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PROTOPLASMA

Effects of Nitrogen Deficiency and Light of High Intensity on *Cryptomonas rufescens (Cryptophyceae)*

I. Cell and Photosynthetic Apparatus Transformations and Encystment

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Summary

Cryptomonas rufescens is an alga which can adapt itself to environmental variations created by nitrogen deficiency and high levels of illumination. The cell ultrastructure changes and starch and storage lipids accumulate in the cytoplasm. Progressively, the cell reaches a resting stage. The differentiation of a complex cell wall leads to cyst formation. This change obtained through controlled conditions, can be associated with an *in vivo* minimal value of the ratio phycoerythrin/chlorophylls. In the two treatments the amounts of chlorophyll A and phycoerythrin decrease, while the amounts of chlorophyll C and carotenoids appear more stable. At the same time, the plastid ultrastructure changes, the internal thylakoids break down and are disorganized, while the peripheral thylakoids become narrower in correlation with their phycoerythrin content.

Keywords: Cryptomonas; Encystment; High light intensity; Nitrogen deficiency.

1. Introduction

Cryptomonas rufescens is a bright red fresh-water alga. In addition to photosynthetic pigments chlorophylls A and C and carotenoids, as in *Chromophyceae* (ALLEN *et al.* 1959, JEFFREY 1976), *C. rufescens* contains phycoerythrin. This biliprotein is similar to those of *Rhodophyceae* and *Cyanophyceae* (O'HEOCHA and RAFTERY 1959, BOGORAD 1975, MACCOLL *et al.* 1976). When the cells are grown without nitrogen compounds, or exposed to high levels of continuous illumination, they turn to cyst formation. In this work, the conditions under which these cysts appear, the different stages of their formation

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and their ultrastructural organization are especially considered. We studied particularly the storage phenomena, the modifications of the photosynthetic apparatus and the differentiation of the cyst wall.

2. Material and Methods

2.1. Cultures

The strain of *Cryptomonas* was originally obtained from Gif/Yvette collections. The species "*rufescens*" has been assigned owing to the red colour of the alga (SKUJA 1939).
The axenic control cultures (T) were grown on S_2T_2 medium (pH 6.7) at room temperature, in erlenmeyer flasks. This medium is derived from L + C medium described by LEFEVRE *et al.* (1952).

$Ca(NO_3)_2$, 4 H_2O	100 mg
KNO_3	100 mg
$MgSO_4$, 7 H_2O	30 mg
K_2HPO_4	40 mg
Extracts of *Sphagnum*	20 ml
Extracts of soil	40 ml
$FeCl_3$	traces
Bidistillated water	1,000 ml

The extracts of soil were prepared according to the methods of PRINGSHEIM (1946). The extracts of soil and *Sphagnum* represent about 0.5 mg/l of nitrogen (calculated from LEFEVRE *et al.* 1952), and they may be considered as an oligonutrient supply.
For the nitrogen deficient cultures (SN), the extracts of soil and *Sphagnum* were reduced (about 25 µg/l), and the nitrogen compounds were omitted from S_2T_2 medium and substituted for the following compounds

$CaCl_2$	50 mg
K_2SO_4	87 mg
Extracts of *Sphagnum*	1 ml
Extracts of soil	2 ml

The pH of the nitrogen deficient medium is 6.7.
The control cultures (T) and the nitrogen deficient cultures (SN) were exposed to daylight (about 4 $Js^{-1}m^{-2}$) and darkness (12 h/12 h).
The cells grown at high light intensity (FL) were continuously exposed to fluorescent tubes (ITT Claude 40 RS) at about 10 $Js^{-1}m^{-2}$.
The cell population density was periodically determined by cell countings on several samples of cultures with a Thoma haematocytometer.

2.2. Electron Microscopy

Cells were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8), postfixed in 1% osmiumtetroxyde and embedded in Araldite. Thin sections were stained with uranyl-acetate plus lead citrate, or potassium permanganate, and examined with a Philips 300 Electron Microscope.
Thylakoid widths were measured in enlarged photographic prints (×90,000), in cross sections where pair-associated thylakoids have the sharpest images. The average width was determined from 50 measurements per treatment.

To determine the area of the constituants, we used a simple gravimetric method. We made a tracing of each organelle on an enlarged micrograph, cutted and weighed it, and then compared with a surface unity.

2.3. Pigment Determination

The *in vivo* absorption spectra was determined by a Cary Model 14 Spectrophotometer. The cells were maintained in suspension in S_2T_2 medium or in 75% glycerol. [The extracts of chlorophylls were made in a mixture of acetone-methanol in darkness, at 4 °C, till the supernatant became colorless, and then evaporated. Concentrations of chlorophylls A and C were calculated by using the equations of JEFFREY and HUMPHREY (1975).]
The extracts of carotenoids obtained after saponification of chlorophylls, were chromatographed in darkness. Their identification and concentration were established using the methods of GOODWIN (1965).
The extracts of phycoerythrin were prepared by ultrasonic disruption of cells in 0.1 M phosphate buffer, and then freezing. Phycoerythrin content was calculated using the extinction coefficient communicated by MACCOLL: E $1\%_{565}$ (mg/ml) = 9.9. The quantitative estimation of isolated pigments was always made on cultures five weeks old.

3. Results

3.1. Culture Conditions and Growth

The experiments which are carried out over a period of seven to eight weeks, enable us to determine several periods on the growth curves of the lots deficient in nitrogen compounds (SN) and exposed to strong light (FL) (Fig.1).
No visible modifications appear during the first week of experiment. Along the next three weeks, the number of cell divisions increases (Preparatory phase: P), the development is more accentuated in FL than in SN. Then the number of cell divisions decreases and the cultures enter a stationary phase of growth (Pre-encystment phase: PE). This period begins about the fourth week for FL and the fifth week for SN. The encystment phase (E) begins after six weeks for FL and later for SN. As soon as cysts appear, the number of cells remains constant in the cultures.
In nitrogen deficiency, the increase in population is always very slow.

3.2. Preparatory and Pre-Encystment Phases

The fine structure of *C. rufescens* is similar to the other *Cryptomonas* species (MIGNOT *et al.* 1968, LUCAS 1970, WEHRMEYER 1970 a, b). It possesses specific organelles like gullet, trichocysts and periplast (Fig. 2). During the preparatory phase, the cell keeps its elongated shape and its mobility. The nucleus, the flagella and the cytoplasmic organelles remain identical. Two kinds of compounds appear in the cell:
Large clear grains of starch; their polysaccharidic composition is confirmed by their positive reaction with the cytochemical test of polysaccharides (PATAg) (THIERY 1967), (Figs. 4 and 5).

Very large osmiophilic droplets, which are often localized at the posterior region of the cell. These non membrane-bound droplets are extracted by an acetone-methanol mixture an represent lipids (Fig. 3).

When the number of cell divisions decreases (phase PE), the cells become completely spherical, motionless and yellowish. The cytoplasm fills with starch and lipid droplets. The plastid area decreases and this organelle becomes re-

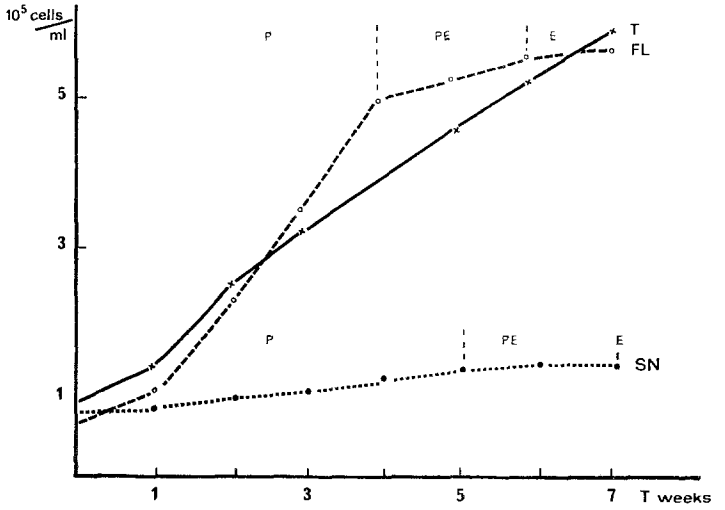


Fig. 1. Kinetics of growth of *Cryptomonas rufescens* ——— *T*; ····· *SN*; - - - - - *FL*. Definition of growth periods: *P* = preparatory phase; *PE* = pre-encystment phase; *E* = encystment phase

stricted to a narrow band applied to the side of the cell (Fig. 6). On the other hand, the Golgi apparatus is always well developed in the central region of the cell and produces numerous vesicles.

We have calculated from electron micrographs the different structure areas. The absolute interpretation of these data is critical (NOURTIER 1971), but the results are nevertheless significant (Tab. 1).

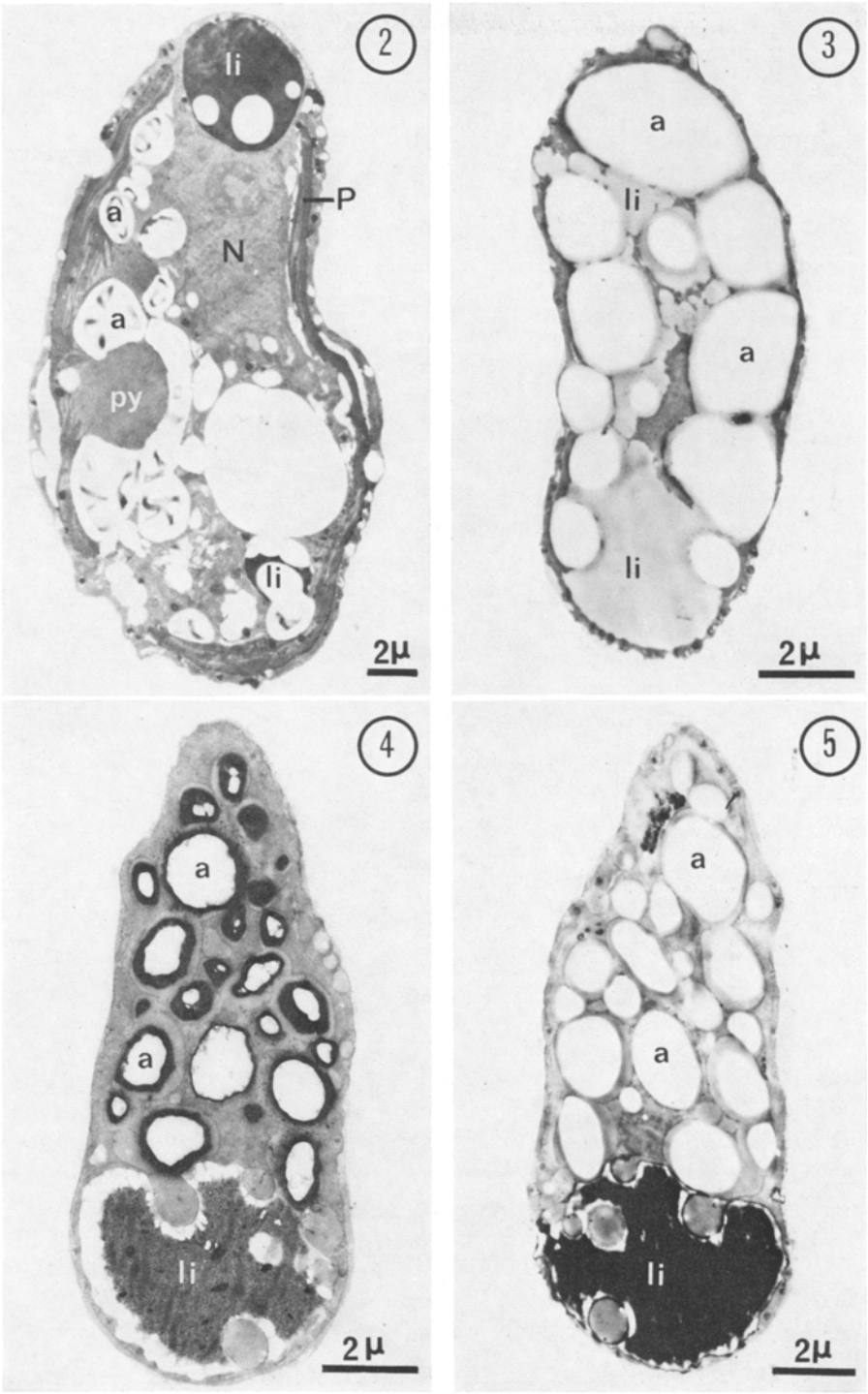
Aspect of the cell during the preparatory phase

Fig. 2. General view of *Cryptomonas*; the cell presents its habitual appearance, but starch (*a*) and lipids (*li*) are more abundant in the cytoplasm (*N* nucleus; *P* plastid; *py* pyrenoid) ×3,400

Fig. 3. Lipid extraction by a mixture of acetone-methanol; (*a* starch; *li* lipids) ×6,500

Fig. 4. Cytochemical test of polysaccharides (PATAg); starch (*a*) presents a positive reaction. ×6,500

Fig. 5. Control of the cytochemical test of polysaccharides. The polysaccharides are not stained (*a* starch), while lipids (*li*) keep the same non typical reaction as in Fig. 4; ×5,300



Figs. 2–5

Table 1. *Different Structure Areas (S.) in the Cell, According to Culture Conditions*

LOTS	% S. plastid / S. cell	% S. starch / S. cell	% S. lipids / S. cell	% ΣS. reserve compounds / S. cell
T	42 à 47	9 à 11	±	9 à 11
SN	15 à 17	25 à 29	32 à 39	57 à 68
FL	12 à 15	35 à 39	25 à 30	60 à 69

Under high light intensity, the reserve compounds are more abundant, and the plastid area is more restricted than in nitrogen deficiency.

The plastid area and the number of thylakoids diminish by two distinct but complementary processes:

1. The reduction in width of peripheral thylakoids (Figs. 8, 9, and 10). The external thylakoids remain pair-associated and regularly arranged, but they become narrower. The thickness of a pair of thylakoids initially measured as 450–480 nm for T, drops to 350–380 nm for SN and 300–330 nm for FL. The intrathylakoidal space where phycoerythrin is localized (GANTT *et al.* 1971) becomes limited, but keeps a strong density. These ultrastructural variations can be associated with the reduction of the phycoerythrin content (Tab. 2).

Table 2. *Pigment Content of the Different Lots of C. rufescens.* Amount in μg pigment/10^6 cells; 10^6 cells = 1.04 mg dry weight (*Chl. A, C* = chlorophylls A, C, *Pe* = phycoerythrin, *Car* = carotenoids)

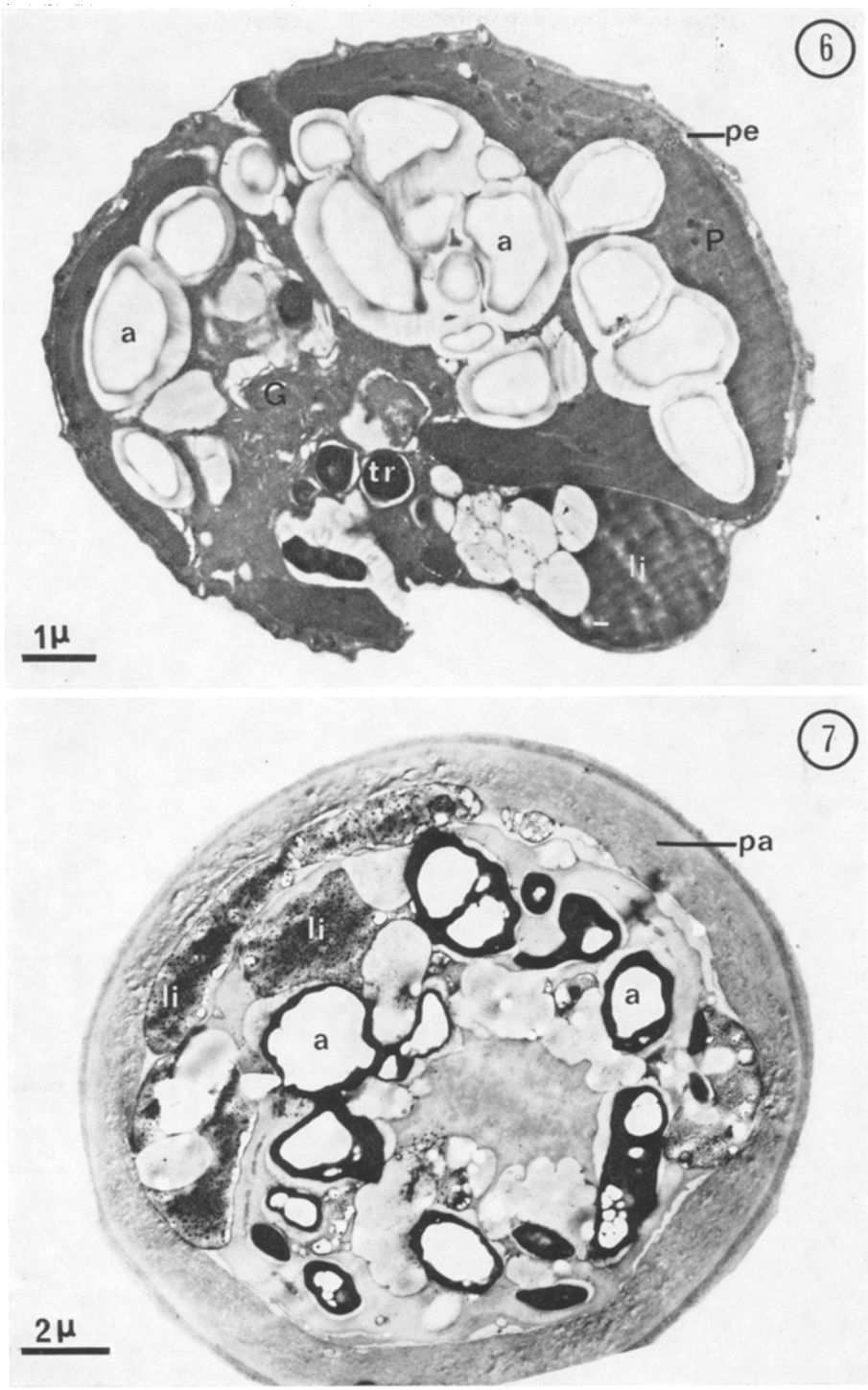
LOTS	Chl. A	Chl. C	% Chl. C / Chl. A	Pe	Pe / Chl. A + C	Car.	Car. / Chl. A + C
T	11	0.6	5.45	42	3.6	2.2	0.18
SN	6	0.35	5.83	17	2.7	1.8	0.28
FL	2.4	0.15	6.25	5	2	1.2	0.47

2. The disorganization of internal thylakoids (Fig. 11). These thylakoids break down into little pieces and single membranes which then disperse (Fig. 11). As soon as internal thylakoids disappear, lipids compounds increase and accumulate in the cytoplasm.

As experimentation time progresses, the algae become yellowish for the two cases. After a five weeks treatment (Fig. 12), there is an inversion of the

Fig. 6. Pre-encystment phase. The cell becomes spherical and is full of reserve compounds: starch (*a*) and lipids (*li*). (*G* Golgi apparatus; *P* plastid; *tr* trichocyst). ×10,000

Fig. 7. Cytochemical test of polysaccharides on encysted cell. The polysaccharidic compounds [starch (*a*)] and the wall (*pa*) present a positive reaction. ×6,200



Figs. 6 and 7

relative height of the chlorophyll absorption peak (678 nm) to the phycoerythrin absorption peak (565 nm). The ratio $\frac{\text{O.D. 565}}{\text{O.D. 678}}$ estimated on *in vivo* spectra, decreases from 1.7 or 1.5 (initial stage), to 0.7 in SN and 0.5 in FL.
The total pigment content becomes very low (Tab. 2), but the loss of phycoerythrin is always greater than that of chlorophylls, especially for FL which contains eight times less phycoerythrin and five times less chlorophylls than T. The chlorophyll C always found in small quantity (see T), decreases relatively less than chlorophyll A. The carotenoids also, appear more stable.

3.3. Encystment Phase

The cysts appear in the two treatments when the *in vivo* ratio phycoerythrin/chlorophylls falls to 0.6 or 0.7. As this value is reached more quickly in high illumination, the encystment is earlier in FL (Fig. 12). About 60 to 70% of the total cells are encysting, their aspect is the same in the two treatments.
The mature cysts are spherical (13 to 17 μm in diameter), and surrounded by a thick wall (2 to 3 μm). The cellular modifications already noticed in the pre-encystment phase are more important (Fig. 13 *b*), and especially the reduction of the plastid area. The nucleus is often difficult to distinguish. The flagella, the gullet and the gullet trichocysts disappear, while the periplast and the peripheral trichocysts are still present. The Golgi cisternae are surrounded by numerous vesicles. Some of them, full of fibrillar materials are localized in the peripheral region of the cell (Figs. 13 *b* and 14), supporting the hypothesis that they participate to the elaboration of the cyst wall.
The first fibrils of the wall lay down around the cell and rest on the trichocyst vesicles (Fig. 15). They form a continuous layer whose thickness increases by contribution of new materials transported by the Golgi vesicles. The cytochemical test of polysaccharides is very low (Fig. 7).
The cyst wall organization is complex, and several parts of unequal thickness are distinguishable: (Figs. 16 and 17).
1. A very thick internal part, (2.5 to 3 μm), near the periplast, formed by fibrillar groups, which lie parallel to the plasmalemma. The longitudinal fi-

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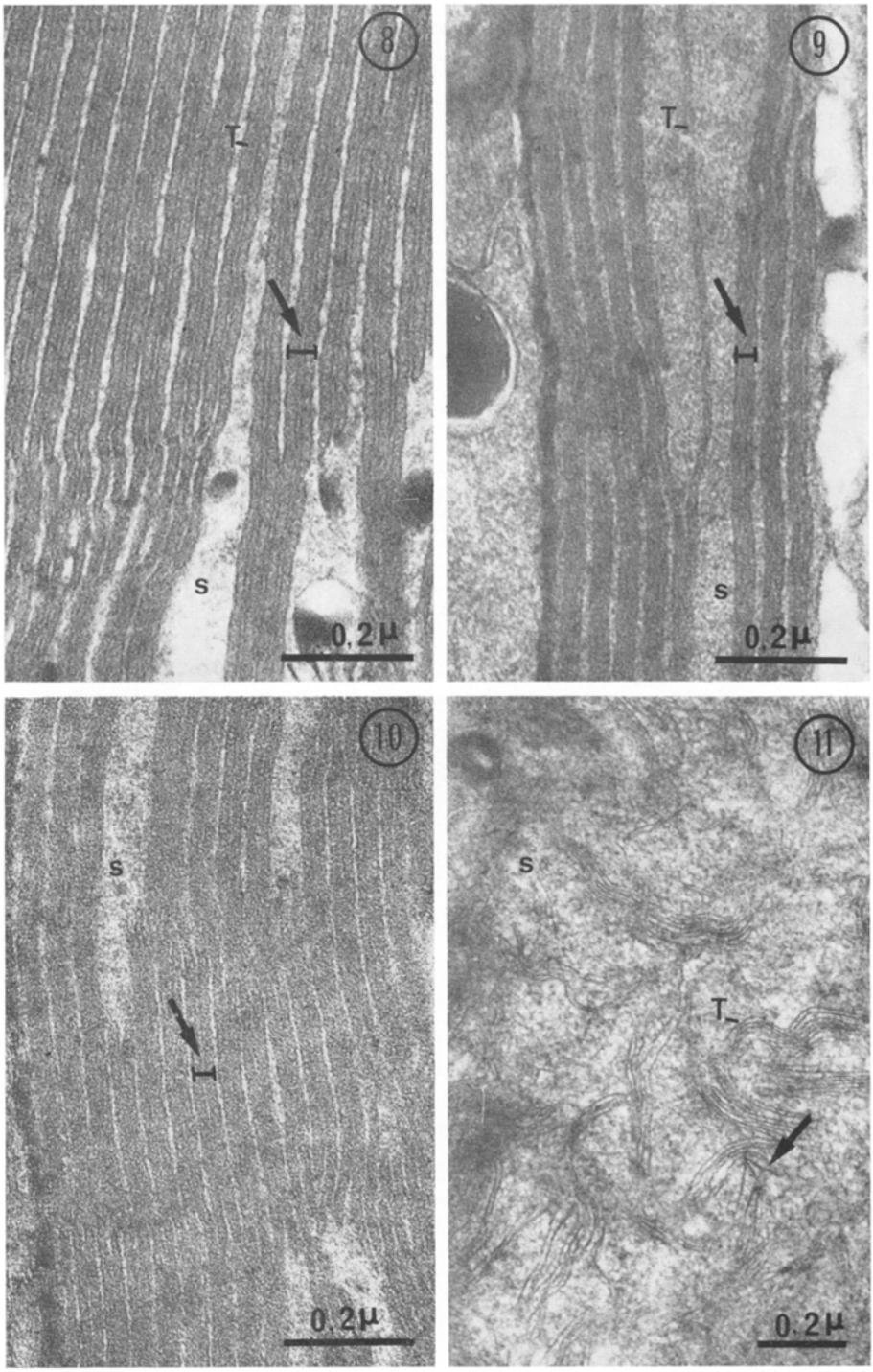
Section of the peripheral thylakoids of the plastid of *Cryptomonas rufescens*
Note the intrathylakoidal width (arrows) which decreases with the phycoerythrin content (Table 2) (*T* thylakoid; *s* stroma)

Fig. 8. *T*; ×90,000

Fig. 9. *FL*; ×90,000

Fig. 10. *SN*; ×90,000

Fig. 11. Transformation of internal thylakoids in *SN* and *FL*. Thylakoids break down into simple membranes (arrow) which are dispersed in the stroma. ×60,000



Figs. 8–11

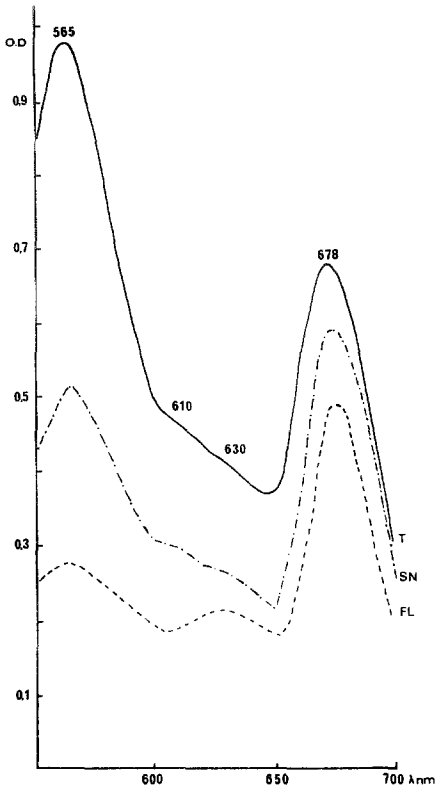


Fig. 12. *In vivo* absorption spectra of *Cryptomonas rufescens* after five weeks of experiments ——— *T*; –·–·– *SN*; ------- *FL*

brils are regularly separated by transverse and oblique fibrils, and exhibit a periodicity which progressively decreases towards the periphery (Fig. 17). They are comparable to the twisted fibrous materials described in some plant cell walls.

2. A thin, dense, external layer (0.2 μm) forming a border. It originates from the condensation of the internal fibrils (Figs. 16 and 17).

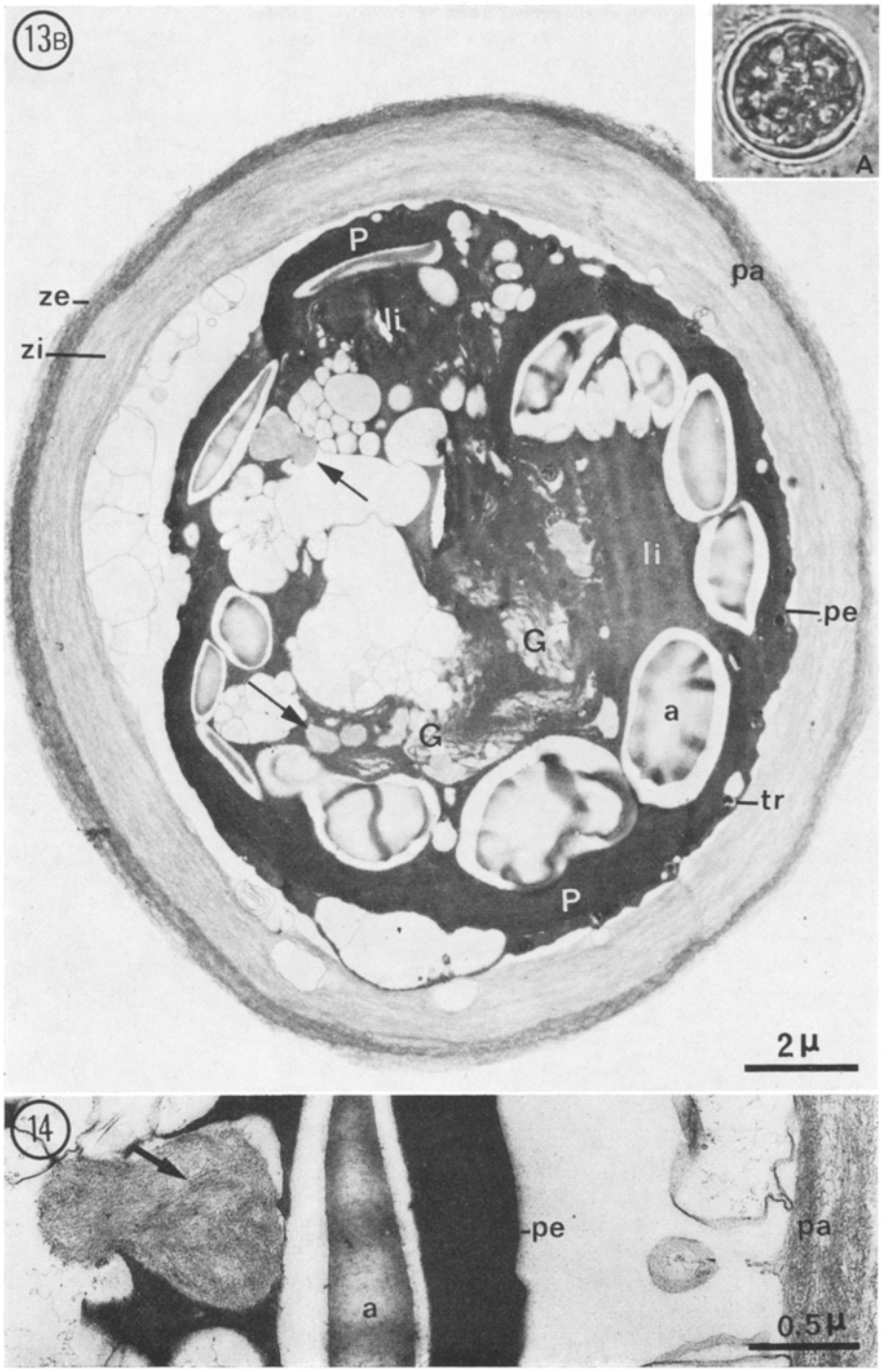
3. As there is no cuticle, the sides of the cyst are very diffuse. Outside the border, many disorganized fibrils seem to be released from the wall and then dispersed in the medium.

Encystment phase

Fig. 13 *A*. General view of a cyst in light microscopy; ×1,000

Fig. 13 *B*. General view of a cyst in electron microscopy. The cell contains abundant reserve compounds, starch (*a*) and lipids (*li*). It is surrounded by a thick wall (*pa*) formed by an external border (*ze*) and an internal layer (*zi*). Some vesicles of the Golgi apparatus (*G*) are filled with fibrillar materials (arrows). (*pe* periplast; *P* plastid; *tr* trichocyst) ×7,300

Fig. 14. Detail of the Golgi vesicles full of fibrillar materials (arrow) which will be incorporated into the wall (*pa*). (*a* starch; *pe* periplast) ×31,500



Figs. 13 and 14

4. Discussion

4.1. Encystment Conditions

The encystment state appears in algae and Protozoa in response to unfavourable environmental conditions (BIBBY and DODGE 1972, CORLISS and ESSER 1974). The induction of cyst formation depends on numerous factors and it has been difficult to find inductive factors in an experimental study (BIBBY and DODGE 1972, BROWN *et al.* 1976). The primary stimulus is often unknown.

In the two treatments, the encystment process of *C. rufescens* coincides with a group of modifications appearing in the medium and the cells. The cultures are in a stationary phase of growth, and the cells are filled with abundant reserves. The membranous systems, and especially plastid, are reduced, and the pigment content is very low. It seems that the ratio $\frac{\text{O.D. 565}}{\text{O.D. 678}}$ has a minimal value beyond which the physiological activity of algae is considerably reduced. Although specific nitrogen compounds are not necessary in *Cryptophyceae* (ANTIA and CHORNEY 1968, CLOERN 1977), the removal of nitrogen from the medium changes the growth and development of these algae, and seems to start encystment, as in *Chlorophyceae* (BERKALOFF 1967, 1975).

The growth of *C. rufescens* is stimulated during the first weeks of high illumination. Many authors (BROWN and RICHARDSON 1968, FAUST and GANTT 1973, CLOERN 1977) have already mentioned this phenomenon. We would like to emphasize that the prolongation of the high light treatment also induces encystment.

4.2. Cell and Photosynthetic Apparatus Transformations

The experimentally induced cysts of *C. rufescens* look like natural cysts first described in light microscopy by DANGEARD (1889), and also those of *C. frigoris* which is always in the cyst form in the snow, its natural medium (JAVORNICKÝ and HINDÁK 1970). In *C. rufescens* we shall point out some peculiarities:

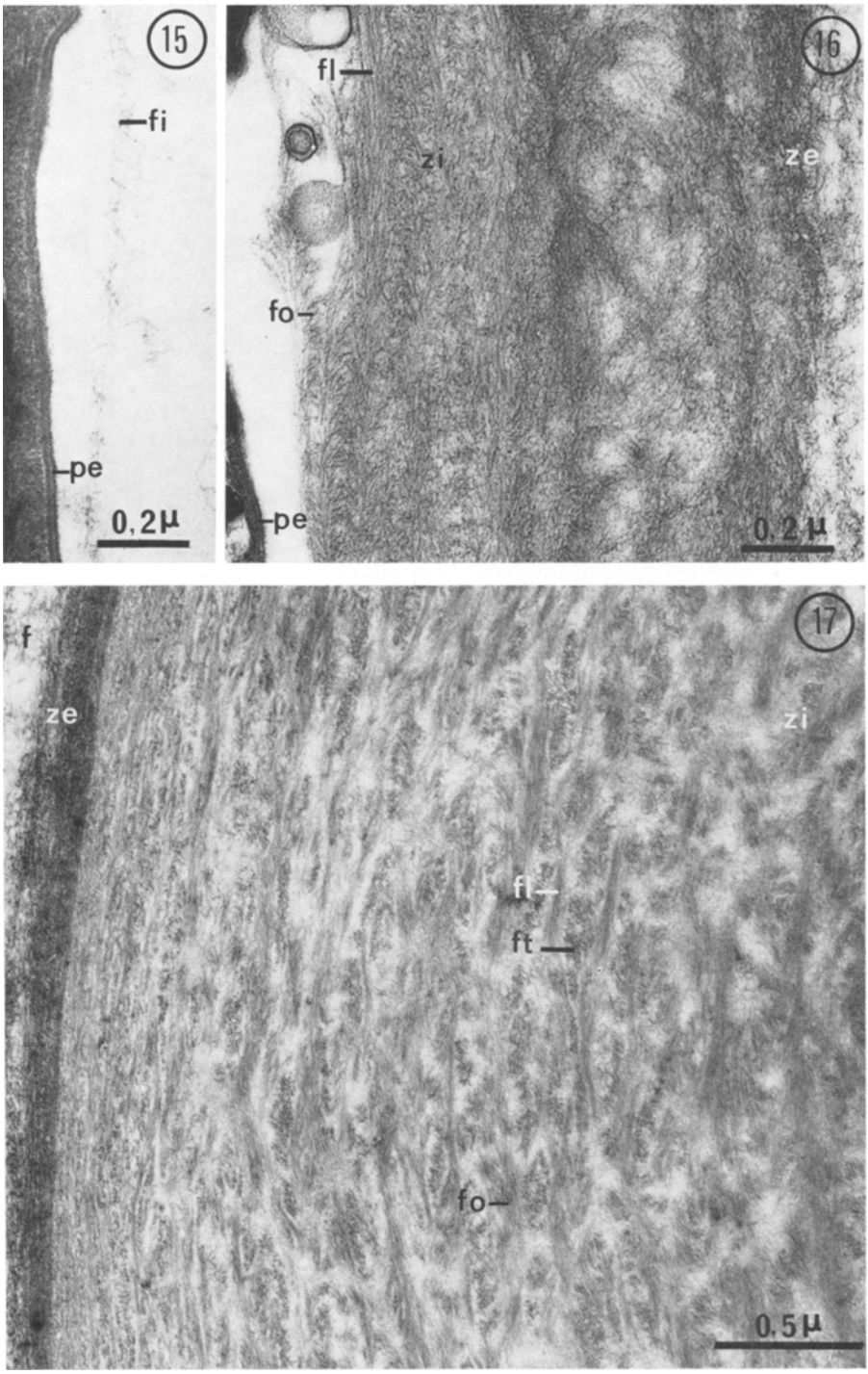
The cysts are formed exogenously, contrary to *Ochromonas* (HIBBERD 1977).

Ultrastructure of the cyst wall

Fig. 15. The first fibrils (*fi*) are deposited around the cell, outside the periplast (*pe*). ×62,500

Fig. 16. The thickness of the wall increases by the depositing of groups of fibrils oriented in different planes (*fl* longitudinal fibrils; *fo* oblique fibrils; *pe* periplast; *ze* external zone of the wall; *zi* internal zone of the wall). ×62,500

Fig. 17. Ultrastructural organization of differentiated cyst wall in cross section. The groups of fibrils of the internal zone (*fl* longitudinal fibrils; *fo* oblique fibrils; *ft* transversal fibrils) have a periodical disposition. The fibrils of the external zone are packed together and form a dense layer (*ze*), with some disorganized fibrils (*f*) outside. ×41,500



Figs. 15–17

As the loss of the gullet trichocysts was still referred to by SCHUSTER (1970) in the case of a *Chilomonas* starved in carbon, let us suppose that these organelles are used as proteinic substrate, in the same manner as phycobiliproteins.
Starch and lipids are formed in the two treatments. Yet their proportions are different: starch is more abundant in high illumination, and lipids in nitrogen deficiency. Starch is distributed throughout the cell, but is always included in the plastid membranes as in colorless *Cryptophyceae* with a leucoplast (MIGNOT 1965, SEPSENWOL 1973). The starch accumulation under high illumination is also mentioned in other *Cryptophyceae* (FAUST and GANTT 1973), and more particularly in photoheterotrophy (ANTIA *et al.* 1969, 1973, FAUST and GANTT 1973). Yet, it has not been observed in nitrogen deficiency. Lipids develop as neutral lipids which are in part formed from membranous systems (LICHTLÉ and DUBACQ in preparation). These lipids do not have a specific location in the cell as in *Woloszynskia* (BIBBY and DODGE 1972), and they do not contain carotenoids as in *Protosiphon* (BERKALOFF 1975).
C. rufescens keeps the same total pigment composition in the two treatments, but the pigment content diminishes, especially for FL. *C. rufescens* displays the same adaption for chlorophyll A as others phycobiliprotein algae grown in high illumination (BROWN and RICHARDSON 1968, GUERIN *et al.* 1973 b, WAALAND *et al.* 1974, FAUST and GANTT 1973), or under nitrogen deficiency (GUERIN *et al.* 1973 a). The relative stability of the chlorophyll C, and the increase of the ratio chlorophyll C/chlorophyll A was also observed by FAUST and GANTT (1973) in a brightly exposed *Chroomonas*. *C. rufescens* seems to be enriched in carotenoids because these compounds are more stable than chlorophylls, but this phenomenon appears to be a secondary one. In fact, new carotenoids are not synthesized more abundantly to supply the loss of chlorophyll A as in *Chlorophyceae* starved in nitrogen compounds (BERKALOFF 1975).
C. rufescens has a very high level of phycoerythrin. As for the phycocyanin of *Chroomonas* (FAUST and GANTT 1973), the amount of phycobiliprotein is directly related to the light intensity and varies in the opposite way of it. We have never obtained a total disappearance of phycoerythrin under prolonged illumination. The behaviour of the phycoerythrin of *C. rufescens* is the same as those of *Rhodophyceae* (BROWN and RICHARDSON 1968, GUERIN *et al.* 1973 b, WAALAND *et al.* 1974).
The phycobiliproteins disappear in many *Rhodophyceae* (GUERIN *et al.* 1973 a, WEHRMEYER and SCHNEIDER 1975) under conditions of nitrogen deficiency. This possibility was not mentioned in the case of *Cryptophyceae*. The phycoerythrin of *C. rufescens* may be used as proteinic substrate in the starved cells as in *Rhodella* (WEHRMEYER and SCHNEIDER 1975), since when nitrogen deficiency stops, this pigment reappears.
The fragmentation of the internal thylakoids may explain the reduction of the plastid area. These observations agree with results obtained with many *Rho-*

dophycea grown in high illumination (GUERIN *et al.* 1973 b, WAALAND *et al.* 1974), or without nitrogen compounds (WEHRMEYER and SCHNEIDER 1975). The peripheral thylakoids of the plastid are always arranged in pairs, but they do not form pseudo-grana as in *Protosiphon* (BERKALOFF 1975), or *Woloszynskia* (BIBBY and DODGE 1972). Yet the loss of phycoerythrin involves the reduction of the thylakoid width, the narrowest thylakoids being associated with FL which contains the lowest amount of phycoerythrin. The same behaviour has been mentioned for a *Chroomonas* with phycocyanin (FAUST and GANTT 1973). However the adaptation of *Cryptophyceae* thylakoids seems particular. In the same experimental conditions, *Rhodophyceae* thylakoids keep the same width, and the loss in phycoerythrin changes only the phycobilisome arrangement (GUERIN *et al.* 1973, WAALAND *et al.* 1974, WEHRMEYER and SCHNEIDER 1975). The modifications of *Cryptophyceae* thylakoids obtained both in high light intensity and nitrogen deficiency, appear independent of the nature of phycobiliproteins.

4.3. Cyst Wall

It seems that all the cellular synthesis are progressively reduced in the encystment process, and become restricted to the building and the maintenance of the new wall structure. The wall comes together with the periplast without changing it. These two structures seem independent and often separated by vesicles. Yet the periplast keeps its organization in contrast to *Dinophyceae* which lose their theca when encysting (BIBBY and DODGE 1972). The *C. rufescens* cyst wall is composed exclusively of fibrils and devoid of amorphous compounds. The regular periodical disposition of groups of fibrils reminds the twisted fibrous materials described in some algae walls (THOMAS 1972, PENG and JAFFE 1976). But it is only a resemblance because the changes in the orientation of the fibrils are not regular between each plane (BOULIGAND pers. com.). The formation of the wall depends principally on the Golgi apparatus as in other cyst walls (BROWN *et al.* 1976, HIBBERD 1977). In normal *C. rufescens* cells, the Golgi vesicles play a role in the formation of trichocysts (MIGNOT 1965, SCHUSTER 1970, WEHRMEYER 1970 b), but do not synthesize the precursors of the wall. By the encystment process, modifications are induced in the biosynthetic pathways of this organelle. The polysaccharide formation seems to be in direct relation to the physiological activity of the alga as in *Porphyridium* (RAMUS 1972). The extracellular fibrils lead us to suppose that there is a turn-over of polysaccharidic compounds and it is possible to consider the cyst wall as a dynamic structure.

5. Conclusions

Under unfavourable environmental conditions, *C. rufescens* progressively differentiates into a resistant form: the cyst, surrounded by a thick wall and containing storage compounds, which allows a survival period.

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