Phytochemistry, 1972, Vol. 11, pp. 1077 to 1082. Pergamon Press. Printed in England.

LIPIDS OF THE THERMOPHILIC ALGA *CYANIDIUM CALDARIUM*

RAPHAEL IKAN and JOSEPH SECKBACH
Department of Organic Chemistry, Hebrew University, Jerusalem, Israel

(*Received* 16 *June* 1971; *in revised form* 21 *August* 1971)

Abstract—A series of fatty acids (C_8–C_{20}), both, saturated and unsaturated and sterols (C_{27}–C_{29}) were identified in the alga, *Cyanidium caldarium*, using chromatographic and spectroscopic methods. Some phylogenetic consequences of the results are discussed.

INTRODUCTION

THE UNICELLULAR alga *Cyanidium caldarium* is of particular interest because it is able to tolerate extreme environmental conditions.[1] *Cyanidium* is found in nature in hot springs (up to 56°), it grows in acidic medium as in 1 N sulphuric acid,[1] thrives under an atmosphere of pure CO_2[2] and resists gas compression for several days.[3] Its systematic position is undetermined among the algal classes. These and other extraordinary features of *C. caldarium* distinguish this organism from other unicellular algae. A study of its lipids could be expected to throw light on the taxonomic relationships and furthermore explain its tolerance to extreme conditions.

Since lipids compose a substantial portion of cellular membranes, we examined the fatty acids and sterol composition in order to elucidate the controversial taxonomy of *C. caldarium*.[4,5]

RESULTS

Algal sterols and fatty acids, as determined by GLC and MS, are summarized in Tables 1 and 2, respectively.

TABLE 1. GAS CHROMATOGRAPHY AND MASS SPECTRA OF *C. caldarium* STEROLS*

Assigned structure	Retention time relative to cholesterol†	Abundance (%)	Molecular ion
Cholesterol	1·00	0·3	386
Ergosterol + 5,6-dihydroergosterol	1·18	55·0	396, 398
Campesterol	1·35	5·6	400
β-Sitosterol	1·54	38·9	414
7-Dehydrositosterol	1·87	0·2	412

*Analysed as trimethylsilyl ethers.
† Retention time of cholesterol, 8·0 min.

[1] T. D. BROOK, *Microbial Growth* (edited by P. MEADOW and S. J. PIRT), p. 15, Cambridge University Press (1969).
[2] J. SECKBACH, F. A BAKER and P. M. SHUGARMAN, *Nature, Lond.* **227**, 744 (1970).
[3] J. SECKBACH and W. F. LIBBY, *Space Life Sciences* **2**, 121 (1970).
[4] R. M. KLEIN and A. CRONQUIST, *Quart. Rev. Biol.* **42**, 105 (1967).
[5] P. C. SILVA, *Physiology and Biochemistry of Algae* (edited by R. A. LEWIN), p. 827, Academic Press (1962).

The UV spectrum of the sterol mixture had peaks at 264, 273, 283 and 295 nm, identical to that of ergosterol.[6] This was supported by the deep blue color it gave in the Liebermann–Burchard test. If one assumes that the major sterol is ergosterol, then the other sterols (except the phytosterols) are probably the homologous compounds of the 24 β-series, but the possibility exists that they belong to the 24 α-series.

TABLE 2. ALGAL FATTY ACIDS AS DETERMINED BY GLC AND MS OF THEIR METHYL ESTERS

Acid No. of carbons	Percentage FFA	Percentage BFA	Molecular ion
8	0·6	—	158
10	0·4	4·1	186
10(1)	0·3	0·3	184
11	2·4	0·1	200
12	1·2	1·0	214
12(1)	0·1	0·2	212
13	0·3	0·1	228
14	1·8	2·6	242
14(1)	1·0	1·4	240
15	1·6	1·5	256
16	47·0	41·4	270
16(1)	1·5	2·6	268
16(2)	0·4	1·0	266
17	1·8	2·0	284
18	5·8	4·8	298
18(1)	12·1	10·5	296
18(2)	20·4	24·8	294
18(3)	0·3	0·4	292
20	0·4	0·8	326
20(1)	tr.	tr.	—
20(2)	tr.	tr.	—
20(3)	tr.	tr.	—

Numbers in parentheses refer to the number of double bonds.
tr.—traces; FFA—free fatty acid fraction; BFA—combined fatty acid fraction.

On a dry weight basis, the lyophilized algal cells contain 7·5% of the lipids and 3·25% fatty acids, the most abundant acids being palmitic ($C_{16:0}$), linoleic ($C_{18:2}$) and oleic ($C_{18:1}$). Both FFA and BFA have about the same composition. Odd-carbon-numbered acids of *C. caldarium* represents *ca.* 5% each of the corresponding even-carbon-numbered acids. It is interesting that most alkanes of green and blue–green algae are odd-numbered;[7] this suggests that they are formed by decarboxylation of even-numbered acids. The structure of the unsaturated acids, which represent *ca.* 40% was confirmed by hydrogenation and GLC analysis of the saturated acids thus obtained.

The ratio of the total C_{16}/C_{18} fatty acids in *Cyanidium* is 1·26 and 1·11 for the FFA and BFA, respectively; this value could be related to the phylogenetic position or morphological complexity of the alga.[8] The data show a strikingly high concentration of $C_{18:2}$, while $C_{18;1}$ and $C_{18:3}$ acids are less abundant (Tables 2 and 3, column A). *Cyanidium caldarium*

[6] F. R. SMITH and E. D. KORN, *J. Lipid Res.* **9**, 405 (1968).
[7] E. GELPI, H. SCHNEIDER, J. MANN and J. ORÓ, *Phytochem.* **9**, 603 (1970).
[8] H. SCHNEIDER, E. GELPI, E. O. BENNETT and J. ORÓ, *Phytochem.* **9**, 613 (1970).

TABLE 3. THE MAJOR FATTY ACIDS OF *C. caldarium* AS DETERMINED BY GLC AND MS OF THEIR METHYL ESTERS AND COMPARABLE DATA FROM PUBLISHED WORK

Acids No. of carbon atoms	Fatty acids (%) A*	B†	C‡
16:0	44·0	53·0	43·4
18:0	4·9	5·0	4·9
18:1	15·4	21·0	29·3
18:2	23·8	21·0	20·0
18:3	0·2	—	2·3
Ratio 16/18	1·0	1·1	0·77
Ratio 18:2/tot. 18	0·53	0·44	0·35
Sat./unsatur.	1·24	1·38	0·935

* Average of 3 determinations.

† Total lipids of cells grown at 55°, mineral medium was supplemented with galactose. Data from Kleinschmidt and McMahon.[10]

‡ Average value of (residue + total) fatty acids as calculated from the data of Allen *et al.*[18] Cells were obtained from Kettering Research Laboratories, Yellow Springs Ohio. Cultures were grown at 42–45° and aerated with 5% CO_2 in air.

differs in this respect from other algae[8,9] which exhibit the reverse order in the distribution of these fatty acids. The ratio $C_{18:2}$/total C_{18} acids is 0·53. A similar ratio was obtained[10] with *C. caldarium* harvested from Yellowstone hot spring (U.S.A.), as also shown in Table 3.

The molecular weights of the fatty acid methyl esters isolated by GLC were determined by MS. Further evidence of their structure was obtained by IR and NMR spectra.

DISCUSSION

It has been suggested that the composition of the algal lipids and sterols may serve as a tool in the solving of phylogenetic problems.[4,9,11,12] The position of the anomalous unicellular thermophilic alga *Cyanidium caldarium* is still in dispute. At one time or another it has been classified as being a cyanophyte, chlorophyte, rhodophyte, cryptophyte,[4,5] or even a symbiotic form between a blue–green alga and a primitive eulcaryotic cell.[13]

The analysis of the fatty acids of *Cyanidium* shows unusual features such as a wide range of acids, a number of odd-numbered acids and greater amounts of $C_{18:2}$ acid than usual. In these respects the *Cyanidium* fatty acids are different from those of other algae and higher plants. Blue–green,[8,11] green,[8] and brown[14] algae have a narrow range of fatty acids than the C_8–C_{20} observed in *Cyanidium*. On the other hand, the red algae[14] (rhodophytes) exhibit higher chains of fatty acids (up to C_{24}).

We have found that 19% of the lipid fraction consisted of free fatty acids. Miller[15]

[9] C. N. KENYON and R. Y. STANIER, *Nature, Lond.* **227**, 1164 (1970).
[10] M. G. KLEINSCHMIDT and V. A. MCMAHON, *Plant Physiol.* **46**, 286 (1970).
[11] R. W. HOLTON, H. H. BLECKER and T. S. STEVENS, *Science* **160**, 545 (1968).
[12] G. W. PATTERSON, *Lipids* **6**, 120 (1971).
[13] P. H. RAVEN, *Science* **169**, 641 (1970).
[14] E. KLENK, W. KNIPPRATH, D. EBERHAGEN and H. P. KOOF, *Z. Physiol. Chem.* **334**, 44 (1963).
[15] J. D. A. MILLER, *Physiology and Biochemistry of Algae* (edited by R. A. LEWIN), p. 357, Academic Press (1962).

stated that the algal FFAs represented 80% of the total lipids of *Nitzschia dosterium* (marine diatom), while other algae show a smaller FFA content.

The occurrence of odd-carbon fatty acids in *Cyanidium* might suggest certain phylogenetic relationships. Klenk *et al.*[14] reported traces of odd numbered C_{15} and C_{17} acids in some green algae, while C_{15}–C_{21} acids were detected mainly in red algae. Minute amounts of C_{17}, $C_{17:1}$ fatty acids were recently[10] reported to be present in *C. caldarium*. The odd-numbered acids in *Cyanidium* may of course, derive from the even-numbered higher homologs through decarboxylation. *Cyanidium* resembles most other algae in having a high degree of saturation in the C_{16} group and a relatively high degree of unsaturation in the C_{18} series. The upper range of the *Cyanidium* unsaturated fatty acids resembles that in some forms of the cyanophytes,[8,9,11] while other algae such as the chlorophytes,[8,14] rhodophytes,[14] or diatoms[16] synthesize $C_{18:4}$ to $C_{20:5}$ or even $C_{22:6}$ polyunsaturated fatty acids.

If one considers the group of the blue–green algae as a whole, the C_{16} acids are prominent, while the C_{18} acids are the major fatty acids in the green algae[8] as the C_{16} and C_{20} are in the red algae[14] and diatoms.[16] *Cyanidium* shows a ratio of total C_{16}/C_{18} (Table 3, column A), which lies between the values of the (freshwater and marine) green and red algae,[14] but according to recent data,[8,11] it is adjacent to several blue–green algae. The deviation of *Cyanidium* with respect to the high content of linoleic and oleic acid is noteworthy. Thermophiles and organisms cultured at elevated temperatures usually have a low unsaturated acids content (see ref. 10). The mesophilic species of fungi (25°) show[17] higher amounts of $C_{18:2}$ than $C_{18:1}$ acid, while the reverse is true for the analogous thermophilic genera (45°). In addition, these mesophilic species contained up to 18·5% linolenic acid, while the thermophilic grown cells of the same genera did not have appreciable amounts.[17] *Cyanidium* differs in that respect, since it was grown at elevated temperatures and nevertheless synthesized linoleic acid in an appreciable amount. Contrary to other algae and higher plants (see ref. 16) which contain major amounts of unsaturated C_{16}, $C_{18:3}$ and C_{20}-polyenoic acids,[8,9,11,14,16] *Cyanidium* contains only small quantities, if any, of these acids.

Recent studies by Kleinschmidt and McMahon[10] with *Cyanidium* have confirmed the general idea that environmental conditions (see refs. 4, 15, 17) affect the fatty acid composition of organisms. They[10] cultured *Cyanidium* at low (20°) and elevated (55°) temperatures and detected a 3-fold decrease of unsaturated/saturated ratios with the increase of temperature. For example, *Cyanidium*[10] may synthesize 30% linolenic acid only at 20°, while this acid was not detected at 55°. The total fatty acid[10] (Table 3, column B) of cells grown at 55° with the supplement of galactose and malt extract is quite similar to our data (Table 3, column A) obtained from cells grown on mineral medium and aerated with 5% CO_2 in air. Our data vary slightly from the analysis by Allen *et al.*[18] (Table 3, column C). They[18] obtained, with a different strain of *Cyanidium caldarium*, a higher content of oleic than linoleic acid; however, their ratio of $C_{18:1}$/total C_{18} is similar to our $C_{18:2}$/total C_{18} ratio. Thus the total ratio of saturation/unsaturation of the C_{16}, C_{18} fatty acid groups is quite similar in all analyses (Table 3).

Although the fatty acids of *Cyanidium* are somewhat similar to those of the blue–green algae, we are tempted to place this paradoxical alga closer to the red algae. Our present data agree with the earlier proposal[4] that *Cyanidium* is a transitional or bridge form and is to be placed between the cyanophytes (prokaryotes) and the eukaryotic red algae.

[16] M. KATES and B. E. VOLCANI, *Biochim. Biophys. Acta* **116**, 264 (1966).
[17] R. O. MUMMA, C. L. FERGUS and R. D. SEKURA, *Lipids* **5**, 100 (1970).
[18] C. F. ALLEN, P. GOOD and R. W. HOLTON, *Plant Physiol.* **46**, 748 (1970).

TABLE 4. *Cyanidium* NUTRIENT MEDIUM*

Compound	Concentration (mg/l.)	Level of element (mg/l.)
$(NH_4)_2SO_4$	$2{\cdot}6 \times 10^3$	552 N
KH_2PO_4	$0{\cdot}56 \times 10^3$	161 K; 128 P
$MgSO_4.7H_2O$	$0{\cdot}49 \times 10^3$	48·5 Mg
$CaCl_2.2H_2O$	$0{\cdot}15 \times 10^3$	40·3 Ca
Fe^{3+} (Sequestrene NaFe)	38·5	5·0 Fe
$MnCl_2.4H_2O$	3·6	1·0 Mn
$Na_2B_4O_7.10H_2O$	8·8	1·0 B
$ZnSO_4.7H_2O$	0·44	0·1 Zn
$CuCl_2.2H_2O$	0·10	0·04 Cu
$VSO_4.2H_2O$	0·60	0·02 V
$Na_2MoO_4.H_2O$	0·60	0·02 Mo

* The mineral solution was modified after Allen's composition (see refs. 2, 3), and was acidified with 50% H_2SO_4 to pH 2–3. All solutions were autoclaved and kept in sterile conditions during the culturing period.

The sterol composition of *Cyanidium* appears to agree with the above proposal. The results, obtained with alga culture under either 5% or pure CO_2 at 45–50°, show that *Cyanidium* sterol content resembles that of the rhodophytes by possessing cholesterol, the widely distributed sterol of the red algae[12] and campesterol which was suggested to be in that group.[12,19] Although *Cyanidium* contains also sitosterol and ergosterol which were reported[15] to be common in green algae, some red algae possess the former one too.[15] The tolerance of *Cyanidium* to acidity[1] and to pure CO_2[2,3] might be an adaptive feature, while thermophily was suggested elsewhere to be a primitive character, because living survivors of the very hot spring are mainly the prokaryotes (blue–green algae and bacteria). Thus *Cyanidium* should be considered a blue–green rhodophyte. Further evidence for this point can be obtained from the fine structure of this organism. Some of the cellular ultrastructures of *Cyanidium* resemble those of the red algae (Seckbach, unpublished). Based on morphological data, Hirose[20] also proposed *Cyanidium* to be a member of the Rhodophyta. Recent phylogenetic implications from pigment studies[21] also support this hypothesis.

EXPERIMENTAL

Algal culturing. The algal cells were grown in a modified mineral acid medium (pH 2–3) as described by Allen (see Table 4). The algae were cultured in 2-l. flasks, their suspension was agitated with magnetically driven stirring bars and kept at 45–50° during the growth period. Continuous illumination was provided by fluorescent tubes which supplied an intensity of about 4800 lx. We aerated the culture with 5% CO_2 in air which was prefiltered and humidified by passing it through water. After a few weeks of growth under the above conditions, the cells were harvested by centrifugation at 5000 rev/min for 10 min, and the pellets were washed in deionized water to remove traces of soluble nutrients. Then the cells were frozen and lyophilized for overnight or longer.

Extraction of lipids. Lyophilized algae (2·3 g) were extracted with $CHCl_3$–MeOH (2:1) in a Soxhlet for 7 hr. The extract was filtered and concentrated under reduced pressure, yielding 154 mg of a green viscous residue.

[19] A. ALCAIDE, M. BARBIER, P. POTIER, A. M. MARGUER and J. TESTE, *Phytochem.* **8**, 2301 (1969).
[20] H. HIROSE, *Bot. Mag. Tokyo* **71**, 347 (1958).
[21] D. J. CHAPMAN, W. J. COLE and H. W. SIEGELMAN, *Am. J. Bot.* **55**. 314 (1968).

Isolation of free fatty acids (FFA). The viscous residue was dissolved in $CHCl_3$ and extracted several times with 2% aq. NaOH. The aqueous layer was treated with 1 N HCl and extracted thoroughly with ether. The combined ethereal extracts were dried over $MgSO_4$ and evaporated to dryness, leaving 29 mg of fatty acids.

Saponification of the neutral fraction. Evaporation of $CHCl_3$ (from which the FFA had been extracted) left 98 mg of a neutral fraction, which was refluxed for 6 hr with 7% methanolic KOH. The solvent was removed *in vacuo*, water was added, and the residue extracted with ether. Evaporation of the ether left 33 mg of unsaponifiable fraction (USF). The aqueous layer was treated with 1 N HCl and extracted thoroughly with ether. The combined ethereal extracts were dried over $MgSO_4$ and evaporated to dryness, leaving 37 mg of fatty acids.

Isolation of sterols. The unsaponifiable fraction was dissolved in light petroleum and chromatographed on a silica gel column (height 15 cm, dia. 1·5 cm). The compounds were eluted with 40 ml of each of the following concentrations of light petroleum in benzene, 10, 25, 50% (v/v), followed by ether and 25% MeOH in ether, and finally with 20% MeOH in $CHCl_3$. The fractions (15 ml each) were evaporated to dryness. Examination of each fraction by qualitative TLC (on silica gel GF-254, developing with light petroleum–Et_2O–HOAc, 20:4:1, spraying with 50% H_2SO_4 and charring at 180°), revealed the presence of sterols at R_f 0·43 in fractions 15–17. These fractions were pooled and subjected to PLC (preparative layer chromatography), the zones of sterols were scraped off the plates and extracted with $CHCl_3$–Et_2O (1:1). The weight of the sterols was 1·7 mg.

TLC on plates of silica impregnated with silver nitrate. The plates were prepared as described by Ikan.[22] The samples of sterols in $CHCl_3$, were spotted on the plate and developed with $CHCl_3$. Observation under UV light revealed the presence of two fluorescent spots; one of them had the same R_f as ergosterol (0·15), and the other 0·17. Spraying with H_2SO_4 and charring revealed another spot at 0·22 (the phytosterols region).

GLC of sterols and fatty acids. A Packard Model 7400 gas chromatograph, equipped with an hydrogen flame ionization detector was used with a 6 ft × 0·25 in. glass column. For sterol analysis it was filled with 3% XE-60 on Gas Chrom Q, 100–120 mesh. The column was maintained at 245°, injector, 260° and the nitrogen flow 30 ml/min. For the methyl esters of fatty acids the column was packed with 15% of diethylene glycol succinate (DEGS) coated on Chromosorb W, 80–100 mesh. The column temperature was maintained at 120° and 190°. The esters were also analysed on a column packed with SE-30 5% on Gas Chrom Q, 100–120 mesh. The column was maintained at 170°. The identity of the compounds was established by comparison of the retention distances with those of the methyl esters of fatty acids and of sterols, and by co-injection with these standards.

Hydrogenation of unsaturated esters. In order to prove the presence of the unsaturated esters, 10 mg of the esters in 10 ml MeOH was hydrogenated catalytically in the presence of platinum oxide for 6 hr, and again examined by GLC.

Acknowledgements—We thank Miss Ora Selictar for her skilful assistance. Special appreciation is due to Professor E. D. Bergmann for his interest and encouragement.

[22] R. IKAN, *J. Chromatog.* **17**, 591 (1965).

Key Word Index—Cyanidium caldarium; alga; fatty acids; sterols; algal phylogeny.