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The Effects of Nitrogen Deficiency on Pigments and Lipids of Cyanobacteria

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ABSTRACT

In contrast to what happens in higher plants and eukaryotic algae, a nitrogen deficiency during growth causes a change in pigment composition but no significant changes in whole cell lipid and fatty acid composition of the two Cyanobacteria, *Pseudanabaena* sp. (strain M2) and *Oscillatoria splendida* (strain L3). Nitrogen deficiency does not affect the cellular content in chlorophyll *a*, but it causes a selective loss in phycobiliproteins; carotenoid content increases with phycocyanin depletion. The major cellular lipids in both Cyanobacteria studied are monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol, and phosphatidylglycerol. The fatty acid composition is particularly interesting as both these filamentous Oscillatoriaceae show important contents in α- and γ-linolenic (18:3) and parinaric (18:4) acids. This seems to be very unusual in Cyanobacteria.

The blue-green algae (Cyanobacteria) are prokaryotic organisms which perform an eukaryotic photosynthesis (10, 16). They share, therefore, with bacteria, their prokaryotic nature. At the same time, their photosynthetic metabolism and the organization of their photosynthetic apparatus somewhat resemble those of eukaryotic algae and vascular plants (10, 17). Unlike eukaryotic algae, Cyanobacteria possess only one kind of Chl, Chl *a*; the other photosynthetic pigments of Cyanobacteria are various phycobiliproteins (*c*-phycoerythrin, *c*-phycocyanin, and allophycocyanin) and carotenoids (β-carotene, echinenone, and myxoxanthophyll) (9, 10).

The major cellular lipids of Cyanobacteria include the three glycolipids characteristic of chloroplasts, namely MGDG[1], DGDG, and SQDG, as well as the phospholipid PG (19), but they do not synthesize phosphatidyl choline, phosphatidyl ethanolamine, or phosphatidyl inositol, which are found in all other classes of algae (12, 13). In contrast, the predominant lipids of bacteria are phospholipids and, in some cases, MGDG (7, 12, 13). Finally, while the chloroplast fatty acids of eukaryotic algae and vascular plants are essentially polyenoic acids, bacteria contain almost exclusively saturated and monounsaturated fatty acids in their cellular lipids (12, 13). Especially interesting is the fact that few blue-green algae so far studied seem to have polyunsaturated fatty acids whose degree of unsaturation is higher than 3, and even those containing the γ-linolenic acid are very rare (12–15, 18, 26).

Studies on the effects of nitrogen deficiency on Cyanobacteria have rarely been made, to our knowledge, and have been dealt exclusively with the effects of such deficiency on the integrity of the photosynthetic apparatus. Allen and Smith, in 1969, showed that nitrogen deficiency causes a selective loss of phycocyanin from *Anacystis nidulans* (1, 16). From various temperature dependent and light dependent studies, it seems that deviations toward phycocyanin deficiency are accompanied by a decrease in unsaturated fatty acids (8, 11, 21–23). The lipid content generally decreases parallel to decreasing pigment content, especially that of phycocyanin and, in some cases, membrane lipids are turned to storage lipids (12).

The purpose of the following work is to determine whether a nitrogen deficiency during growth affects the pigment composition and cellular organization, and to see if this deficiency affects in some way the lipid and fatty acid composition of the filamentous Cyanobacteria *Pseudanabaena* sp. (strain M2) and *Oscillatoria splendida* (strain L3).

MATERIALS AND METHODS

Organisms and Growth Conditions. *Pseudanabaena* sp. (strain M2 from the Culture Collection of the Laboratoire de Botanique, Ecole Normale Supérieure, Paris) and *Oscillatoria splendida* (strain L3 from the same Culture Collection) were grown at 20°C under 12 h light (50 $\mu E\ m^{-2}\ s^{-1}$) in culture medium 'Z' pH 6.8 (25). Cells were analyzed in their late exponential growth phase (1 week).

Nitrogen deficiency was obtained by diluting cells in their late growth phase in modified Z medium in which NaCl replaced $NaNO_3$ and $CaCl_2$, $2H_2O$ replaced $Ca(NO_3)_2$, $4H_2O$. Cells were analyzed after 5 weeks deficiency. Light intensity was augmented up to 100 $\mu E\ m^{-2}\ s^{-1}$.

Cellular Preparation for Analytical Procedures. Cells were centrifuged and washed twice in 30 mM K-phosphate, pH 7, then resuspended in 30 mM K-phosphate-sucrose buffer, pH 6.8, and ruptured at 8000 p.s.i. with a French Pressure Cell Press. The homogenate, on which all further analytical measures were performed, was then suspended in 10 mM Hepes buffer, pH 7.

Pigment and Protein Assay. Chl *a* was extracted with 90% methanol and determined from its *A* at 665 nm according to Arnon (2).

Carotenoids were extracted by ether and ethanol following saponification. Crude extracts were analyzed spectrometrically, and the extinction coefficient $E^{1\%}_{1cm}$ 2500 was used, at the absorption maximum 450 nm.

Phycobiliproteins were extracted by centrifugation at 100,000*g* for 2 h. Absorption maxima of the supernatant were determined spectrometrically and a quantitative estimation was done according to Bennett and Bogorad (3).

[1] Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TFA, total fatty acids; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol; PG, phosphatidylglycerol; NL, neutral lipids; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 18:4, parinaric acid.

Table I. *Dry Weight, Pigment, and Protein Composition of Normal and Nitrogen Deficient Cells of Both Strains (Pseudanabaena sp. and O. splendida)*

Strain	Protein	Dry Weight	Chl *a*	Carotenoids	Phycobiliproteins
			μg/ml of cell culture		
Pseudanabaena sp.					
Normal	377	524	9.18	78.76	122.13
N_2 deficient	184	1226	8.84	120.50	0.61
O. splendida					
Normal	216	642	10.05	86.70	55.51
N_2 deficient	190	1454	10.03	154.28	2.98

Protein content was estimated with Bio-Rad Protein Assay using BSA as a standard.

Absorption Spectra. Absorption spectra were obtained on an Aminco dual wavelength spectrophotometer. Low temperature absorption spectra were measured according to Bennoun and Jupin (4).

Electron Microscopy. Samples were fixed by glutaraldehyde 2% (buffered with 0.1 M phosphate during 3 to 4 h at 20°C, then rinsed and postfixed with osmium tetroxide 1%, in the same buffer, for 16 h at 4°C. Cells were washed with water and treated with uranyl acetate (1% aqueous solution) for 1 to 2 h, at room temperature. Samples were dehydrated by ethanol and then embedded in Spurr low viscosity medium (24). Sections were contrasted by aqueous uranyl acetate and lead citrate and examined using a Philips M300 electron microscope.

Lipid Extraction and Analysis. Total lipids were extracted according to Bligh and Dyer (5) with a mixture of methanol-chloroform-water (final proportions 1:1:0.9, v/v). The chloroform phase was then removed and evaporated to dryness under N_2, at room temperature. The dry extracts were solved in a known volume of ethanol-benzene (1:4, v/v) and stored under N_2 at −20°C.

Individual galactolipids and phospholipids were separated by TLC on silica gel plates (Merck) using chloroform-methanol-acetic acid-water (85:15:8:2.5, v/v) and located by spraying with 0.01% Primuline in acetone-water (80:20, v/v), then visualized under UV light.

Fatty Acid Analysis. Fatty acid methyl esters of the total lipids and of individual lipids were prepared with 14% boron trifluoride in methanol or according to Carreau and Dubacq (6). The methyl esters were extracted using petroleum ether as solvent. GLC was performed on a silica capillary column (25 m long) coated with 20 M PEG. Helium was the carrier gas. The quantitative internal standard was margaric acid, C17:0.

The degree of unsaturation of the fatty acids in C18 was also determined by TLC on silica gel plates (Merck) previously treated for 30 s by a $AgNO_3$ solution (8 g $AgNO_3$ in 90% ethanol), using petroleum ether-diethylether-acetic acid (70:30:0.4, v/v) as solvent. After separation on the $AgNO_3$ silica gel, the different bands, which contained fatty acid methyl esters separated according to their double bond number, were extracted with petroleum ether and analyzed by GLC as described above.

RESULTS

Pseudanabaena sp. (strain M2) and *O. splendida* (strain L3) were grown in normal and in nitrogen deficient media. Under these conditions, phycobilin content decreased sharply, as expected, in nitrogen deficient cells of both strains, as shown in Table I. Chl content did not change with nitrogen deficiency in *O. splendida* cells and showed only a slight decrease in *Pseudanabaena* sp. deficient cells compared to normal cells. As for carotenoids, an increase (about a twofold increase) was observed in nitrogen deficient cells compared to normal cells. Dry weight shows a twofold increase in nitrogen deficient cells. This is most probably due to the production of cellular polyphosphates and glycogen in response to the nitrogen deficiency (see "Discussion"). The Chl to dry weight ratio is thus 0.016 in *O. splendida* normal cells and 0.007 in *O. splendida* nitrogen deficient cells; in *Pseudanabaena* these ratios are 0.018 in normal cells and 0.007 in deficient cells. Carotenoid to Chl ratios were as follows: 8.58 for normal and 13.81 for deficient *Pseudanabaena* cells, and 8.63 and 15.38 for *O. splendida* normal and deficient cells, respectively.

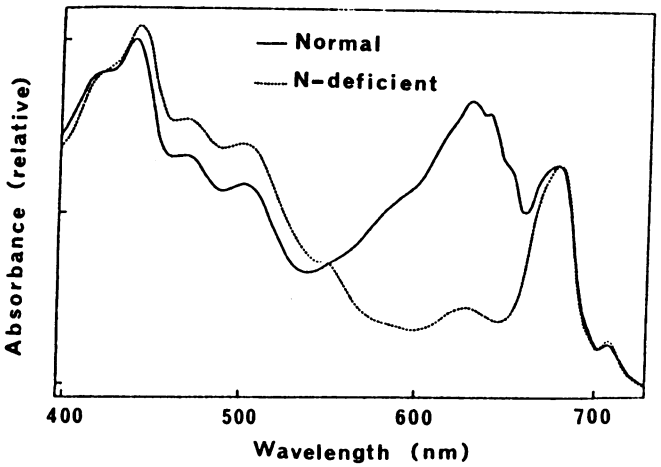

FIG. 1. 77 K Absorption spectra (from 400 to 730 nm) of normal and nitrogen deficient *Pseudanabaena* sp. cells. (——) Normal cells; (· · · · ·) deficient cells.

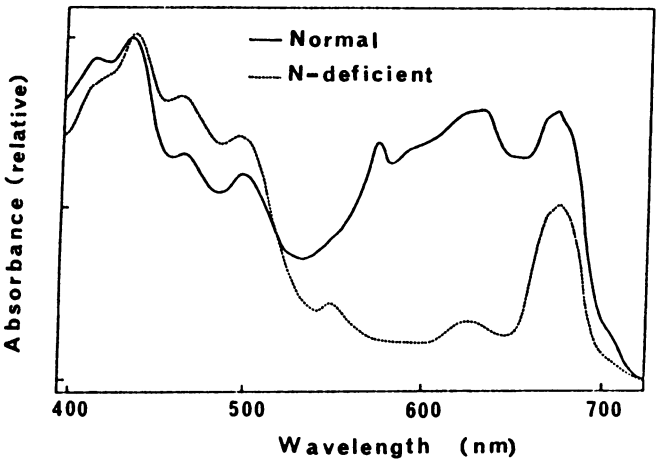

FIG. 2. 77 K Absorption spectra (from 400 to 730 nm) of normal and nitrogen deficient *O. splendida* cells. (——) Normal cells; (· · · · ·) deficient cells.

Protein content decreased in nitrogen deficient cells of both strains, as shown in Table I.

Low temperature, 77 K, absorption spectra are presented in

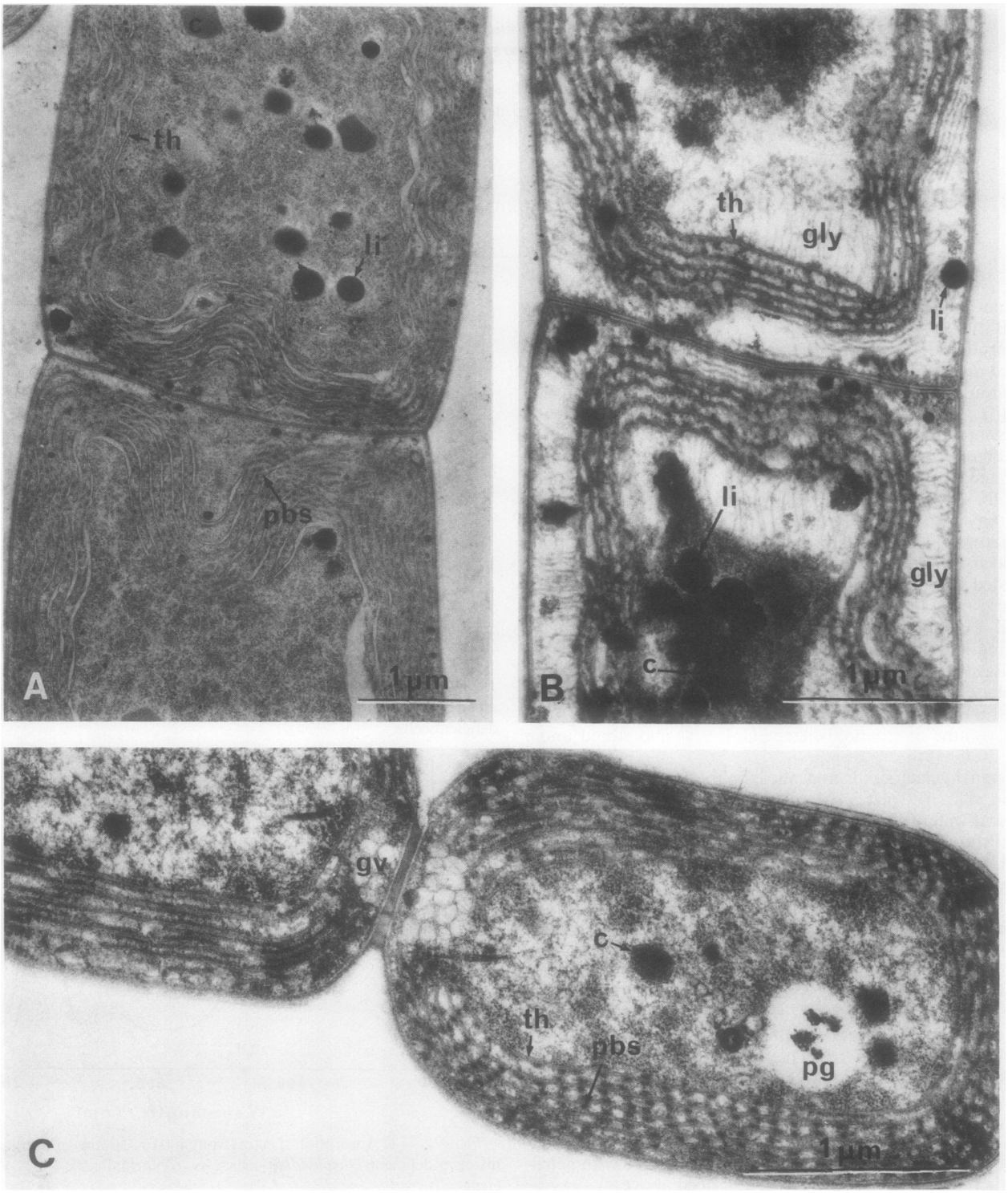

FIG. 3. A, Electron micrograph of a normal *O. splendida* cell. B, Electron micrograph of a nitrogen deficient *O. splendida* cell. C, Electron micrograph of a normal *Pseudanabaena* sp. cell. th, thylakoids; pbs, phycobilisomes; c, polyhedral bodies (carboxysomes); li, lipid inclusions; pg, polyphosphate granules; gly, glycogen; gv, gas vacuoles.

Figures 1 and 2. Chl *a* maxima at 437 nm and at 677 to 682 nm were observed in *Pseudanabaena* normal as well as deficient cells. Nitrogen deficient cells also present a peak at 626 nm, while normal cells present a peak at 707 nm due to a Chl *a* long wave absorption species. Carotenoids absorb at 468 nm and at 502 nm in normal *Pseudanabaena* sp. cells, but these peaks are slightly shifted toward shorter wavelengths (466 nm, 500 nm) in deficient cells. The latter also present a carotenoid absorption maximum at 548 nm. Only normal cells contain phycobiliproteins, which absorb from 582 to 651 nm. These two spectra (Fig. 1) have been adjusted at their 677 to 682 nm peak.

The same spectra can be observed for *O. splendida* (Fig. 2) with very slight but specific changes in maxima. Normal cells show Chl *a* absorption maxima at 436 nm and 676 nm, as well as a Chl *a* long wave absorption peak at 705 nm. Nitrogen deficient cells also present the long wave and the 676 nm peaks,

Table II. *Percent Lipid Composition of Pseudanabaena and O. splendida Normal and Deficient Cells*

Results presented are the mean values from 8 independent analyses.

Lipid	*Pseudanabaena* sp.		*O. splendida*	
	Normal	Deficient	Normal	Deficient
	%		%	
MGDG	50	45	54	53
DGDG	22	26	24	30
SQDG	12	11	4	5
PG	11	14	12	10
NL	5	4	6	2

together with a peak at 626 nm, and an absorption maximum at 447 nm instead of 436 nm. Carotenoids absorb at 465 nm and 497 nm (in deficient cells) or 500 nm (in normal cells), with a peak at 548 nm in nitrogen deficient cells. As in *Pseudanabaena*, only normal *O. splendida* cells have phycobiliproteins. These show three major peaks: the first one at 575 nm due to phycoerythrocyanin (PEC), then a peak at 627 nm due to phycocyanin (PC), and a peak at 650 nm due to allophycocyanin (APC).

Figure 3A presents an electron micrograph of a normal *O. splendida* cell. The cell wall is well visualized and distinguishable layers can be found. Thylakoids are regularly distributed near the cell wall, although some invaginations do take place. Many phycobilisomes are associated with thylakoids. A few polyhedral bodies (carboxysomes) are randomly dispersed within the cell and some lipid inclusions are found toward the center of the cell. Polyphosphates are also present in small amounts.

A normal *Pseudanabaena* sp. cell is shown in Figure 3C. The cell wall is well visualized and thylakoids with associated phycobilisomes are regularly distributed at the cell periphery, giving a clearly defined nucleoplasmic region where a few polyhedral bodies and lipid granules as well as polyphosphates can be seen. Gas vacuoles are found at one of the cell poles.

Nitrogen deficiency of *O. splendida* cultures induced the main differences (Fig. 3B), namely, a slight loss or disruption of thylakoids and a total loss of phycobilisomes (granular inclusions seen close to thylakoids that are probably glycogen). Nitrogen deficiency caused an enormous increase in the content of cellular glycogen which pushed lipid inclusions and carboxysomes toward the center of the cell.

Very similar changes occurred in *Pseudanabaena* sp. nitrogen deficient cells (not shown).

Total lipid composition of both types of cells (normally grown and nitrogen deficient) of both strains studied is shown in Table II.

Qualitatively, thus, as seen from Table II, the total acyl lipids of both types of cells of either of the two Cyanobacteria are identical and, notwithstanding some slight variations, the quantitative composition of both types of cells in major lipids also seems to be relatively similar. By far, MGDG is the major lipid in these cells. The MGDG to DGDG ratio is 2.27 in *Pseudanabaena* sp. normal cells and 1.73 in nitrogen deficient *Pseudanabaena* sp. cells. Comparable ratios are found in *O. splendida* (2.25 and 1.76 in normal and deficient cells, respectively). The DGDG content of nitrogen deficient cells of both algae is slightly higher than that of normal cells, 4% in *Pseudanabaena* and 6% in *O. splendida*. In *Pseudanabaena* sp. PG is also slightly more abundant in nitrogen deficient cells, whereas the galactolipid SQDG is approximately in the same percentage in both normal and deficient cells. In *O. splendida*, however, SQDG content is low in either normal and nitrogen deficient cells and, in contrast to what happens in *Pseudanabaena* sp., nitrogen deficiency slightly decreases PG level in *O. splendida* cells. Neutral lipid content is relatively low; it remains constant in *Pseudanabaena* sp. cells with nitrogen deficiency, but it decreases in *O. splendida* deficient cells (a 4% decrease).

The fatty acid compositions of the total lipid extracts and of the major lipids of both types of cells are shown in Tables III and IV, for *Pseudanabaena* sp. and *O. splendida*, respectively.

The quantitative fatty acid composition of total lipid extracts and of each acyl lipid of both algae does not change considerably with nitrogen deficiency. Both types of cells contain, in all their lipid classes, essentially palmitic acid (16:0), which, together with palmitoleic acid (16:1), account for about 50% of total fatty acid composition in all analyses. This fits very well with the classical data indicating that in Cyanobacteria all the lipids are 1.C18-2.C16 glycerolipids. Total extracts as well as the two major glycolipids, MGDG and DGDG, contain an important amount of the highly polyunsaturated parinaric acid (18:4). Yet, some important differences exist between the two strains studied. In fact, while in *Pseudanabaena* sp. normal cell MGDG and DGDG

Table III. *Fatty Acid Composition of Lipids from Normal and N_2 Deficient Pseudanabaena sp. Cells*

Results presented are the mean values from 16 independent analyses. t = traces (lower than 1%).

Lipid and Culture	Fatty Acid							
	16:0	16:1cis	18:0	18:1	18:2	18:3γ	18:3	18:4
Total extract				%				
Normal	40	10	1	1	8	10	7	23
Deficient	40	8	1	2	10	15	5	20
MGDG								
Normal	43	9	1	1	3	13	1	29
Deficient	42	8	1	1	3	20	1	24
DGDG								
Normal	34	14	2	3	6	14	3	24
Deficient	38	12	2	1	7	15	2	23
SQDG								
Normal	47	4	6	7	12	t	24	t
Deficient	49	4	2	3	15	2	21	2
PG								
Normal	46	6	2	6	21	3	14	3
Deficient	48	7	5	3	23	2	9	3
NL								
Normal	38	18	6	23	6	3	1	3
Deficient	35	20	13	22	4	1	t	3

Table IV. *Fatty Acid Composition of Lipids from Normal and N_2 Deficient O. splendida Cells*
Results presented are the mean values from 16 independent analyses. t = traces (lower than 1%).

Lipid and Culture	Fatty Acid							
	16:0	16:1cis	18:0	18:1	18:2	18:3γ	18:3	18:4
Total extract				%				
Normal	46	2	1	2	4	4	7	32
Deficient	50	3	t	4	3	2	6	32
MGDG								
Normal	47	2	t	1	3	5	3	39
Deficient	48	2	t	2	2	3	4	39
DGDG								
Normal	44	3	1	1	1	2	1	46
Deficient	48	3	1	3	3	2	4	36
SQDG								
Normal	45	2	8	18	7	1	14	4
Deficient	47	3	15	15	5	1	9	5
PG								
Normal	45	2	2	6	7	t	36	2
Deficient	43	4	2	10	4	t	18	9
NL								
Normal	60	8	9	15	3	t	1	4
Deficient	61	5	5	11	3	1	2	9

are rich in parinaric (29% in MGDG) and γ-linolenic acid (14% in DGDG), *O. splendida* cells have a higher parinaric acid content (up to 46% in DGDG) but a lower γ-linolenic acid percentage in all lipid classes. On the other hand, *Pseudanabaena* sp. cellular SQDG is rich in α-linolenic and in linoleic acids (24 and 12%, respectively, in normal cells), whereas *O. splendida* SQDG is mainly enriched in α-linolenic and oleic acids (14 and 18%, respectively, in normal cells). Finally, while in *Pseudanabaena* sp. PG is essentially rich in both linoleic and α-linolenic acids, in *O. splendida* this lipid is enriched in α-linolenic acid only, with a rather low content in linoleic acid. In contrast to what happens with galactolipids, in both PG and SQDG of these two strains of Cyanobacteria γ-linolenic and parinaric acids account for a very low percentage.

DISCUSSION

In plants, deficiencies of nitrogen and minerals will lead to a decline in nutritional status, metabolism, and therefore, lipid synthesis. In general, the largest effects are on fatty acid desaturation and the level of polyunsaturated fatty acids (12, 13). In bacteria, the ionic composition of the growth medium generally has little effect on the lipid composition unless there is a very marked deficiency. Still, in those bacteria that facultatively fix nitrogen there may be large changes in lipid metabolism depending upon the nitrogen source (12).

In the two Cyanobacteria studied (strain M2 of *Pseudanabaena* sp. and strain L3 of *O. splendida*) nitrogen deficiency during growth causes a complete loss of phycocyanin, with no considerable change in the Chl *a* content. This is in agreement with the results of Allen and Smith (1) concerning *Anacystis nidulans*. The decrease in the Chl to dry weight ratio in nitrogen deficient cells observed in our results is most probably due to the considerable production of glycogen and polyphosphates in response to the deficiency (as proved by cytochemical test), thus increasing the dry weight of deficient cells compared to normal cells. The carotenoids to Chl *a* ratio increased with nitrogen deficiency; this agrees with the results of Datz and Dohler (8) concerning *Synechococcus*, and the results of Oquist (20), where a higher carotenoid to Chl *a* proportion is observed in the absorption spectra of phycocyanin free photosynthetic lamellae from *A. nidulans*.

In terms of their lipid and fatty acid composition, these two Cyanobacteria strains do not seem to be particularly affected by nitrogen deficiency. The major acyl lipids found were MGDG, DGDG, SQDG, and PG. As in chloroplasts, the galactolipids are the main lipid components. But, in contrast to what has normally been reported by other authors (12), nitrogen deficiency did not increase the neutral lipid content of the cells. In *Pseudanabaena* sp. nitrogen deficiency decreased the percent composition in cellular galactolipids, with a corresponding increase in the phospholipid PG. The inverse was true in *O. splendida*, with a 2% decrease in PG content in response to the deficiency. In plants (12), nitrogen deficiency decreases synthesis of PG, phosphatidylcholine (PC), and phosphatidylethanolamine (PE). Specific answers to nitrogen deficiency can therefore occur even in very closely related species.

When looking at the fatty acid composition of individual lipids, it is obvious that SQDG and PG may be separated from MGDG and DGDG in either normal or deficient cultures. This separation is based on differences in composition due to increased levels of C18:3 and C18:1 in *Pseudanabaena* (and C18:2 in *O. splendida*) and reduced proportions of C18:4 in SQDG and PG. These findings do agree with those presented for *Tolypothrix* (14, 15), the only Cyanobacterium so far mentioned in the literature, to our knowledge, which contains both the γ and α C18:3 acids as well as the C18:4 acid in all major lipids.

It would be of interest to relate these findings to lipid metabolism, especially to desaturation processes in plants. In the case of the two strains studied, the results here obtained would indicate that the third step of desaturation (*i.e.*, 18:2 → 18:3) is performed by different enzymes which recognize the polar head of the lipid (galactose or phosphoglycerol and sulfoquinovose) and introduce the double bond in a different position, α (ω_3) or γ (ω_6) in C18:2 to produce C18:3. Very low cross affinity of these enzymes would occur, since traces of C18:3γ were found in PG and SQDG. Another hypothesis would be that the same desaturation enzyme would introduce the double bond differently according to the polar head of the lipidic substrate. Investigation using these organisms, which possess different desaturation products according to the lipid polar head, will certainly help the knowledge of the specificities of lipid synthetizing enzymes.

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