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Effect of Growth Temperature on the Lipid Composition of *Cyanidium caldarium*

I. CLASS SEPARATION OF LIPIDS[1]

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ABSTRACT

***Cyanidium caldarium* was cultured at 20 and 55 C and harvested during exponential growth phase. Comparative lipid studies on each cell type show a decrease by one-half of the total lipid in cells grown at 55 C over cells grown at 20 C. While the distribution of lipid into each of five lipid classes was not influenced by high temperature (55 C), the degree of unsaturation was greatly affected. Ratios of unsaturated to saturated fatty acids in these cells decreased 3-fold with increased temperature in the growth environment. Cells cultured at 20 C contained 30% of their fatty acids as linolenic while this fatty acid could not be detected in cells cultured at 55 C.**

In order to develop and function properly, organisms must be capable of adjusting to their environment. Of the environmental factors, temperature is an important parameter determining the lethal ranges of survival. Since temperature affects all biological reactions, the over-all effect of temperature is rather complex; therefore, this investigation is limited to a narrower set of biological reactions: lipid metabolism.

It has been well documented that an increase in temperature causes a corresponding decrease in the amount of unsaturated fatty acids (3, 6, 8, 10, 13). Farkas and Herodek (6) investigated the effect of temperature on lipids in crustacean plankton. The melting point of lipids from copepods remained somewhat lower than the water temperature during a yearly cycle. Utilizing iodine values of fat, these workers demonstrated that the changes in melting point were due to variations in the degree of unsaturation in the long chain fatty acids. Holton *et al.* (10) investigated the effect of growth temperature on the fatty acid composition of the blue-green alga, *Anacystis nidulans*. Cell density, illumination, aeration, medium composition, and growth rate were carefully controlled. Their results showed that at all temperatures palmitic acid (16:0) and a hexadecenoic acid (16:1), presumed to be Δ^9-palmitoleic, comprised approximately 90% of the total fatty acids, but the ratio of the 16:1 to 16:0 decreased with increasing temperature. The approximate ratio of 1 between total unsaturated and saturated fatty acids remained unchanged for the alga grown at 26, 32, and 35 C, but at 41 C the ratio changed to 0.7, indicating an increase in the saturation of the fatty acids.

Cyanidium caldarium is a thermophilic eucaryote which can grow at temperatures below 20 C and has been reported growing at temperatures up to 56 C (5). This acid-tolerant and heat-tolerant unicellular alga is a regular member of the microflora found in acidic hot springs throughout the world (2, 4). Electron microscopic investigations show a highly differentiated cell containing: a thick cell wall, plasmalemma, ground matrix, endoplasmic reticulum, chloroplast, mitochondria, nucleus, and vacuoles (14). Cell division occurs by formation of four daughter cells within the mother cell wall, and after rupturing the four daughter cells are released. These morphological characteristics make it quite suitable for studying the effect of temperature on lipid composition and its possible role in the molecular mechanism of thermophily.

METHODS AND MATERIALS

Culturing of Algal Cells. A thermophilic strain of *C. caldarium* was collected from the Amphitheater Springs in Yellowstone National Park. At this site the alga was growing at 53 C and pH 2.8. Pure cultures were obtained by treatment with penicillin, streptomycin, and chloramphenicol. With the medium described by Ascione *et al.* (1) *C. caldarium* was grown in the laboratory at two different temperatures, 20 and 55 C, but at a constant pH of 2.2. Batch cultures were grown in 12-liter Pyrex carboys with vigorous aeration in constant temperature water baths with a thin layer of mineral oil layered on top of the water in the bath to retard evaporation. A continuous light source of 300 ft-c was supplied by 15-w fluorescent lamps. Growth of the algal suspensions was assayed by absorbance measurements at 550 nm. When growth reached exponential phase (0.64 OD), the cells were harvested with a Sharples centrifuge at 50,000*g*. The resulting paste of cells was lyophilized, and the dry weight was obtained.

Lipid Extraction and Separation. The lyophilized cells were homogenized in chloroform with a Duall tissue grinder and transferred to a Soxhlet extraction apparatus. Extraction was continued until the chloroform extract became colorless (10–12 hr). The extract was evaporated on a rotary evaporator previously flushed with nitrogen and separated into nonlipid and lipid fractions by Sephadex chromatography (20). The lipid fraction was taken to dryness and dissolved in *n*-hexane-diethyl ether (99:1), and an aliquot (10 mg) was placed on a silicic acid column for separation. Separation was achieved by modification of the methods described by Lis *et al.* (12) and Rouser *et al.* (18). Five fractions were obtained from the following series of elutions: 15 ml of *n*-hexane–diethyl ether (99:1); 15 ml of *n*-hexane–diethyl ether (96:4); 15 ml of *n*-hexane–diethyl ether (50:50);

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75 ml of acetone; and 15 ml of methanol; hereafter these are referred to as fractions 1, 2, 3, 4, and 5, respectively. Typical lipids found in these fractions are, respectively, waxes, hydrocarbons, and sterol esters; triglycerides; free sterols, free fatty acids, monoglycerides, and diglycerides; glycolipids and sulfolipids; and phospholipids. The dry weight of each fraction was determined by gravimetric analysis.

Identification and Analysis of Fatty Acids. Fatty acid analyses were carried out on the total lipid, the fractions eluted from the silicic acid column, and the major spots on the thin layer chromatograms. In all cases the lipids were saponified, and the methyl esters of the separated fatty acids were prepared according to Morrison and Smith (15). Fatty acid methyl esters were analyzed with the Aerograph model 600-D gas-liquid chromatograph. Ten per cent diethylene glycol succinate on Anakrom ABS, 80/90 mesh, was used as the stationary phase with nitrogen (50 ml/min) as the mobile phase at a column temperature of 180 C. A standard curve was obtained by injecting quantitative standards (Applied Science), and the relative peak areas were measured as peak height × retention time (11).

For infrared assay of the presence of *cis* and *trans* configurations in the unsaturated fatty acids, the methyl esters of such acids were collected after gas chromatographic separation (Aerograph model A-90-P with flame ionization detector and equipped with a stream splitter [40:1]). The collected esters were then concentrated by a KBr Wick-Stick (Harshaw Chemical Co.) prior to infrared assay (Perkin-Elmer, model 221, fitted with a beam condenser).

An aliquot of fatty acid methyl esters was placed on a thin layer plate impregnated with silver nitrate (19). Separation according to the number of double bonds was achieved by developing in *n*-hexane-diethyl ether (90:10). The areas were scraped from the plate, extracted, and subjected to ozonolysis. The resulting aldehydes and aldesters were identified by gas-liquid chromatography. The column employed for separation of aldehydes was the SE-30 while the diethylene glycol succinate column was employed for the separation of aldesters. A programmed temperature series from 60 to 190 C at 5 C/min (16) was used in both separations.

RESULTS

Since environmental growth conditions are known to affect both qualitative and quantitative values in lipid composition (5, 13, 17), all conditions were kept essentially constant while only temperature was varied. Typical growth curves of *C. caldarium* at 20 and 55 C are presented in Figure 1. The specific growth rates (log 10 units/day) were 0.08 and 0.20 for cells grown at 20 and 55 C, respectively. Apparently, specific growth rates do not affect fatty acid compositions in other microorganisms (5, 10, 13), and specific growth rates might not be an important factor in influencing the lipid composition of this alga. While cells grown at 55 C had a linear growth curve, those cultured at 20 C showed a discontinuous curve (Fig. 1) similar to that reported for *C. caldarium* at 25 C (1). When cells grown at 20 C were transferred to 55 C, and vice versa, a short lag period was observed before cells entered the exponential phase of growth. It was further noted that cells grown at 20 C appeared dark green in color while those grown at 55 C were yellowish green.

Data compiled in Table I show that the lipid content of the cells cultured at 20 C is greater by more than 2-fold than that of those cells grown at 55 C. The total lipid from the cells cultured at 20 C varied little in four replications (±0.5 mg/g dry wt) while greater variation (±5.0 mg/g dry wt) was observed in those cells grown at 55 C. Separation of the total lipids from the cells grown at the two temperatures into five lipid classes by silicic acid column chromatography did not result in any apparent change in their over-all distributions (Table I), for the total lipid of the two cell types is distributed nearly equally within each class.

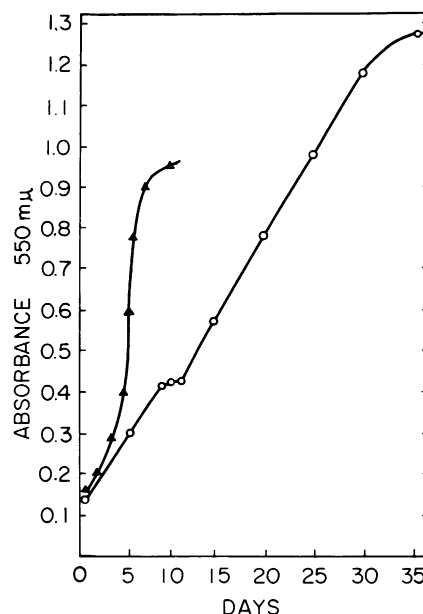

FIG. 1. Growth curves of *C. caldarium* at 20 (O) and 55 (△).

Table I. *Total Lipid and Class Separation of Lipids from Cyanidium caldarium*

These data are the average of four experiments.

	Total Lipids		Total Fatty Acids	
	20 C	55 C	20 C	55 C
	mg/g dry wt			
Total lipid	50.0	20.0	21.0	7.2
	% of total			
Lipid fractions				
1	9	10	7	3
2	12	8	21	11
3	15	22	14	17
4	25	22	13	21
5	36	38	45	48

Variation in total fatty acids from each cell type can be realized (Table I) if these values are expressed in mg/g dry weight, but if the total fatty acid values are expressed as a percentage of the total lipid, then less disparity exists with cells grown at 20 and 55 C (values are 42 and 36%, respectively). Similarly, if comparisons are made between cell types for the fatty acids in each of the five fractions, these distributions are not very dissimilar (Table I). Therefore, it would appear that only the total lipid content is varied in cells grown at the two temperatures, while percentages of lipid distributed into the five lipid classes as well as their resulting fatty acids remain fairly constant.

Preliminary identification of fatty acid methyl esters was accomplished with gas-liquid chromatography by comparing the retention times of known methyl esters. Infrared spectral studies on the unsaturated fatty acid methyl esters did not show strong absorption in the region of 10.00 to 10.34 μ as expected for the *trans* configuration (7). Methyl elaidate was subjected to the same procedure as the algal fatty acids, and a strong band with a maximum at 10.34 μ was observed, as expected. This evidence was used to establish that the unsaturated fatty acids were all in the *cis* configuration.

Table II. *Major Fatty Acids of Cyanidium caldarium*
These data are the average of two experiments.

	Fatty Acids[1]									
	20 C					55 C				
	16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
	%	%	%	%	%	%	%	%	%	%
Total	18	7	34	11	30	53	5	21	21	0
Lipid fraction										
1	5	5	55	14	22	17	8	44	30	0
2	8	7	53	11	22	28	5	45	22	0
3	22	2	22	14	40	39	6	34	21	0
4	21	2	8	12	57	52	2	28	17	0
5	21	11	33	10	24	65	7	6	23	0

[1] Minute amounts of 12:0, 14:0, 16:1, 17:0, 17:1 also present.

Table III. *Ratios of Unsaturated to Saturated Fatty Acids in Lipid Fractions and Polar Lipid Components*
These data are the average of two experiments.

	Ratio of Unsaturated to Saturated Fatty Acids	
	20 C	55 C
Total	3.0	0.9
Lipid fractions		
1	10.0	3.0
2	6.2	2.0
3	3.2	1.2
4	3.4	2.1
5	2.1	0.4

The monoenoic, dienoic, and trienoic fatty acid methyl esters were separated by argentation thin layer chromatography and ozonized, and their major products were identified. The products were: for monoenoic fatty acid methyl esters, nonanal and methyl azelaaldehydate; for dienoic fatty acid methyl esters, hexanal and methyl azelaaldehydate; and for trienoic fatty acids methyl esters, propanal and methyl azelaaldehydate. Therefore, the principal unsaturated fatty acids present in cells grown at 20 C were *cis*-9-octadecenoic, *cis,cis*-9,12-octadecadienoic, and *cis,cis,cis*-9,12,15-octadecatrienoic.

Fatty acid analysis demonstrated that the major fatty acids found in *C. caldarium* were palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). Lauric, myristic, heptadecanoic, and heptadecenoic acids comprised less than 3% of the total fatty acids present. Table II shows that the total lipids in the cells grown at 55 C lack detectable amounts of linolenic acid while the total lipids of cells grown at 20 C contain appreciable levels of this acid. While linolenic acid is lacking, palmitic comprises 53% of the fatty acids at the higher temperature. After fractionation with silicic acid columns, the ratios of the individual fatty acids change (Table II) within each of the five fractions. In the cells grown at 55 C the amount of palmitic acid increases as the polarity of the lipid fractions increases. Oleic acid decreases in the same order; therefore, the increase of palmitic acid seems to occur at the expense of oleic acid. Although the cells grown at 20 C show some trend in this manner, it is not so pronounced as in cells grown at the higher temperature.

Table III expresses the fatty acid compositions in terms of ratios of the unsaturated to the saturated. In the total lipid the ratio was 3-fold greater in the cells grown at the lower temperature. The first three fractions show similar ratios in the cells grown at the lower temperature. However, in fraction 4 the ratio is less than 2-fold greater while in fraction 5 the ratio is greater than 5-fold in the cells grown at the lower temperature. The ratios of total amount of unsaturated fatty acids are higher at both temperatures for the less polar fractions (fractions 1–3), but these ratios decrease as the polarity of lipid components increases (fractions 4–5).

DISCUSSION

The data show that the total lipid in terms of mg/g dry weight was greater in the cells grown at 20 C. Since after fractionation there was no gross change in the over-all composition, it seems more likely that some nonlipid cell constituent is increased at 55 C rather than there being an increase in lipid. This is also suggested by an increase in the cell wall thickness at 55 C (unpublished observations), which would result in an apparent decrease in lipid composition when values are expressed on a dry weight basis.

The ratio of unsaturated to saturated fatty acids in the total lipid as well as in the five fractions decreased at the higher temperature; this finding agrees with earlier work (6, 8, 10, 13). Though the concentration of linolenate was expected to be diminished, it was surprising to find it absent at 55 C. It could be that the enzyme which converts linoleate to linolenate is being rapidly denatured, or, on the other hand, the solubility of oxygen, which is decreased at high temperature, may influence the rate of linolenate synthesis (11).

With an increase in temperature the solubility of carbon dioxide is decreased. Since the solubility of carbon dioxide is very low at low pH and further decreased at high temperature, the cells grown at 55 C were probably forced to utilize the galactose present in the media as an energy source rather than carrying on photosynthesis. Therefore, it is possible that the observed effect of temperature on the lipid composition in *C. caldarium* is an indirect one caused by changes in the solubility of atmospheric gases at the two temperatures studied. Current investigations are under way to study this possibility further.

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