyl diglyceride and monogalactosyl diglyceride standards. GLC and GLC-MS analyses of the acetylated acid hydrolysates of each isolated component showed that the sugar was indeed galactose. No sulfur containing components were detected in these chromatograms although trace amounts of ^{35}S were de-

Table II
Proportions of Lipid Components Recovered From
Thin-Layer Chromatograms of Total Lipids

Spot No.	R_f Values	$\%^{14}C$ Activity	Tentative Identification
0	0.00	34.0	polar lipids
1	0.20	0.9	free fatty acids + sterols
2	0.31	1.8	yellow pigment
3	0.35	0.8	yellow pigment
4	0.39	11.0	orange pigment
5	0.41	2.6	yellow pigment
6	0.45	6.1	yellow pigment
7	0.49	13.0	yellow pigment
8	0.64	2.2	yellow pigment
9	0.73	15.4	yellow pigment
10	0.83	2.7	isoprenoid hydrocarbon
11	0.89	4.3	isoprenoid hydrocarbon
12	0.93	5.2	aliphatic hydrocarbon

Data obtained from a thin-layer chromatogram developed with neutral lipid solvent A. R_f values for authentic lipids were found to be: eicosane, 0.93; tetrahydrosqualene, 0.90; squalene, 0.82; β-carotene, 0.73; cholesteryl oleate, 0.50; methyl stearate, 0.45; tripalmitin, 0.41; 1,3 dipalmitin, 0.31; myristic acid, 0.20; cholesterol, 0.18.

tected in the samples by scintillation counting. Further characterization of the lipids was performed with two dimensional chromatography in solvent system C. In this chromatogram 19 compounds were detected with a possible 5 additional ones existing in relatively trace quantities. In this system, the number of components detected were approximately twice that visualized by unidirect-

Table III
Proportions of Lipid Components Recovered From
Thin Layer Chromatograms of Total Lipids

Spot No.	R_f Value	$\%^{14}C$ Act.	$\%^{32}P$ Act.	Tentative Identification
0	0.00	3.6	7.6	?
1	0.01	0.9	0	?
2	0.13	1.4	10.7	?
3	0.18	2.5	15.0	phosphatidyl choline
4	0.25	3.6	19.8	phosphatidyl glycerol
5	0.28	3.5	0	diglycosyl diglyceride
6	0.33	8.2	35.0	phosphatidyl ethanolamine
7	0.37	4.9	5.1	phosphatidyl-amino acid
8	0.43	2.7	5.9	diphosphatidyl glycerol
9	0.51	2.0	0	?
10	0.62	1.0	1.0	phosphatidic acid
11	0.80	7.4	0	monoglycosyl diglycerides
12,13,14	0.88-0.97	58.5	0	neutral lipids

Data obtained from thin layer chromatogram developed in one dimension with polar solvent B. R_f of authentic st'ds: mixture non-polar lipid, 0.90-0.99; monogalactosyl diglyceride, 0.80; phosphatidic acid, 0.62; sterol glucoside, 0.52; diphosphatidyl glycerol, 0.43; phosphatidyl serine, 0.38; phosphatidyl ethanolamine, 0.33; digalactosyldiglyceride, 0.28; phosphatidyl glycerol, 0.23; phosphatidyl choline, 0.81; phosphatidylinositol, 0.08.

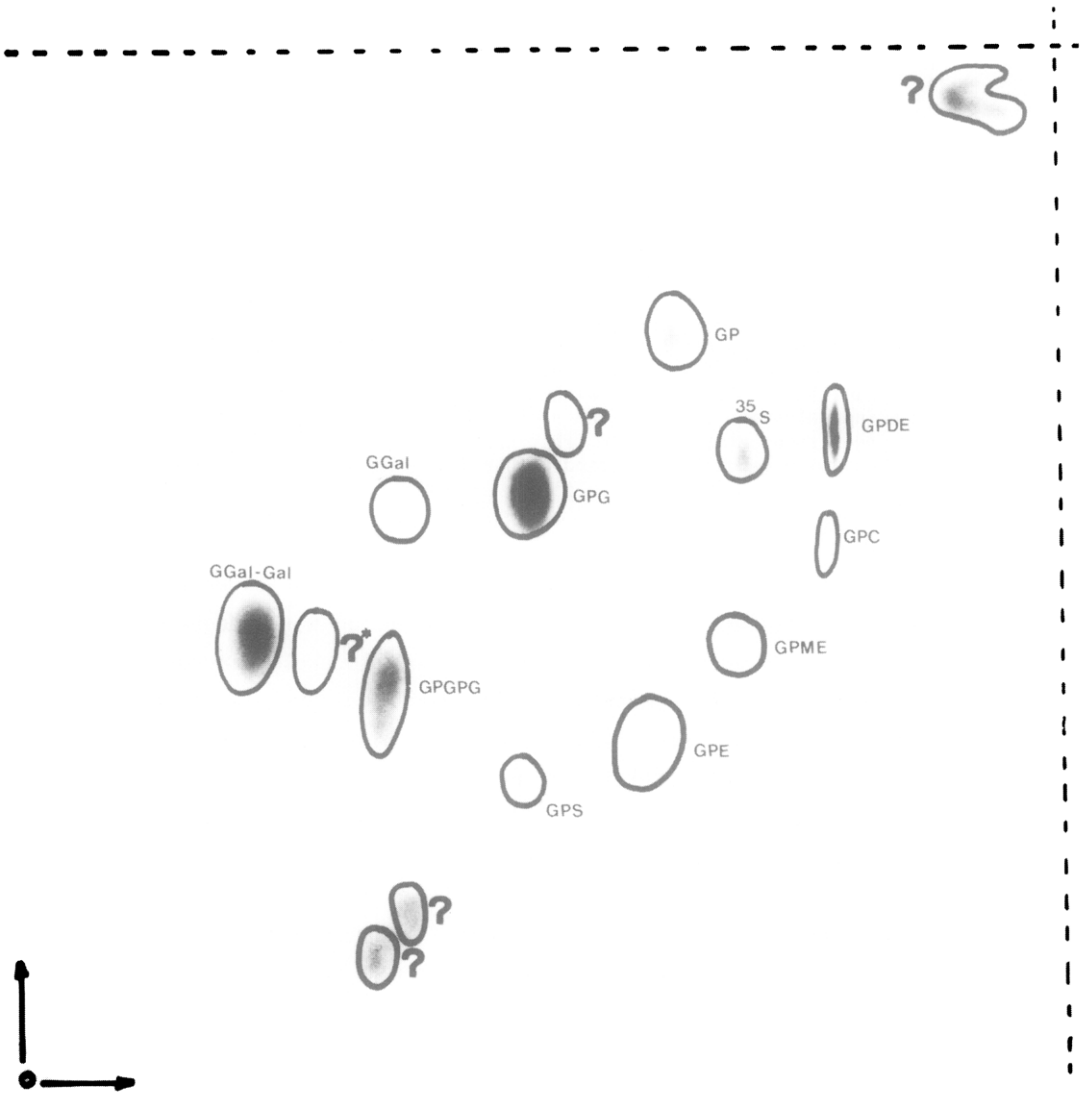

Figure 1. Radioautogram of water-soluble fraction of deacylated ^{14}C-polar lipids (fraction IV and V, Table 1) after separation over cellulose thin-layer plates in a previously described solvent system (29).

ionally developed TLC. The components that co-chromatographed with known standards in this system were the same as those identified in the unideveloped TLC

Table IV
Percentage Composition Data of Lipid Components

Fatty Acids	Rel. % Composition	Fatty Acids	Rel. % Composition
8:0	1.9	17:2	1.5
11:1	1.2	18:0	trace
11:2	0.9	18:1	3.8
13:2	0.7	18:2	6.0
15:2	0.7	18:3	12.5
16:0	20.6	20:0	trace
16:1	7.8	20:5	1.8
16:2	10.7	22:0	trace
16:3	5.8	22:2	3.1
16:4	trace	22:5	4.9
		unidentified	5.8

Resolution of the components were determined on 93 m x 0.975 stainless steel capillary columns coated with 5% Igepal Co990 or 3% OV-17, and a 10 m x 0.2 mm glass capillary column coated with OV-101. Identification was obtained by comparing retention times to those of authentic standards and by their mass fragmentation patterns. The first number indicates the number of carbons in the chain length; the second number indicates number of double bonds.

described in Table III. The water soluble deacylated lipids were chromatographed on cellulose-TLC. All the components (Fig. 1) were recovered from the methanol eluate subfraction with the exception of glycerol-galactose-galactose, glycerol-galactose and the component designated ?* which eluted in the acetone eluate. The tentatively identified deacylated lipids corresponded to the specific intact lipids (Table III) in addition to glycerol phosphatidylmonomethylethanolamine and glycerol phosphatidyldimethylethanolamine which were not discernable in previous chromatograms. The components of the 6 remaining spots were not identified. One of them, designated ^{35}S, was determined as a derivative of a sulfolipid from the results of the ^{35}S incorporation studies. The most relatively intense component as determined from ^{32}P incorporation studies was GPE corresponding to phosphatidyl ethanolamine. The lipid soluble fraction separated in solvent system D consisted of a spot (89%) that co-chromatographed with fatty acid methyl esters and a spot that remained at the origin (16%).

The fatty acid composition of the total extracted lipids consisted primarily of unsaturated fatty acids (Table IV). The fatty acids were predominantly even-number carbon chains containing from 0 to 5 unsaturated bonds like those reported for other Dunaliella spp. (10). The fatty acid pool of the lipids of this alga, however, also contained a broad range of odd-numbered carbon fatty acids. The even-numbered carbon fatty acid methyl esters gave typical fragmentation patterns, similar to those previously published (32).

The unsaponifiable lipids of the benzene, chloroform and methanol eluates each provided complex patterns with each having 8 common compounds. Principal spots were yellow pigmented ones with R_f values of 0.98, 0.25, and 0.00 (at the origin). The largest quantity of components co-chromatographed with hydrocarbons standards. Two minor components co-chromatographed with diphytanyl glycerol diether and dibiphytanyl diglycerol tetraether standards. The isolated components, however, did not hydrolyze in HI and did not yield the expected phytane (C-20) and biphytane (C-40) components (6, 8). These lipid subcomponents were not further studied at this time. One of the components found only in the methanol eluate co-chromatographed with a cholesterol standard (R_f = 0.48) Six major sterol components were detected by GLC and GLC-MS. Since the sterols isolated were recovered from the methanol eluate, and practically none of them were detected in any of the other eluates (Table I), one must assume that they are associated with polar components, such as sugars. The sterols and their derivatives will be identified in detail in another report. Several other hydroxylated ring structures were also detected in both the benzene and methanol eluates after saponification, but they were not fully identified.

The hexane-benzene fractions (Table I) contained more than 49% of all the ^{14}C incorporated into the extractable lipids. The principal aliphatic hydrocarbon components in these two eluates were a $C_{17}H_{34}$ straight chain monoene (56%) and methyl branched $C_{17}H_{36}$ (14%) and $C_{19}H_{40}$ (15%). The C-17 branched hydrocarbon had a parent ion of 240 with diagnostic peaks at m/e 168,169 and m/e 98,99. The fragmentation pattern indicated an internally branched structure tentatively identified as 6-methyl hexadecane. The branched C-19 hydrocarbon with a parent ion at m/e = 268 and a strong peak at M^+-43 was tentatively identified as 4-methyl octadecane. The mass spectral pattern of the major component was typical for a straight chain C-17:1 hydrocarbon. Two additional hydrocarbons were identified as di- and tri- unsaturated C-17 hydrocarbons. Relatively trace quantities of other components were detected but not identified due to relative sample sizes. The principal hydrocarbons in the benzene eluate were the pigmented carotenoids. A relatively small quantity of squalenes were also detected similar to those previously described (33).

DISCUSSION

More than 50% of the organic material of D. salina was lipid. The principal lipids were non-polar ones with hydrocarbons comprising slightly greater than 30% of the total lipid composition. The unsaponifiable lipids obtained from acid and alkaline hydrolysates were a viscous yellowish oil that was soluble in petroleum ether. The unsaponifiable fraction was extremely complex

with some 30 compounds readily discernable by TLC alone. The components consisted of mixtures of aliphatic hydrocarbons, acyclic and cyclic isoprenoid hydrocarbons, sterols, a variety of hydroxylated cyclic structures and many other unidentified components. Because of the tremendous ability of this wall-less eukaryotic alga to produce such large quantities of lipids without the cultivation system yet being maximized for lipid production, this organism is potentially valuable for biotechnological developments.

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