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Lipid Composition of *Cyanidium*[1]

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C. FREEMAN ALLEN, PEARL GOOD, AND RAYMOND W. HOLTON
Department of Chemistry, Pomona College, Claremont, California 91711; and Department of Botany, The University of Tennessee, Knoxville, Tennessee 37916

ABSTRACT

The major lipids in *Cyanidium caldarium* Geitler are monogalactosyl diglyceride, digalactosyl diglyceride, plant sulfolipid, lecithin, phosphatidyl glycerol, phosphatidyl inositol, and phosphatidyl ethanolamine. Fatty acid composition varies appreciably among the lipids, but the major ones are palmitic acid, oleic acid, linoleic acid, and moderate amounts of stearic acid. Trace amounts of other acids in the C_{14} to C_{20} range were also present. Moderate amounts of linolenic acid were found in two strains, but not in a third. The proportion of saturated acid is relatively high in all lipids ranging from about a third in monogalactosyl diglyceride to three-fourths in sulfolipid. This may be a result of the high growth temperature. Lipases forming lysosulfolipid, and lysophosphatidyl glycerol are active in ruptured cells; galactolipid is degraded with loss of both acyl residues. Thus the lipid and fatty acid composition of *Cyanidium* more closely resembles that of green algae than that of the blue-green algae, although there are differences of possible phylogenetic interest.

Cyanidium caldarium is a monotypic genus with several characteristics of a blue-green alga but others typical of the higher algae. Its various attributes and peculiarities have recently been reviewed (14) and can be briefly summarized as follows: cytologically, it possesses mitochondria, a single chloroplast, and a nucleus with enclosing membrane in which chromosomes have not been visualized; biochemically, it contains chlorophyll *a* as its sole chlorophyll and contains C-phycocyanin and allophycocyanin, β-carotene, lutein, and zeaxanthin although it lacks the myxoxanthophyll found in many blue-green algae. Its aldolase resembles that of *Chlorella* (18). This mosaic of characteristics defies a convenient classification of *Cyanidium* into the blue-green, red, green, or cryptophycean groups of algae, into each of which it has at one time been placed. Physiologically, it is most interesting in that it is both acidophilic and thermophilic with growth occurring at temperatures as high as 60 C (6) and at acidities of pH 2.0 (10).

Knowledge of the lipid and fatty acid composition of green plants, and particularly microorganisms, has been of considerable use in considering the phylogeny and interrelationships in these organisms. Thus, while α-linolenic acid is found in all photosynthetic higher algae and green plants, it is by no means present in all blue-green algae and is absent completely from photosynthetic bacteria (12). While the only lipid common to the photosynthetic bacteria is phosphatidyl glycerol, algae and green plants all contain monogalactosyl diglyceride, digalactosyl diglyceride, and sulfoquinovosyl diglyceride (plant sulfolipid) as well. The blue-green algae do not contain phosphatidyl choline (lecithin) which is characteristic of higher algae. The object of these experiments was to ascertain the lipid and fatty acid composition of *Cyanidium* with hope that these data might enable us to understand better its relationshlp to other algae.

MATERIALS AND METHODS

Cultures of *Cyanidium caldarium* Geitler were obtained from three sources: lot I from M. B. Allen, who also described the culture medium used for the alga (5); lot II from the culture collection of the Kettering Research Laboratory, Yellow Springs, Ohio; and lot III of strain 006 from Nymph Creek, Yellowstone National Park, as cultured and provided to us by T. D. Brock and W. N. Doemel, Indiana University. Lot I was grown in 16-liter carboys illuminated by fluorescent lights and an incandescent light and aerated with 5% CO_2 in air. The temperature was maintained at approximately 45 C. The cells were harvested in a Sharples centrifuge and stored as a frozen paste on Dry Ice until used for analysis.

Lots II and III were grown in 250-ml batches in 500-ml Erlenmeyer flasks aerated with 5% CO_2 in air and placed above a panel fluorescent lamp which provided illumination while maintaining the culture temperature at 42 to 45 C. Cells were centrifuged from the medium and used at once for the extraction of lipids. The pH of the culture medium at the time of harvest was 2.5 to 3.0.

The culture from the Kettering collection (lot II) grew more slowly than either of the other two and prodced cells with markedly lower chlorophyll content. The cells in all three lots appeared similar under the light microscope except for the lighter green color of lot II.

Cells labeled with ^{35}S and ^{32}P were grown in test tubes at pH 3 under the same conditions as lots II and III except that unlabeled sulfate and phosphate, respectively, were replaced by millicurie amounts of $H_2{}^{35}SO_4$ and $H_3{}^{32}PO_4$.

Extraction of Lipids. Exposure of lipids to oxygen was minimized by use of a nitrogen atmosphere whenever practical in all subsequent operations.

Cyanidium is exceedingly resistant to the usual lipid extraction procedures, probably because of an impervious cell wall. Neither repeated extraction with chloroform-methanol mixtures at room temperature followed by sonication with a Branson Instrument Co. model LS-75 sonifier with a ½ inch diameter probe for 1 min, nor extraction with boiling ethanol followed by chloroform at 20 C released more than a small amount of chlorophyll or lipid.

The first lot of cells, used for isolation of lipid in quantity, was ruptured by grinding at high speed in a VirTis homogenizer with

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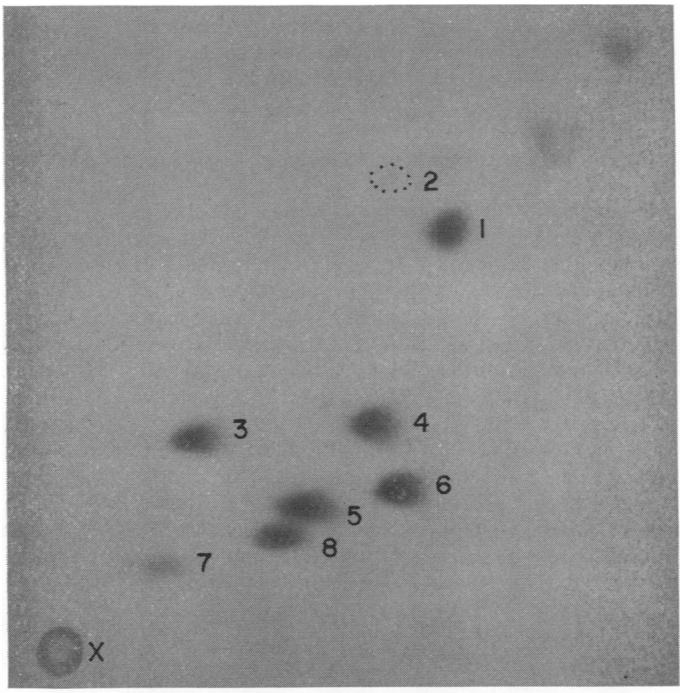

FIG. 1. Thin layer chromatogram of lipids from *Cyanidium caldarium*, lot III, photographed under ultraviolet illumination with the lipid spots fluorescent after spraying with alkaline Rhodamine 6G. The chromatogram was developed in chloroform-methanol-water (65:25:4, v/v) vertically and in chloroform-methanol-isopropylamine-15 M ammonium hydroxide (65:25:0.5:5, v/v) horizontally. The spots are: 1: monogalactosyl diglyceride; 2: fatty acid; 3: digalactosyl diglyceride; 4: phosphatidyl ethanolamine; 5: sulfolipid; 6: phosphatidyl glycerol; 7: phosphatidyl inositol; 8: phosphatidyl choline; and X: uncharacterized.

0.2 mm glass beads in chloroform-methanol (2:1, v/v) for 40 min. The mixture was cooled with ice during homogenization, but in spite of this the isolated lipids contained appreciable quantities of lysolipids and fatty acids indicative of enzymatic degradation. These extracts were evaporated in a rotary evaporator and taken up in a small portion of chloroform-methanol (2:1) for further investigation.

The cells (about 100 mg wet wt) labeled with 1 mc of either ^{35}S or ^{32}P were dropped into a few milliliters of boiling methanol, heated for 1 min, and then brought to room temperature. Little lipid was extracted by this treatment. The methanol was separated by centrifugation, and the cells were suspended in cooled glacial acetic acid. An equal volume of chloroform was added, the cells were separated, and additional lipid was extracted with chloroform-methanol (2:1, v/v). Very little lipid remained in the cell residues at this point, as judged by the small quantity of fatty acid liberated on hydrolysis of the cell residues. Water was added to the pooled methanol-chloroform-acetic acid extracts equal to the combined volume. The resultant two phases were washed with chloroform and water, respectively. The combined chloroform-rich phase was evaporated to dryness with a jet of nitrogen, and residual lipids were dissolved in chloroform-methanol prior to analysis of labeled sulfo- and phospholipids.

Lots II and III were also extracted in this manner, except that acetic acid was replaced by formic acid on some cultures from lot III.

The insoluble cell residues remaining after lipid extraction were dried under vacuum over silica gel and weighed to permit calculation of lipid content per gram of extracted residue for lots II and III (Table I).

Table I. *Lipid Composition of Cyanidium caldarium*

Lipid	Lot I[1]	Lot II[2]	Lot III[3]			
			A	B	C_1	C_2
	weight %	*μmoles lipid/g dried cell residue*				
Monogalactosyl diglyceride	(10)	9.3	19.7	26.7	23.4	27.0
Digalactosyl diglyceride	22	9.0	20.2	26.0	26.4	28.8
Phosphatidyl glycerol	(6)	2.0	2.5	5.6	11.3	11.8
Plant sulfolipid	(10)	6.3	14.1	9.9	13.6	13.6
Phosphatidyl ethanolamine	9	11.0	16.0	7.9	10.6	10.8
Phosphatidyl choline	14	17.8	14.8	16.7	16.9	16.7
Phosphatidyl inositol	2	3.8	3.3	3.1	2.9	3.8
Fatty acids (free)	(15)	1.5	2.5	4.2	3.4	4.0
Lyso phosphatidyl glycerol	(5)					
Lyso sulfolipid	(8)					
Unextracted lipids		4.4				

[1] From M. B. Allen. Extensive degradation of lipids during isolation renders the values in parentheses quite different from the composition *in vivo*.

[2] From the C. F. Kettering Research Laboratory algae collection. This culture grew at about 1/10 the rate of the other strains.

[3] From Yellowstone National Park. Cells in culture A appeared to lose all color before growth became apparent after about a week of incubation under the conditions described. The cells then grew rapidly. B was a subculture of A, and C was a subculture of B. All cells were harvested near mid log phase of growth. C_1 and C_2 are duplicate runs.

Separation and Quantitative Determination of Lipids. Lipids from lot I were chromatographed on DEAE-cellulose[2] and on silicic acid columns by the procedure of Allen *et al.* (2), but enzymatic degradation of the lipids during isolation did not permit determination of composition *in vivo*. In addition to the parent lipids, lysophosphatidyl glycerol, lysosulfolipid, and fatty acid were identified as major artifacts.

Lipids of cells from lots II and III were separated by two-dimensional thin layer chromatography and analyzed by the method of Allen and Good (1). Areas of silicic acid-containing lipids were removed, mixed with a known quantity of heptadecanoic acid internal standard, and heated at 70 C for 2 hr with 5% sulfuric acid in methanol (v/v). Methyl esters were separated from the diluted reaction mixture with hexane and analyzed by gas chromatography on Reoplex 400 and diethylene glycol succinate columns. Acyl group composition and molar quantities of lipid were calculated from peak areas relative to the internal standard.

Identification of Lipids. Purified lipids were identified by infrared spectra (2), and by comparison with authentic standards on silicic acid plates developed in acidic, basic, and neutral solvent systems.

Further confirmation of the identification of plant sulfolipid and its lyso analogue was made by paper chromatography of the water-soluble acidic hydrolysis products (8). Both yielded sulfoquinovose with chromatographic properties identical to a synthetic sample supplied by A. A. Benson.

The presence of phosphorus was confirmed in lipids identified as phospholipids, and its absence in all others, by color tests based on phosphomolybdate formation.

Radioautography. Phospho- and sulfolipids from the ^{32}P- and ^{35}S-labeled *Cyanidium* were chromatographed separately on silicic acid plates in two dimensions, first with chloroform-

[2] Abbreviation: DEAE-: diethylaminoethyl.

methanol-water (65:25:4), then with chloroform-methanol-15 M NH_4OH (65:35:5). Lipids were located by radioautography. Only one radioactive lipid was present in the ^{35}S-labeled material, and this corresponded to R_F values of plant sulfolipid.

Radioactive areas on the chromatogram of ^{32}P-lipids corresponded to phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl inositol.

The absence of lysosulfolipid and lysophosphatidyl glycerol in these chromatograms confirms their absence in appreciable amounts in intact cells and supports the conclusion that they are artifacts produced during the prolonged grinding procedure used with the first lot of cells.

Additional confirmation of phospholipid identity was obtained from relative migration rates of lipids deacylated with methanolic potassium hydroxide at 37 C (9), and from the rate of migration of the deacylated lipids during paper electrophoresis at pH 3.6 in pyridine-acetic acid buffer.

RESULTS AND DISCUSSION

Lipid composition and the fatty acid content of *C. caldarium* are shown in Tables I and II. Among the lipids are mono- and digalactosyl diglyceride, phosphatidyl glycerol, and sulfolipid, typically present in eucaryotic algae and higher plants as well as in blue-green algae. Also present are lecithin, phosphatidyl inositol, and phosphatidyl ethanolamine, not found in the blue-green algae but present in more highly evolved green plants (4, 13). Smaller amounts of other uncharacterized lipids are also present (Fig. 1).

Cyanidium lipids contain polyunsaturated acids, as is typical of eucaryotic algae and some blue-green algae, but not the unicellular blue-green algae (12). However, the percentage of unsaturated acids and degree of unsaturation are lower than is common in higher plants (17). This is probably a consequence of the relatively high temperature at which the *Cyanidium* was grown, for the ratio of saturated to unsaturated acids has been shown to increase in other algae at higher temperatures (11). *Cyanidium* contains in phosphatidyl glycerol, phosphatidyl choline, and phosphatidyl ethanolamine a very small amount of an acid with the retention time close to *trans*-3-hexadecenoic acid. This acid is a major constituent of phosphatidyl glycerol in typical higher green plant photosynthetic systems and in algae such as *Chlorella* grown on a mineral medium, but is not found in blue-green algae, nor in *Chlorella* grown heterotrophically (3, 4, 15–17, 19). Linolenic

Table II. *Fatty Acid Composition of Cyanidium Lipids*[1]

Lipid	Lot	16:0	18:0	18:1	18:2	α-18:3	Other Acids (less than 1%)[2]
Monogalactosyl diglyceride	II	37.1	Tr	15.3	40.3	7.3	X, 16:1
	IIIA	36.5	Tr	4.0	59.6		
	$IIIC_1$	30.7	Tr	1.4	67.9		
Digalactosyl diglyceride	II	52.5	Tr	18.1	29.4	Tr	X, 20:2
	IIIA	44.2	Tr	3.7	52.1		
	$IIIC_1$	35.5	Tr	1.3	63.2		
Phosphatidyl glycerol	II	49.4	1.8	28.0	20.8	?	X, Y
	IIIA	44.5	2.0	10.8	42.7		
	$IIIC_1$	46.3	1.0	2.3	50.4		
Plant sulfolipid	II	75.8	2.6	15.8	5.8	?	16:2
	IIIA	70.2	7.0	4.1	18.7		
	$IIIC_1$	73.9	5.3	1.2	19.6		
Phosphatidyl ethanolamine	II	20.9	6.3	57.7	15.0	Tr	X, Y, 16:1, 16:2, 20:0, 20:2
	IIIA	31.3	8.3	39.2	21.1		
	$IIIC_1$	36.7	7.3	20.0	36.1		
Phosphatidyl choline	II	33.8	4.5	38.2	23.5	Tr	X, Y, 16:1, 16:2, 20:0, 20:2
	IIIA	28.3	7.1	31.8	32.9		
	$IIIC_1$	24.0	6.4	22.9	46.7		
Phosphatidyl inositol	II	60.3	2.2	32.4	5.2	Tr	16:1, 16:2, 20:2
	IIIA	32.8	16.4	33.7	17.1		
	$IIIC_1$	31.1	18.8	21.5	28.5		
Fatty acids (free)	II	40.8	20.0	27.1	12.1		16:1
	IIIA	45.3	23.7	13.2	15.4		
	$IIIC_1$	28.5	16.5	24.5	27.6		
Residue	II	43.8	6.6	29.2	19.4	1.1	16:1, 20:0.:20:1
Total lipid	II	43.1	3.2	29.5	20.7	3.5	X, Y, 20:0, 20:1

[1] Fatty acids are indicated by number of carbons:number of olefinic bonds.

[2] All lipids contain trace amounts of 14:0 and 14:2. Other fatty acids were found in trace amounts in various lipids as indicated, but not all were present in each lot. X has a retention time between 14:2 and 14:3 on Reoplex 400. Y has a retention close to, but just short of, *trans*-3-hexadecenoic acid. 20:2 is present in somewhat greater than 1% concentration in phosphatidyl choline and phosphatidyl inositol in lot III, but was not detected in lot II.

acid is a component of the lipids in two of the *Cyanidium* strains investigated, but it could not be detected in the third.

As is apparent from Tables I and II, the quantitative molar lipid content and acyl group composition do change with the strain and growth state of the culture. However, phosphatidyl choline, phosphatidyl inositol, and phosphatidyl ethanolamine, which are at most minor components of photosynthetic lamellae but are present in mitochondrial and other cell membranes, do remain at a nearly constant level. Mono- and digalactosyl diglyceride and plant sulfolipid are the major lipids specific to photosynthetic lamellae, and all of these increase uniformly, presumably with the content of these membranes. Phosphatidyl glycerol, which is also a major lipid in the photosynthetic apparatus, shows a similar but more erratic increase. The concentration of this lipid may be dependent on the metabolic state of the cells (7).

The pattern of lipase activity in *Cyanidium* as indicated by the degradation of lipids during extraction is similar to that of higher plants and green algae (17). Phosphatidyl glycerol and sulfolipid are actively deacylated to their lyso analogue, whereas both acyl residues are cleaved from monogalactosyl diglyceride without accumulation of its lyso analogue. Such activity is not found in *Anacystis nidulans* (4, 17) and is apparently lacking in other blue-green algae.

Thus the lipid and fatty acid composition of *Cyanidium* parallel the other biochemical and cytological information. This organism resembles the green algae and higher plant groups, but there are certain minor, yet distinct differences from the "typical" green algae and higher plants studied to date. Our results tend to substantiate the idea that *Cyanidium*'s phylogenetic position is of an "intermediate" nature; this position has been formalized by Klein and Cronquist's suggestion (14) of a separate line of algae, the "Uralgae."

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