

PHYSIOLOGICAL, ULTRASTRUCTURAL AND CYTOCHEMICAL STUDIES ON
THE UTILIZATION OF VARIOUS INTERMEDIATES OF THE PURINE
CATABOLISM PATHWAY AS SOLE SOURCES OF NITROGEN BY MARINE
PHYTOPLANKTERS

By

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ABSTRACT

Eleven species of marine microalgae belonging to six different taxonomic divisions were tested for their ability to grow on allantoin, allantoic acid, hypoxanthine and urea as sole sources of nitrogen. All species were able to utilize the nitrogen atoms of urea but only six of these were able to grow on allantoic acid, while five showed moderate to good growth in hypoxanthine. None was able to utilize allantoin. The study of nickel requirements for the growth of these microalgae on the different sources of nitrogen, together with the results of inhibitor tests suggest that those species capable of utilizing both hypoxanthine and allantoic acid catabolize purines through the standard pathway of purine oxidation described in other microorganisms and higher plants. This pathway leads to the production of urea and its subsequent conversion to utilizable ammonium. In the case of one species, Pavlova lutheri, growth on urea is inhibited by urease inhibitors, while growth in allantoic acid or hypoxanthine occurs in the presence of urease inhibitors. The results suggest that in this case the catabolic oxidation of purines and their derivatives does not involve urea production and occurs through a pathway different from that observed in the other species.

Cells of Amphidinium carterae grown on hypoxanthine undergo major ultrastructural changes. These affect the

perichromatinic granules, the dictyosomes and dictyosome-derived vesicles, the distribution of the endoplasmic reticulum, the number of mitochondria and microbodies, and the size and distribution of the vacuolar compartment. Some of these ultrastructural changes, such as increase in endoplasmic reticulum and the number of microbodies, along with the cytochemical demonstration of both uricase and catalase activities within microbodies, support the occurrence in these microalgae of the standard pathway for the catabolic degradation of purines. Cells of both Dunaliella tertiolecta and Pavlova lutheri grown on hypoxanthine also undergo major ultrastructural changes. These affect mainly the endoplasmic reticulum, mitochondria and vacuoles. The effect on mitochondria is particularly interesting since cytochemical tests reveal the presence of both uricase and catalase activities in these organelles. When one takes into consideration that no microbodies are observed in these microalgae and that uricase controls the key step of the formation of allantoin and H_2O_2 through the oxidation of urate, it becomes apparent that in these microalgae mitochondria participate in the oxidative degradation of purines and their derivatives and play a major role in the organic N-budget of these microorganisms.

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INTRODUCTION

The availability of nitrogen for phytoplankton growth in coastal and open - ocean waters is often assessed by measuring the concentrations of inorganic nitrogen compounds. As pointed out by Antia et al. (1975) such an approach overlooks the large pool of organic nitrogen present in seawater. Apart from ammonium and nitrate, the two most common sources of inorganic nitrogen (Syrett, 1962; Morris, 1974), many algae can utilize various organic compounds as sole nitrogen sources for phototrophic growth. Although organic-N compounds are not always as effective as nitrate, many of these show the potential for supporting significant growth (see Antia et al., 1989, for a review).

Urea is an important organic nitrogen source for phytoplankton growth (Oliveira and Antia, 1984) that can also be endogenously produced through metabolic pathways such as the aerobic degradation of purines (Naylor, 1970). Urea utilization by algae involves the production of two alternative urea - degrading enzyme - systems: Urease or ATP:urea amidolyase. The evidence shows that a given algal species may produce either urease or ATP:urea amidolyase but not both enzymes and that ATP:urea amidolyase is restricted in occurrence to some Chlorophycean algae (Bekheet and Syrett, 1977; Al-Houty and Syrett, 1984). Recent studies on the phototrophic growth of several marine phytoplanktonic algae with urea serving as sole source of nitrogen showed

that growth could only occur, in most cases, on addition of minute amounts of Ni^{2+} to the growth media (Oliveira and Antia, 1984, 1986a). Of particular interest to these findings is the discovery that nickel is a constituent of jackbean (Canavalia ensiformis L.) urease (Dixon et al., 1975) and is required for urease activity in soybean tissue cultures (Polacco, 1977). A nickel requirement for urease activity was also reported for some microalgae (Syrett, 1981; Rees and Bekheet, 1982; Oliveira and Antia, 1986b).

Antia et al. (1989), in their review on the role of dissolved organic nitrogen in phytoplankton nutrition show widespread ability on the part of microalgae to utilize purines and/or their derivatives as sole sources of nitrogen. The utilization of purines and purine derivatives such as hypoxanthine, xanthine, uric acid and allantoin as nitrogen sources for the phototrophic growth of marine microalgae has now been established for organisms from different taxonomic divisions (Antia et al., 1980a; Prasad, 1983; Shah and Syrett, 1984a). Some of these compounds are considered to be important organic N sources in certain marine environments, particularly inshore areas (van Baalen and Marler, 1963). The evidence suggests the existence in microalgae of a pathway of purine oxidation similar to that described for other microorganisms (Vogels and van der Drift, 1976) and higher plants (Reynolds et al., 1982). This pathway leads to the formation of urea and its subsequent conversion to utilizable ammonium (Antia et al., 1989).

However, as pointed out by Prasad (1983) and Antia et al. (1989), the information available for the algae is at best fragmentary and restricted in most cases to tests dealing exclusively with growth studies (see also Antia et al., 1980b). The validity of the growth tests is further complicated by the fact that inappropriate concentrations of Ni^{2+} in the culture medium may lead to misinterpretations regarding the ability of the algae to utilize the intermediates of the pathway as sole sources of nitrogen. Consequently, the pathway of catabolism of purines and their derivatives in algae is far from understood. Equally unknown is the importance of the intermediates of this pathway on the organic N-budget of both coastal and estuarine areas which are characterized by the seasonal occurrence of large pools of unidentified organic - N (Butler et al., 1979; Valiella and Teal, 1979).

In this thesis, I report on the Ni^{2+} requirement of 11 species of marine phytoplankters belonging to 6 different taxonomic divisions for growth on hypoxanthine, allantoin, allantoic acid and urea as sole sources of N. Since recent evidence suggests that in higher plants, purine N utilization may also proceed without the production of urea (Winkler et al., 1987, 1988), I have also conducted growth inhibition and enzymatic studies to help elucidate the mechanism(s) of oxidation of purines and their derivatives responsible for the growth of these 11 species of microalgae.

I have also come to realize that while some of the ultrastructural implications of the catabolic oxidation of purines have been well studied in higher plants, particularly in higher plants relying on nodular nitrogen fixation (Newcomb et al., 1985; Kaneko and Newcomb, 1987; Webb and Newcomb, 1987), no such studies exist for microorganisms particularly the microalgae. Therefore, I also report on the ultrastructure and cytochemistry of the marine dinoflagellate Amphidinium carterae, the Chlorophycean alga Dunaliella tertiolecta and the Prymnesiophyte Pavlova lutheri grown on nitrate, urea, allantoate and hypoxanthine in order to gain further insight into the mechanism(s) of purine- and purine derivatives - N utilization by these microalgae.

MATERIALS AND METHODS

Algal species

Stock cultures of the algae listed in Table 1 were routinely maintained on nitrate (as nitrogen source), under standard axenic conditions, according to Antia and Cheng (1970).

Growth tests

All tests were carried out in culture tubes at 18 °C under continuous illumination (irradiance 95 - 100 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) from cool - white fluorescent lamps. The standard test medium was that of Oliveira and Antia (1984) with the salinity adjusted to 26‰. In the case of Olisthodiscus luteus and Dunaliella tertiolecta, final salinity of the test medium was retained at 14‰. Unless otherwise stated, Ni^{2+} , nitrate, urea, allantoinic acid, allantoin, hypoxanthine, citrate, allopurinol, 2,6,8-trichloropurine and hydroxyurea additions were made to 4 mL of test media before inoculation with 0.2 mL aliquots of the algal stock culture was carried out. The concentrations utilized in the experiments are given in Tables 2 through 6. All test media were filter - sterilized (0.2 μm pore size presterilized Nalgene filters) and aseptic techniques were used throughout the tests to ensure axenic growth conditions.

Growth was monitored directly in culture tubes by periodically reading their optical density at 600 nm (OD_{600})

on a spectrophotometer after brief vortex mixing. Every growth test included a control without any added nitrogen source. The growth measured on experimental nitrogen sources was corrected for the residual growth, if any, measured under those circumstances. Plots of the corrected growth against the incubation time period were used to calculate the following three parameters: (1) adaptation period = number of days from inoculation to the first significant increase in OD₆₀₀, (2) exponential growth rate = maximum increase in OD₆₀₀ per day during exponential phase of growth, expressed as percentage of corresponding rate from nitrate control, and (3) maximum yield = maximum OD₆₀₀ on the growth curve for a test, expressed as percentage of similar yield from growth on nitrate. The nitrogen stoichiometry for equivalent growth was defined by the equation 0.5 mM hypoxanthine = 0.5 mM allantoin = 0.5 mM allantoic acid = 1 mM urea = 2 mM nitrate (Oliveira and Antia, 1984).

Cell free extracts

Preparation of cell - free extracts of the microalgae for determination of enzyme activities was conducted according to the procedure described by Shah and Syrett (1984b). Enzyme assays were conducted for xanthine dehydrogenase (Stirpe and Della Corte, 1969), uricase (Theimer and Beevers, 1971), allantoinase and allantoicase (Reinert and Marzluf, 1975). Whenever an enzyme activity was measured it

was established that the rate was linear with time over the period of assay and directly proportional to the amount of cell - free extract added to the assay mixture. Further details are given in the text (Table 7).

Electron Microscopy

Fixations for electron microscopic observations were always carried out at the same time of the day and stage of growth to minimize the impact of the culture conditions on the cellular ultrastructure. Samples were concentrated by gentle centrifugation and fixed for 3 hours at 4°C with 2% to 2.5% (v/v) glutaraldehyde in a saline phosphate (0.17 M) buffer (PBS-pH 7.4) for the cells of Amphidinium carterae or in a saline sodium cacodylate buffer solution (0.1M, pH 7.4) for Dunaliella tertiolecta and Pavlova lutheri. The cells were then post-fixed for 1.5 to 2 hours at 4°C with 1% (v/v) OsO₄ in the same buffer and rinsed thoroughly in the appropriate buffer after each fixation. Final concentration of the samples, prior to dehydration in a graded methanol series and final embedding in Polybed (Epon) 812 (Luft, 1961), were conducted using the bovine serum albumin (BSA) technique (Oliveira et al., 1989). Ultrathin sections were stained with a saturated solution of uranyl acetate in 50% methanol and lead citrate (Reynolds, 1963).

Cytochemistry

Uricase cytochemistry. Cells were concentrated by centrifugation and fixed briefly for 5 minutes in 0.25% (v/v) formaldehyde. They were preincubated in the control medium for 5 minutes. This medium contained 3 mM CeCl₃, 50 mM 3-amino-1,2,4- triazole and 0.001% Triton X-100 in 0.1M Pipes buffer (pH 9.4). The cells were then incubated for an extra 30 minutes at 37°C in the experimental medium which was identical to the control one, except for the addition of 0.1 mM uric acid (Angermuller and Fahimi, 1986). After washing the cells thoroughly with Pipes buffer, they were fixed in 2% formaldehyde for 2 hours at 4°C with the same buffer, followed by post fixation with 1% osmium tetroxide for 1.5 hours at 4°C. The samples were rinsed in buffer after each fixation, dehydrated with a graded methanol series and embedded in Polybed 812. Control experiments were conducted in the presence of 2,6,8 - trichloropurine or oxypurine (2 mM), two competitive inhibitors of uricase.

Catalase cytochemistry. For the cytochemical localization of catalase, the cells were fixed with 2.5% glutaraldehyde in the appropriate buffer, i.e. in a saline phosphate (0.17 M) buffer (PBS - pH 7.4) for Amphidinium carterae and in a saline sodium cacodylate buffer for Dunaliella tertiolecta and Pavlova lutheri, for 2.5 hours and rinsed in the same buffer for 30 minutes at 4°C. The cells were then incubated in the standard 3,3' - diaminobenzidine (DAB)

incubation medium for 60 minutes at 37°C on a rotary shaker (Frederick and Newcomb, 1969). This medium contained 20 mg DAB (Sigma Chemical Co., St. Louis, Mo.), 10 ml of 0.05 mM propanediol buffer (2-amino-2-methyl-1,3-propanediol) at pH 10.0, and 0.2 ml of 3% H₂O₂. The pH was adjusted to 9.0 prior to incubation of the cells. Following the incubation, the cells were rinsed in buffer for 30 minutes and post-fixed in 1% osmium tetroxide in the appropriate buffer for 1.5 hours at 4°C. After rinsing, the samples were processed for electron microscopic observation as described above. Control experiments were conducted by eliminating H₂O₂ from the incubation medium or by adding 3-amino-1,2,4-triazole, a catalase inhibitor.

Morphometry

Calculation of the volume densities (i.e. the volume of individual components per total cytoplasm volume) of the endoplasmic reticulum, mitochondria, peroxisomes and vacuoles was carried out in electron micrographs enlarged to a final magnification of 36,000 X following the stereologic procedures described by Oliveira and Fitch (1988). To compare means of parameters obtained from different nitrogen regimes, Student's t test was used (Snedecor and Cochran, 1967). Two means were considered to differ significantly if the probability of error was $p < 0.05$.

RESULTS

I) Physiological studies

Eleven species of microalgae representing different classes of phytoplankton were tested for their ability to utilize different components of the purine catabolism pathway, i.e. hypoxanthine, allantoin, allantoic acid or urea, as the sole source of nitrogen (Table 1).

Table 2 shows the results of the growth on urea of all species tested. Pavlova lutheri, Thalassiosira nordenskioldii, Dunaliella tertiolecta, Agmenellum quadruplicatum, Hymenomonas elongata, Amphidinium carterae, Isochrysis galbana and Nannochloropsis oculata showed good growth on urea. Both their growth rates and cell yields were approximately equal to those from equivalent nitrate. Thalassiosira pseudonana and Olisthodiscus luteus were also able to grow on urea but less efficiently than the other species, while Prymnesium parvum showed very poor growth rates and cell yields.

Nickel - dependency for growth on urea was demonstrated in Amphidinium carterae and Olisthodiscus luteus since no growth was observed in the absence or at low nickel concentrations (up to 0.01 μM). Growth on urea was improved by 63% for Thalassiosira nordenskioldii by increasing the nickel supplementation. Growth was also improved (20 to 25%) in urea-grown cells of Hymenomonas elongata and Thalassiosira pseudonana when nickel was added into the

culture medium. In contrast, Pavlova lutheri, Dunaliella tertiolecta, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata showed good growth on urea without nickel supplementation. Prymnesium parvum displayed very poor growth on urea with or without the addition of nickel to the culture medium (Table 2).

Maximum growth rates and cell yields were recorded at 1 μM Ni^{2+} for Amphidinium carterae and these values started to decrease above this nickel concentration, especially at 10 μM Ni^{2+} . However, in Olisthodiscus luteus, both maximum growth rates and cell yields were approximately equal at 1, 5 and 10 μM Ni^{2+} . In Thalassiosira nordenskioldii, maximum values for both growth rates and cell yields were obtained at 1 μM Ni^{2+} , with a very slight decrease in these parameters occurring at 5 and 10 μM nickel. Only a small improvement in cell yield values (20%) was observed in urea-grown cells of Thalassiosira pseudonana with 1 μM nickel compared with higher concentrations. In this case no major differences were noted in the values of the growth rate. In Hymenomonas elongata, maximum values for growth rate and cell yield were obtained at 1 μM Ni^{2+} and they started to decrease at higher concentrations (Table 2).

In all of the species tested, urea utilization requires only a short adaptation lag period (between 2 to 4 days), except for Prymnesium parvum where an adaptation period of 10 days was observed. Dunaliella tertiolecta, Agmenellum quadruplicatum and Nannochloropsis oculata showed a lag

period at least twice as long in nickel-supplemented medium compared with cultures grown on urea without nickel additions. The adaptation period decreased by 40 to 100% with increases in nickel supplementation in both Amphidinium carterae and Olisthodiscus luteus (Table 2).

Table 3 shows the growth results of the same microalgae on allantoic acid. Hymenomonas elongata, Pavlova lutheri and Nannochloropsis oculata showed good growth on allantoic acid. Both maximum growth rates and cell yields of Hymenomonas elongata were approximately equal to those from equivalent urea. Although the maximum yields were similar for Pavlova lutheri and Nannochloropsis oculata when they were grown on urea or allantoic acid, their growth rates were different. The growth rates of allantoic acid-grown cultures of Pavlova lutheri were slightly higher (20%) than those from urea, while the opposite situation was detected in Nannochloropsis oculata, i.e. the growth rates of urea-grown cells were higher (25%) than those from allantoic acid. Dunaliella tertiolecta, Agmenellum quadruplicatum and Isochrysis galbana were also able to grow on allantoic acid but less efficiently than in urea. A decrease of approximately 30% was detected in both maximum growth rates and cell yields of allantoic acid-grown cells of Dunaliella tertiolecta, while a 15% decrease in the value of these parameters was recorded in Isochrysis galbana when compared with those from equivalent urea growth. Although the growth rates remained equal in urea- and allantoic acid-grown

cells of Agmenellum quadruplicatum, the maximum yields decreased by 50% in allantoic acid cultures. Growth was not detected in Prymnesium parvum, Thalassiosira nordenskioldii, Thalassiosira pseudonana, Amphidinium carterae and Olisthodiscus luteus when allantoic acid was added into the culture medium as the sole source of nitrogen.

Both growth rates and cell yields were slightly improved (15 to 20%) in allantoic acid-grown cells of Hymenomonas elongata when nickel was added into the medium. The maximum values for these parameters were obtained at $1 \mu\text{M Ni}^{2+}$, and they started to decrease at higher nickel concentrations (5 and 10 μM). At these higher concentrations, this microalga displayed an adaptation lag period of twice the duration of the one from lower nickel concentrations (1 μM or lower). In contrast, Pavlova lutheri, Dunaliella tertiolecta, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata showed no signs of requirement for nickel supplementation since growth improvement was not recorded when nickel was added into the medium containing allantoic acid as the sole source of nitrogen. In fact, the adaptation lag period increased in duration when nickel was added to allantoic acid-cultures of Dunaliella tertiolecta, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata, while no changes were detected in Pavlova lutheri. These observations are similar to those reported for urea-grown cultures (Table 3 compare with

Table 2). None of the species tested grew on allantoin with or without nickel supplementation.

Table 4 shows the results of the growth of the microalgae on hypoxanthine. Prymnesium parvum displayed higher growth on hypoxanthine when compared with those from equivalent urea. An increase of more than 50% in both maximum growth rates and cell yields was observed. An improvement of 10% and 30% in maximum yields and growth rates, respectively, was also detected in hypoxanthine-grown cells of Pavlova lutheri when compared with urea-grown cultures. Although the maximum yields were approximately the same in urea- and hypoxanthine-growth in Dunaliella tertiolecta, Amphidinium carterae and Nannochloropsis oculata, their growth rates were different. A 15% and 40% decrease in the exponential growth rate values were observed in Nannochloropsis oculata and Dunaliella tertiolecta respectively, while an increase of 25% was noted in Amphidinium carterae. Growth was not observed in Hymenomonas elongata, Thalassiosira nordenskioldii, Thalassiosira pseudonana, Agmenellum quadruplicatum, Olisthodiscus luteus and Isochrysis galbana when hypoxanthine was utilized as the sole source of nitrogen.

Nickel-dependency for growth on hypoxanthine was only demonstrated in Amphidinium carterae, since no growth was observed in the absence or at low nickel concentrations (up to 0.01 μM). Maximum growth rates and cell yields were recorded at 1 μM Ni^{2+} and these values started to decrease

above this concentration, especially at $10 \mu\text{M Ni}^{2+}$. The adaptation lag period of this hypoxanthine-grown culture decreased by approximately 75% in duration when nickel was added into the medium at $1 \mu\text{M}$ or above this optimal level (5 and $10 \mu\text{M Ni}^{2+}$). Prymnesium parvum, Pavlova lutheri, Dunaliella tertiolecta and Nannochloropsis oculata showed no requirement for nickel supplementation since good growth was observed on hypoxanthine without addition of nickel into the culture media. No changes in the duration of the adaptation period were detected in hypoxanthine-grown cells of Pavlova lutheri, while the lag period was twice as long when nickel was added into the cultures of Dunaliella tertiolecta and Nannochloropsis oculata. In Prymnesium parvum, the lag period increased by 50% at 5 and $10 \mu\text{M Ni}^{2+}$ when compared with lower nickel concentrations (Table 4).

The optimal concentrations of urea, allantoic acid, hypoxanthine and nickel that support maximum growth of the microalgae are reported in Table 5. Hymenomonas elongata, Pavlova lutheri, Dunaliella tertiolecta, Agmenellum quadruplicatum and Isochrysis galbana displayed the highest values for growth rate and yield when grown with 1.0 mM of urea. In contrast, 2.0 mM of urea supported optimal growth for Amphidinium carterae and Nannochloropsis oculata. Particularly noticeable in this respect was the growth response of Prymnesium parvum which showed the greatest improvement in growth rate and maximum yield values when supplemented with 4.0 mM urea. The optimal concentration of

allantoic acid for those species able to utilize this organic compound as the sole source of nitrogen was determined to be 0.5 mM for Hymenomonas elongata, Pavlova lutheri, Dunaliella tertiolecta, Agmenellum quadruplicatum, Isochrysis galbana, and 1.0 mM for Nannochloropsis oculata. In the case of hypoxanthine, 0.5 mM was the optimal concentration that supported growth of Pavlova lutheri, Dunaliella tertiolecta, 1.0 mM for Amphidinium carterae, Nannochloropsis oculata, and 2.0 mM for Prymnesium parvum.

II) Chelation studies

Nickel chelation, using citrate as the chelator, was also carried out to study microalgal dependency on this trace metal ion. In those organisms showing improvement or good growth on urea with nickel supplementation, growth was inhibited when citrate (5 mM) was added into the medium containing urea as the sole source of nitrogen. The growth inhibition could be reversed by the addition of excess nickel into the culture media (Figs. 1 and 2). In contrast, in the microalgae that showed no requirement for nickel supplementation, the addition of citrate into the medium did not affect either their growth rates or cell yields. Growth reduction or inhibition was not detected when citrate was added into allantoic acid-cultures of Pavlova lutheri, Dunaliella tertiolecta, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata. However, growth inhibition was recorded in allantoic acid-grown cells

of Hymenomonas elongata. The inhibition was reversed by the addition of excess nickel into the medium (Fig. 1). Growth inhibition was detected in Amphidinium carterae when citrate was added into the culture medium containing hypoxanthine as the sole source of nitrogen. This inhibition was also reversed by the addition of excess nickel into the medium (Fig. 2). No changes in growth were observed when citrate was added into hypoxanthine cultures of Prymnesium parvum, Pavlova lutheri, Dunaliella tertiolecta and Nannochloropsis oculata.

III) Inhibition studies

Growth inhibition studies were also carried out in this investigation. Allopurinol, 2,6,8-trichloropurine and hydroxyurea were added at various concentrations into the culture medium containing the appropriate organic nitrogen source (Table 6). The urea-growth of all species of microalgae, with the exception of Dunaliella tertiolecta, was inhibited by hydroxyurea. Hydroxyurea-dependent inhibition of growth was also observed among those species capable of growing with allantoic acid and/or hypoxanthine, with the exception of Dunaliella tertiolecta and Pavlova lutheri. Growth inhibition occurred when 1.0 μM of allopurinol was added to hypoxanthine (nickel was added where required) cultures of Prymnesium parvum, Amphidinium carterae and Nannochloropsis oculata. Slightly higher concentrations of allopurinol were required for Pavlova

lutheri (5 μM) and Dunaliella tertiolecta (2 μM) in order for growth to be inhibited. A similar pattern of growth inhibition responses was also detected in these species when 2,6,8-trichloropurine was added to hypoxanthine-grown cultures.

IV) Enzymatic studies

Cell-free extracts of Amphidinium carterae, Dunaliella tertiolecta and Pavlova lutheri were examined for xanthine dehydrogenase, uricase, allantoinase and allantoicase activities. No activity could be demonstrated in nitrogen-deprived or urea-grown cells. However, with the exception of uricase activity, it was possible to detect all other activities in hypoxanthine-grown cells of these three species of microalgae (Table 7). No inhibitors are known for allantoinase and allantoicase activities, but in the case of xanthine dehydrogenase, inhibition of activity could be demonstrated by utilizing allopurinol (Fig. 3).

V) Ultrastructural and Morphometric studies

In nitrate-grown control cells of Amphidinium carterae, the nucleus always displays condensed chromosomes completely surrounded by perichromatinic granules which measure 300 ± 30 nm in diameter (Figs. 4a, 5 and 8). In urea- (Fig. 5) and hypoxanthine-grown (Fig. 8) cells, the perichromosomal granules are less abundant and smaller in size, measuring 150 ± 12 and 145 ± 15 nm in diameter, respectively. These

alterations are not an artifact of the plane of sectioning since they persist in serial sections. No obvious morphological differences can be observed in the nucleus and nucleolus of the cells of both Dunaliella tertiolecta and Pavlova lutheri grown in different nitrogen regimes (Figs. 16 to 23).

Dictyosomes are rather numerous in cells of Amphidinium carterae grown on different nitrogen sources. The cis-face of the dictyosomes are closely associated with elements of the endoplasmic reticulum (E.R.) through transition vesicles (Figs. 11, 12 and 13, arrowheads). The trans-face of the dictyosomes is usually characterized by the presence of hypertrophied cisternae and smaller vesicular profiles measuring 70 ± 5 nm in diameter. However, while these are moderately represented in nitrate and urea-grown cultures of Amphidinium carterae (Figs. 4a, 5, 11 and 12), they occur in large numbers in the hypoxanthine-grown cells (Figs. 6 and 13). No significant ultrastructural alterations are detected in the dictyosomes of the cells of both Dunaliella tertiolecta and Pavlova lutheri grown in all four different nitrogen regimes.

In Amphidinium carterae, endoplasmic reticulum-like elements are often observed in close proximity to the trans-face of the dictyosomes (Figs. 12 and 13). In hypoxanthine-grown cells of this microalga, E.R. elements frequently congregate in the immediate vicinity of chloroplasts and mitochondria without establishing direct contact with their

envelopes (Fig. 7, arrows). Although not forming extensive congregations, E.R. elements are also observed in the vicinity of chloroplasts and mitochondria in both urea-grown (Fig. 5, arrow), and nitrate-grown cells of Amphidinium carterae (Fig. 4a, arrow). Morphometric measurements reveal that the volume density of the E.R. expressed per cytoplasm volume is 66% ($p < 0.01$) higher in hypoxanthine-grown cells compared to that in urea or nitrate. In Dunaliella tertiolecta, endoplasmic reticulum (ER) profiles are observed predominantly in the apical cytoplasm, some of these showing a consistent relationship to the cis-face of dictyosomes through the occurrence of transition vesicles. In hypoxanthine-grown cells, congregation of E.R. elements is also observed in the immediate vicinity of the trans-face of dictyosomes. Overall, the E.R. is more developed in hypoxanthine-growth than in cells grown in the three other sources of nitrogen (Fig. 19 cf. with Figs. 16, 17 and 18, arrowheads). Similar situation is detected in Pavlova lutheri, especially in hypoxanthine-grown cells, where extensive development of the E.R. is observed throughout the cytoplasm (Fig. 23 cf. with Figs. 20, 21 and 22, arrowheads). Morphometric measurements reveal that the volume density of the E.R. expressed per cytoplasm volume is 45 and 55% ($p < 0.01$) higher in hypoxanthine-grown cells of Dunaliella tertiolecta and Pavlova lutheri, respectively, compared with growth on the three other sources of nitrogen.

No obvious morphological differences could be observed in the chloroplast and pyrenoid structures of Amphidinium carterae cells grown in the different nitrogen regimes (Figs. 4a, 5 and 6). In allantoic acid-grown cells of Dunaliella tertiolecta, most of the chloroplast volume is occupied by starch granules, while in nitrate-, urea- and hypoxanthine-grown cultures only a few starch granules are observed (Fig. 18, cf. with Figs. 16, 17 and 19). No obvious morphological differences are observed in the chloroplast of cells of Pavlova lutheri grown in the different nitrogen regimes (Figs. 20 to 23).

Mitochondria exhibiting typical dinoflagellate tubular cristae can be seen throughout the cell cytoplasm of Amphidinium carterae. However, the number of mitochondria is strikingly different in nitrate-grown cells (19 ± 3 /section) compared with urea- (35 ± 4 /section) and hypoxanthine-grown (52 ± 4 /section) cultures of Amphidinium carterae. This difference may be accounted for by the fact that mitochondria are more frequently observed undergoing division in urea and hypoxanthine-grown (Figs. 5 and 7) than in nitrate-grown cells (Fig. 4a). Morphometric measurements show that the volume density of the mitochondrial compartment increases by 78% and 47% ($p < 0.01$) in hypoxanthine and urea growth, respectively, compared to growth on nitrate. In Dunaliella tertiolecta and Pavlova lutheri, the mitochondria do not appear to differ morphologically when cells are grown in different nitrogen

sources. However, an increase in the number of mitochondrial profiles (Figs. 19 and 23) was detected in both species during hypoxanthine growth. Morphometric measurements show that the volume density of the mitochondrial compartment of hypoxanthine-grown cells of Dunaliella tertiolecta and Pavlova lutheri increases by 40 and 45% ($p < 0.01$), respectively, in relation to growth on the other sources of nitrogen.

In Amphidinium carterae, microbodies are rarely detected in nitrate-grown cultures, and although present in urea-grown cells, they are far from abundant. Microbodies (415 ± 15 nm in diameter) become readily visible when hypoxanthine is supplied to the growth medium along with nickel (Fig. 6, arrowheads). Morphometric measurements show increases of 72% and 28% in the volume density of the peroxisomal compartment of cells grown on hypoxanthine and urea, respectively, compared to those grown on nitrate. These are single membrane-bound organelles with a granular nucleoid-free matrix, frequently seen in close association with membranous elements resembling endoplasmic reticulum profiles (Fig. 9). On occasion some microbody-like profiles appear as a terminal enlargement of endoplasmic reticulum cisternae (Fig. 6, arrow). Other micrographs show microbodies connected to each other by a narrow tubular system (Fig. 10, arrowhead). No microbodies are detected at any time in cells of Dunaliella tertiolecta and Pavlova lutheri grown in all four nitrogen regimes.

The vacuolar apparatus of Amphidinium is subdivided into two major compartments. Both compartments are distinct and independent from the pusule (Fig. 4b). One of these compartments is represented by peripherally located vacuoles, while the other is made up of several central vacuolar profiles. In nitrate-grown cultures, the peripheral vacuoles are well developed and almost free of membranous inclusions (Fig. 4a). In urea-grown cells, membranous inclusions can be seen in well-developed peripheral vacuoles (Fig. 5). Peripheral vacuoles are, however, less developed in hypoxanthine-grown cells (Fig. 6). In contrast, the central vacuoles in nitrate-grown cells are very small, and again there are no extensive accumulation of inclusions (Fig. 4a). In urea-grown cultures, the central vacuolar compartment is more developed, and contains a few membranous inclusions (Fig. 5). The central vacuoles are extremely well-developed, and contain large membranous inclusions in hypoxanthine-grown cells (Fig. 6). Morphometric studies reveal that the vacuolar compartment of hypoxanthine-grown cells of Dunaliella tertiolecta is 50 to 55% larger than that of cells grown in the other sources of nitrogen (compare also Fig. 19 with Figs. 16 to 18). The vacuolar compartment of hypoxanthine- and allantoic acid-grown cells of Pavlova lutheri seems also consistently larger than that observed in cells of the same organism supplied with nitrate or urea as sole sources of nitrogen (compare Figs. 22 and 23 with Figs. 20 and 21). This is confirmed by morphometric

analysis that shows an increase of 67 to 72% ($p < 0.01$) in the volume density of this cellular compartment.

Lipid granules are frequently observed in the cytoplasm of the cells of Dunaliella tertiolecta. These are particularly abundant in allantoinic acid-grown cultures, compared to those grown in nitrate, urea or hypoxanthine (Fig. 18 cf. with Figs. 16, 17 and 19, "L"). Lipid granules are rarely detected in the cells of Pavlova lutheri (Figs. 20 to 23) or Amphidinium carterae (Figs. 4a, 5 and 6).

VI) Cytochemical studies

Cytochemical studies, using cerium chloride to demonstrate the occurrence of uricase activity, show deposition of reaction product within the microbody-like organelles of hypoxanthine-grown cells of Amphidinium carterae. Accumulation of reaction product is particularly intense in certain regions of the microbodies that could be the morphological equivalent of structureless nucleoids (Fig. 14, arrowheads). Control experiments, carried out in the presence of the inhibitors of uricase activity, 2,6,8-trichloropurine or oxypurine, eliminate the deposition of reaction product. In addition to uricase, these organelles also show intense deposition of reaction product indicative of catalase activity (Fig. 15). Deposition of reaction product is absent from samples incubated with aminotriazole, a catalase inhibitor, or without H_2O_2 . In Dunaliella tertiolecta and Pavlova lutheri, the deposition of reaction

product, indicative of uricase activity, occurs within the cristae and in the outer compartment of the mitochondria (Figs. 24 and 28). Deposition of reaction product, however, occurs only after brief fixation (5 min), in very low concentrations of glutaraldehyde (0.25%) and in the presence of uric acid (Table 8). Control experiments show no deposition of reaction product (Figs. 25 and 29). In addition to uricase, mitochondria also show intense deposition of reaction product indicative of catalase activity in both Dunaliella tertiolecta and Pavlova lutheri (Figs. 26 and 30). Deposition of reaction product occurs only at pH 9.0 in cells incubated at 37°C and it is absent from samples incubated with aminotriazole or without H₂O₂ (Figs. 27 and 31, Table 8). A third type of reaction product deposition in mitochondria of these microalgae is observed at pH 6.0 when the cells are incubated at room temperature in the absence of H₂O₂. Under these circumstances, the deposition of reaction product is strongly inhibited by low concentrations of potassium cyanide which is indicative of cytochrome system-dependent oxidative activity. Furthermore, while the cytochrome system-dependent deposition of reaction product occurs in cells grown in all four sources of nitrogen tested, uricase- and catalase-dependent deposition of reaction products are only observed in mitochondria of hypoxanthine grown cells (Table 8).

DISCUSSION

Growth Studies

The results clearly demonstrate the widespread occurrence of urea - utilizing capabilities on the part of all microalgae tested, with significantly greater growth exhibited by some species relative to the nitrate controls. The facility and efficiency of urea utilization by most of the species surveyed confirm the findings of previous authors that urea is one of the most important sources of organic-N available for growth of marine phytoplankton in the oceans (see Oliveira and Antia, 1984 for review). In a previous study, Prymnesium parvum showed excellent growth on urea, while Amphidinium carterae displayed a low nickel requirement for growth on this organic-N source. It was also shown that nickel supplementation was toxic for urea-grown cells of Olisthodiscus luteus (Oliveira and Antia, 1986a). In contrast, the present study shows, as previously observed by Droop (1955), poor growth on urea for Prymnesium parvum, while nickel is required at higher concentrations to support the urea-growth of Amphidinium carterae and especially Olisthodiscus luteus (Table 2). I have utilized the same culture conditions and media as in the previous study. Therefore, I attribute the discrepancies to the use of different clones of these microalgae.

The available evidence, on the distribution of urea-degrading enzymes, indicates that two alternative enzyme

systems (urease and ATP:urea amidolyase) occur in algae. ATP:urea amidolyase is restricted to some members of the Chlorophyceae, while urease occurs in all other algal classes and some of the chlorophycean algae examined so far (Leftley and Syrett, 1973; Bekheet and Syrett, 1977; Syrett, 1981; Al - Houty and Syrett, 1984). More germane to this problem was the identification of nickel as a constituent of jackbean (Canavalia ensiformis L.) urease (Dixon et al., 1975) and soybean tissue cultures (Polacco, 1977). A nickel requirement for urease activity was also reported for some microalgae (Syrett, 1981; Rees and Bekheet, 1982; Oliveira and Antia, 1986b). Therefore, the strict requirement for Ni^{2+} exhibited by Amphidinium carterae and Olisthodiscus luteus for growth on urea as sole source of organic-N suggests that urease is the enzyme responsible for the conversion of urea into utilizable ammonium (Polacco, 1977). This is confirmed by both the hydroxyurea- and citrate-dependent growth inhibitions observed when these microalgae are supplemented with urea as sole source of nitrogen.

The endogenous nickel content of the sea water used in the preparation of our test media is 3.42 nM and our estimate of endogenous nickel in the final media, excluding contamination from added salts, is in the order of 2.7 and 1.4 nM for the salinities of 26 and 14‰ utilized in the present study, respectively (Oliveira and Antia, 1984). The growth enhancement observed in urea-grown cells of

Hymenomonas elongata, Thalassiosira nordenskioldii and Thalassiosira pseudonana with nickel supplementation, therefore, implies that urease is also present in these microalgae. This is further confirmed by the strong growth inhibition observed when citrate, the natural Ni^{2+} -chelator found in nickel accumulating plants (Kersten et al., 1980), is added to the culture media. In contrast, the growth of Pavlova lutheri, Dunaliella tertiolecta, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata on urea displayed no signs of nickel-requirement. Furthermore, the addition of citrate did not affect the growth of these organisms. These data suggest that either these organisms have the capacity to readily concentrate Ni^{2+} from the nanomole levels normally occurring in seawater (see Oliveira and Antia, 1986a for review) or they use the ATP:urea amidolyase Ni^{2+} -independent system for the conversion of urea directly to utilizable ammonium (Oliveira and Antia, 1986b). In the case of Pavlova lutheri, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata, the first explanation is more likely to be true, since growth of these microalgae in urea as sole source of nitrogen is inhibited by hydroxyurea, a potent inhibitor of urease activity (Reithel, 1971; Carvajal et al., 1982). This compound is without effect on the urea-supported growth of Dunaliella tertiolecta. Instead, this is inhibited by avidin, a known inhibitor of ATP:urea amidolyase activity. Furthermore, addition of

biotin abolishes the avidin-induced inhibition (unpublished results). These results are indicative of the occurrence of ATP:urea amidolyase activity and explains the reason why a nickel requirement is absent from this microalga (Leftley and Syrett, 1973; Carvajal et al., 1982).

The long adaptation lag period observed in urea-grown cells of Prymnesium parvum together with its poor growth on this nitrogen source suggest that this microalga may not be well equipped to utilize the organic nitrogen of urea. This is further confirmed by their inability to grow efficiently on this organic nitrogen source even at higher concentrations (Antia et al., 1975). The problem is not one of nickel dependency since additions of nickel ions to the culture media failed to improve growth. Since hydroxyurea completely inhibits whatever growth is detected in the presence of urea, the more likely explanation for the poor results observed is the impairment of the mechanism of urea uptake by these cells.

According to Antia et al., (1989), allantoic acid has not been previously tested as an organic N-source for the growth of microalgae. Under our test conditions, only 6 of the 11 species studied, showed moderate to good growth on this nitrogen source. The growth observed in 0.5 mM allantoic acid-grown cultures of Hymenomonas elongata, Pavlova lutheri and Nannochloropsis oculata is equivalent to the one from 1 mM urea-supplemented cultures. Since the N-atoms stoichiometry of allantoic acid versus urea is 4:2,

the previous observation suggests that these microalgae utilize the allantoic acid - N as efficiently as that from urea. These results may then be construed to imply that allantoic acid might be catabolized to urea and ureidoglycolate by the enzyme allantoicase (Antia et al., 1989; Shah and Syrett, 1984b). This is supported by the determination of allantoicase activity in cell-free extracts of some of these microalgae (Table 7). The inhibition of allantoate-supplemented cultures of Hymenomonas elongata and Nannochloropsis oculata by hydroxyurea further supports this interpretation. However, the absence of inhibition displayed by allantoate-grown cells of Pavlova lutheri treated with hydroxyurea suggests that in this case urea may not be one of the products of the catabolic action of this enzyme.

The growth recorded in 0.5 mM allantoic acid-grown cells of Dunaliella tertiolecta, Agmenellum quadruplicatum and Isochrysis galbana was less than that from 1.0 mM urea, especially for Agmenellum quadruplicatum (compare Table 3 with Table 2). Antia et al., (1989) suggested that culture factors, such as temperature, pH or illumination could affect the capability of microalgae to decompose the entire structure of allantoic acid and utilize all 4 N atoms present in the molecule; hence, the lower efficiency in the utilization of allantoic acid-N. The inhibition of allantoate-grown cultures of Agmenellum quadruplicatum and Isochrysis galbana by hydroxyurea suggests that conversion of allantoic acid to urea by the catalytic action of the

enzyme allantoicase also occurs in these microalgae. Hydroxyurea showed no effect upon the growth of allantoate-supplemented cultures of Dunaliella tertiolecta. However, these results are not surprising since ATP:urea amidolyase, not urease, was shown to be the enzyme responsible for the utilization of urea-N in this genus (Leftley and Syrett, 1973). The nickel-independence of ATP:urea amidolyase explains the absence of Ni²⁺-requirement for growth on this and other urea-producing sources of organic N (Oliveira and Antia, 1986b). Nickel-independence was also demonstrated in allantoic acid cultures of Pavlova lutheri, Agmenellum quadruplicatum, Isochrysis galbana and Nannochloropsis oculata. In contrast, the addition of citrate to allantoic acid-supplemented cultures of Hymenomonas elongata suppressed growth, suggesting that this metal ion is required for growth on this organic-N source.

Allantoin was also tested for its ability to support the growth of phytoplankton as the sole source of organic-N, but no growth was observed in all species studied. Except for certain benthic-type species which appear to be well equipped for utilizing allantoin, Antia et al., (1980b) suggested that this organic-N is generally a poor N-source for phytoplankton growth relative to the purines. A similar situation was also observed in Chlorella pyrenoidosa (Ammann and Lynch, 1964). These investigators indicated that a cellular permeability barrier to allantoin might possibly be the cause of the absence of growth.

Hypoxanthine was chosen as the prime purine representative for this investigation not only on account of its established stability in natural sea water but also because unlike adenine or guanine, it does not possess any exocyclic nitrogenous group; hence any nitrogen utilization must depend on the ability of a particular species to retrieve the purine-skeleton nitrogen (Antia et al., 1989). Out of the 11 species examined in this investigation, only 5 showed moderate to good growth on hypoxanthine. Prymnesium parvum proved to be rather interesting in the sense that it grows well on hypoxanthine, while poor growth was observed on urea. The situation is similar to the one previously reported by Antia et al., (1975) for Chlamydomonas palla. However, in the case of Prymnesium parvum, a higher substrate concentration (2 mM) in comparison with other hypoxanthine utilizers (0.5 to 1 mM - Table 5) is required for growth to occur. This suggests that this microalga may possess a less effective permease or uptake system for hypoxanthine (Antia et al., 1980a). The growth observed in hypoxanthine-cultures of Pavlova lutheri, Nannochloropsis oculata and Dunaliella tertiolecta was equivalent to the one from allantoinic acid- and also urea-grown cells of these species (Table 5). Since both hypoxanthine and allantoinic acid contain 4 N atoms versus 2 N atoms per molecule of urea, the evidence indicates that these 3 species of microalgae are efficient utilizers of all the hypoxanthine -N. The hypoxanthine growth of these microalgae is not

dependent upon Ni^{2+} supplementation and proceeds in the presence of the Ni^{2+} chelator citrate. Amphidinium carterae also exhibited good growth on hypoxanthine. However, this species requires the addition of nickel into the culture medium. The growth observed, under these circumstances, is similar to the one from equivalent urea plus Ni^{2+} supplemented cultures (compare Table 4 with Table 2). The addition of citrate to hypoxanthine-cultures of Amphidinium carterae suppressed their growth. The growth suppression is reversible, since the addition of excess nickel into the culture medium restores it. These data further support the nickel-dependency of this process and confirms that urease is the enzyme responsible for urea catabolism in this microalga.

Inhibitor Studies and Determination of Enzymatic activities

As reported by Fujihara and Yamaguchi (1978), allopurinol [4-hydroxypyrazolo (3,4-d) pyrimidine], an analogue of hypoxanthine, is a potent inhibitor of the enzyme xanthine dehydrogenase. This enzyme is responsible for the oxidation of hypoxanthine to xanthine, and this to uric acid (Antia et al., 1989). Therefore, the growth inhibition observed in hypoxanthine-grown cultures of Prymnesium parvum, Pavlova lutheri, Dunaliella tertiolecta, Amphidinium carterae and Nannochloropsis oculata when allopurinol was added into the culture medium, implies that the degradation of hypoxanthine \rightarrow xanthine \rightarrow uric acid is dependent on the

activity of xanthine dehydrogenase. The demonstration of the occurrence of xanthine dehydrogenase activity in some of these microalgae confirms this interpretation (Table 7).

Uricase (urate:oxygen oxidoreductase) is a cuproprotein that catalyzes the oxidation of urate in the presence of oxygen, yielding hydrogen peroxide (H_2O_2), carbon dioxide and allantoin (Mahler et al., 1955). A number of substances are known to be effective inhibitors of uricase. These include oxypurines, 2,6,8-trichloropurine and oxonate (Muller and Moller, 1969 and references cited therein). The growth inhibition of hypoxanthine-supplemented cultures of these microalgae, treated with trichloropurine, indicates that uricase is responsible for the uric acid \rightarrow allantoin catalytic conversion. Failure to detect uricase activity in crude cell-free extracts of the microalgae may be related to the high sensitivity of this enzyme to changes in pH, among other factors (Huynh and Oliveira, 1989a, b). Indeed, Shah and Syrett (1984b) also failed to detect uricase activity in crude extracts of the marine diatom Phaeodactylum tricornutum, although activity was measurable in partially purified fractions of the same extract. Urate oxidation proceeds simultaneously with the degradation by catalase of the H_2O_2 formed during the reaction. It is then important to notice that cytochemical studies of Amphidinium carterae, Dunaliella tertiolecta and Pavlova lutheri reveal the occurrence of both uricase and catalase activities (Huynh and Oliveira, 1989a, b). The determination in crude cell

preparations of some of the microalgae of allantoinase and allantoicase activities suggests then that the standard pathway of purine degradation also occurs in microalgae and it is responsible for the utilization of the nitrogen of purines and its derivatives via urea production (Vogels and van der Drift 1976; Reynolds et al., 1982). The fact that urease inhibitors, with the exception of Dunaliella tertiolecta and Pavlova lutheri, block the growth of hypoxanthine-supplemented cultures of the other three species able to grow on this N source further supports this interpretation. The lack of hypoxanthine-growth inhibition of Dunaliella tertiolecta by urease inhibitors reflects the occurrence of ATP:urea amidolyase activity (Leftley and Syrett, 1973). The fact that utilization of hypoxanthine and allantoic acid by Pavlova lutheri remains unaffected in the presence of urease inhibitors suggests that the catabolic oxidation of purines and their derivatives does not involve the production of urea. It is interesting regarding these findings that recently Winkler et al., (1987, 1988) showed that allantoin catabolism in soybean suspension culture cells is carried out by two amidohydrolase reactions, allantoate amidohydrolase and ureidoglycolate amidohydrolase. Under these circumstances the whole process proceeds without detectable production of urea. The ability for urea utilization exhibited by this microalga may then represent a system independent from that of purine catabolism, such as that of the ornithine cycle

involving urea release from arginine by arginase (Naylor, 1970). However, whether or not a two amidohydrolase system similar to the one observed in soybean exists in Pavlova lutheri remains to be determined.

Ultrastructure and Cytochemical Studies on Amphidinium carterae

Recent studies have shown that high levels of the transition metals Fe, Ni, Cu and Zn are found in the condensed chromatin of a variety of dinoflagellates (for review see Spector, 1984). Studies on the uptake of ^{63}Ni into dinoflagellate chromosomes reveal that there is a continuous increase in the mean level of this ion over a 24 hrs period. This was suggested either to be indicative of a continuous change in the balance of transition metals within existing chromatin or to be related to the formation of new chromatin by continuous DNA synthesis (Sigeo, 1982). Although the function of the perichromatinic granules is still unknown, the Spector et al. (1981) model on dinoflagellate chromosome organization suggests that they play a role in the stabilization of the chromosomes. The ultrastructural changes in the perichromatinic granules may then reflect the response of the cells to the presence of Ni^{2+} in the growth medium of both urea and hypoxanthine cultures.

In a recent review on the ultrastructure of dinoflagellates, Dodge and Greuet (1987) point out that it

is usually difficult to differentiate between the forming (cis) face and the maturing (trans) face of dictyosomes in these microalgae. In Amphidinium carterae, the cis- and trans-faces of the dictyosomes are distinct in all three nitrogen nutritional regimes tested. The cis-face shows a clear relationship with endoplasmic reticulum elements which includes transition vesicles (Kristen, 1980). The cisternae of the trans-face are, in turn, always hypertrophied. Another distinctive feature of the trans region of the dictyosomes is the presence of vesicular-like profiles measuring 70 ± 5 nm in diameter. These are particularly abundant in hypoxanthine-grown cells. Studies on the effects of heavy metals on the growth of microalgae have shown increased dictyosomal activity (hypertrophy and vesiculation) at the trans region of the organelles (Smith, 1983; Chan and Wong, 1987). In the case of Skeletonema costatum, this increase in dictyosomal activity was suggested to be related to mechanisms of metal sequestration (Smith, 1983).

The presence of large membranous inclusions within vacuoles is indicative of extensive autophagic activity (Marty et al., 1980). Why such widespread autophagic activity would develop in hypoxanthine-grown cells escapes our present understanding of the nutritional effects of different nitrogen sources on cellular ultrastructure. However, the effect is not a deleterious one, since growth in hypoxanthine-nickel supplemented medium proceeds as

efficiently as in nitrate or urea plus nickel conditions. Recent studies showed that in aquatic organisms several cellular compartments (i.e. Golgi apparatus, lysosomes) play a major role in intracellular metal homeostasis. The extent to which any one of these compartments is involved in metal binding appears to depend on a number of factors, including nutritional regime and interactions with competing metal ions (Fowler, 1987). The increase in the vacuolar apparatus of Amphidinium carterae could then, at least in part, be a reflection of such processes. The fact that an increase also occurs in the vacuolar system of urea plus nickel but not nitrate/nickel-free supplemented cells supports this interpretation.

Based on the distribution of the enzymes of purine catabolism, the ureide allantoin is hydrolyzed to allantoic acid by allantoinase possibly located in the endoplasmic reticulum (Hanks et al., 1981; 1983). The increase observed in the complexity and size of the ER in hypoxanthine-grown cells seems then consistent with the key role this organelle plays in the catabolism of purines. The proliferation in endoplasmic reticulum coincides also in these cells with a large increase in the number of microbodies. Endoplasmic reticulum elements are often closely positioned to the bounding membrane of these organelles and occasionally direct continuity between ER and microbodies can also be observed (Fig. 4, arrow). The situation resembles that of the root nodules of higher plants specialized for ureide

production and suggests that in Amphidinium carterae microbodies may originate from the ER (Newcomb et al., 1985; Kaneko and Newcomb, 1987; Webb and Newcomb, 1987). In some micrographs, microbodies are connected to one another by tubular profiles (Fig. 8, arrowhead). These are indicative of the existence of a peroxisomal reticulum and suggest that microbodies may also be formed by the fission of preexisting ones (Lazarow and Fujiki, 1985).

The functional specialization of microbodies (e.g. peroxisomes) has been investigated extensively in higher plants (Beever, 1979; Tolbert and Essner, 1981) but rarely so with respect to microalgae. Considering the information available, it seems that there are two groups of microbodies in the algae. One group is represented by unspecialized microbodies characterized mainly by the presence of catalase and uricase activities. The other by organelles functionally resembling the glyoxysomes and peroxisomes of higher plants (Stabeneau, 1984). The demonstration of both uricase and catalase activities in Amphidinium could then be taken as indicative of the occurrence of unspecialized microbodies in the cells of this dinoflagellate. However, the proliferation of microbodies is only observed in hypoxanthine-growth which is a strong indicator of some major alteration(s) in the endogenous metabolism of these cells. In ureide-producing root nodules uric acid is oxidized to allantoin in the peroxisomes by uricase. This reaction produces H_2O_2 which is degraded by catalase, also located in the peroxisomes (Hanks

et al., 1981; Schubert, 1986; Kaneko and Newcomb, 1987; Webb and Newcomb, 1987; Vaugh and Stegink, 1987). These observations show that hypoxanthine-growth of Amphidinium very likely occurs via its conversion to urea through catabolic oxidation and that the large increase in the microbody population is related to this type of metabolism. The fact that no uricase and catalase activities are detected in nitrate or urea-grown cells supports this interpretation.

In nitrate-grown cultures microbodies are rarely detected, a fact that confirms observations by other authors (Dodge and Crawford, 1968; Klut et al., 1981). They are also far from abundant in urea-grown cells compared with hypoxanthine-grown cells. These features in conjunction with the absence of enzymatic activity for uricase and catalase suggest that these microbodies are functionally different from those in hypoxanthine-grown material. In a recent study of the catalase-negative microbodies of Amphidinium carterae, Klut et al. (1984) suggested that these organelles might be regarded as atypical glyoxysomes where the marker enzymes of the glyoxylate cycle may be absent or repressed. The evidence supports then the existence in this microalga of two functionally distinct, nutrition-related populations of microbodies. Such a situation seems also to occur in Euglena where microbodies appear to perform like peroxisomes during autotrophic growth and as glyoxysomes

under heterotrophic conditions (Graves et al., 1972; Collins and Merrett, 1975).

Klut et al. (1984) interpreted the lack of catalase in these microbodies as indicative of a corresponding lack of glycolate oxidase. Although little is known of the overall mechanism of photorespiration in dinoflagellates, the available evidence supports the absence of glycolate oxidase from these organelles (Burris, 1977). It suggests instead that glycolate metabolism is initiated by glycolate dehydrogenase. This enzyme cannot transfer electrons directly to oxygen; hence, it does not form H_2O_2 during glycolate oxidation. In algae, glycolate dehydrogenase is usually located in mitochondria rather than microbodies (Stabenau, 1984). In Scenedesmus obliquus, the increase in the number of mitochondria during adaptation to low CO_2 was suggested to reflect an increase in the activity of glycolate metabolism (Kramer and Findenegg, 1978). The occurrence of large numbers of mitochondria in cells of Amphidinium could then reflect, at least in part, their participation in photorespiration (Klut et al., 1981).

Ultrastructural and Cytochemical Studies on Dunaliella tertiolecta and Pavlova lutheri

As previously discussed, the increase in endoplasmic reticulum reported to occur in cells of the dinoflagellate Amphidinium carterae grown on hypoxanthine as sole source of nitrogen, is indicative of the importance of the E.R. in the utilization of hypoxanthine-N (Huynh and Oliveira, 1989a). The larger development of the endoplasmic reticulum (E.R.) in hypoxanthine-grown cells of Dunaliella tertiolecta and Pavlova lutheri seems then also consistent with the key role this organelle plays in the catabolism of purines (Hanks et al., 1981; 1983).

The oxidation of urate and the subsequent degradation by catalase of the H_2O_2 formed in the uricase reaction are usually compartmentalized in microbody-like organelles, the peroxisomes (Schubert, 1981; Huynh and Oliveira, 1989a). The intriguing fact in both Dunaliella tertiolecta and Pavlova lutheri is the location of these reactions, since no microbody-like organelles are detected in cells grown on all four sources of nitrogen tested. It is then significant that cytochemical deposition of reaction products indicative of the occurrence of urate oxidase and catalase activities is observed in mitochondria. It is interesting also to notice that these coincide with an increase in the volume density of the mitochondrial compartment. The cytochemical localization of uricase and catalase activities in mitochondria is an unusual finding, although uricase activity was reported before to be associated with the mitochondrial fraction of soybean seeds (Glycine max A62-1:

nodulating variety). It is interesting to notice that in this case uricase activity was only detected during certain periods of plant development (Tajima and Yamamoto, 1975). The situation resembles that observed in both Dunaliella tertiolecta and Pavlova lutheri, since uricase activity is only detected in hypoxanthine but not allantoic acid, urea or nitrate supported growth.

The question of the specificity of the cytochemical reactions must be carefully considered. The pH optimum for urate oxidase activity was shown to be above 9, although in soybean radicles an optimum pH of 7.0 was reported (Muller and Moller, 1969; Tajima and Yamamoto, 1975). In this last instance, however, the enzymatic activity proved to be due not to uricase but to two other enzymes, diamine oxidase and peroxidase (Tajima et al, 1985). It is important to notice then that the deposition of reaction product indicative of uricase activity in mitochondria of both Dunaliella tertiolecta and Pavlova lutheri occurs at high pH values and rapidly declines with decreasing pH (Table 8). Angermuller and Fahimi (1986 and references cited therein) showed that urate oxidase activity is extremely sensitive to aldehyde fixation. Even short fixation periods of 20 min with 1% aldehyde produces a 90% loss in activity (Yokota and Nagata, 1974). Deposition of reaction product can only be obtained in our material by lowering the concentration of glutaraldehyde to 0.25% (v/v) and using a (very short) 5 min fixation period. It is also important to emphasize that

no deposition of reaction product occurred in mitochondria of these two microalgae when uric acid was omitted from the incubation medium. Trichloropurine has been extensively used in cytochemical studies as a control test for the identification of uricase-dependent reaction product deposition in organelles of a variety of plant cells. Under these conditions staining is almost completely or even totally abolished (Huynh and Oliveira, 1989a, and references cited therein). In the case of both Dunaliella tertiolecta and Pavlova lutheri, no deposition of reaction product occurs in mitochondria when the cells are incubated in the presence of trichloropurine. The results are then consistent with those used by other authors for the cytochemical demonstration of urate oxidase activity in both plant and animal cells (see Angermuller and Fahimi, 1986; Huynh and Oliveira, 1989a for reviews).

3,3'-Diaminobenzidine (DAB) is a widely utilized substrate in the cytochemical localization of peroxidatic or oxidative activities of peroxidase, catalase and the mitochondrial cytochrome system, respectively (Frederick, 1987). Deposition of cytochrome dependent reaction products are known to be optimized at pH 6.0, in the absence or the presence of very low levels of H_2O_2 and at room temperature. The reaction is usually abolished or strongly inhibited by low concentrations of potassium cyanide. Reaction product deposition with these characteristics is also detected in mitochondria of Dunaliella tertiolecta and Pavlova lutheri

grown in all four sources of nitrogen (Table 8). This evidence suggests reaction product to be formed as a consequence of the cytochrome system, including cytochrome c oxidase activity (Silverberg and Sawa, 1974; Taylor and Hall, 1978; Olah and Mueller, 1981).

These reactions are distinct from the reaction observed in mitochondria of both microalgae at pH 9.0 and after longer fixation periods. Under these conditions, deposition of reaction products occurs only in the presence of higher levels of H_2O_2 when the cells are incubated at $37^\circ C$. The deposition of reaction products is completely abolished by aminotriazole, a non-competitive inhibitor of catalase, but it is not affected by potassium cyanide, a non-competitive inhibitor of the mitochondrial cytochrome system or by trichloropurine, an inhibitor of uricase. These characteristics are considered indicative of the occurrence of catalase activity in mitochondria of both microalgae (Novikoff and Goldfisher, 1969; Silverberg and Sawa, 1974; van der Rhee et al, 1977; Olah and Mueller, 1981). Furthermore, as in the case of uricase activity, catalase-dependent deposition of reaction product is only observed in mitochondria of hypoxanthine-grown cells (Table 8). These findings contrast with the cytochrome-dependent deposition of reaction products that is observed in mitochondria of both microalgae grown on all four sources of nitrogen; hence, they suggest that synthesis of uricase and catalase

and their localization in mitochondria is substrate dependent.

An increase in starch granules, cytoplasmic and chloroplast lipids has been shown to occur in association with aging in cells of Dunaliella primolecta (Eyden, 1975) and Dunaliella tertiolecta (Hoshaw and Maluf, 1981) grown photoautotrophically. Similar observations have also been reported in aging cells of both photoautotrophic and photoheterotrophically grown cultures of Chroomonas salina (Antia et al., 1973). However, the increase in starch and lipids observed in allantoic acid-grown cells of Dunaliella tertiolecta can not be attributed to aging since the cells used in this study were obtained from the early exponential growth phase. Why such an increase in starch and lipids would only develop in allantoic acid-grown cells escapes our present understanding of the nutritional effects of different nitrogen sources on cellular ultrastructure. Nevertheless, the effect is not a deleterious one, since growth in allantoic acid cultures proceeds as efficiently as in nitrate, urea or hypoxanthine-supplemented cells. An interaction between chloroplasts and vacuoles with respect to the regulation of nitrogen metabolism was reported in Chlorella (Tischner, 1984). However, whether a similar interaction can account for the increases observed in the volume density of the vacuolar compartments of both Dunaliella tertiolecta and Pavlova lutheri remains to be determined.

Conclusions

In conclusion, it can be said that some microalgae possess the ability to utilize purines and/or their derivatives as sole sources of N. The catabolic oxidation of these compounds seem to occur in most of these microalgae by the standard pathway of purine oxidation described for other microorganisms (Vogels and van der Drift, 1976) and higher plants (Reynolds et al., 1982). However, in the case of the Prymnesiophyte Pavlova lutheri, oxidation of purines seems to take place without final conversion to urea. This indicates the occurrence in this microalga of a modification of the pathway of purine-derived N utilization (Antia et al., 1989) and resembles the situation reported for soybean suspension cell cultures (Winkler et al., 1987, 1988).

The growth of Amphidinium in organic N-sources (urea and hypoxanthine) plus Ni^{2+} produces major ultrastructural changes. Some of the changes (i.e. size of perichromatinic granules, number of dictyosome-derived vesicles, size and distribution of the vacuolar apparatus) can be attributed to mechanisms of metal homeostasis. Other changes (i.e. increases in ER and microbodies) indicate the occurrence of a mechanism for the catabolic degradation of purines similar to that observed in higher plants. The demonstration of both uricase and catalase activities in microbodies of hypoxanthine-grown cells supports this interpretation.

The growth of Dunaliella tertiolecta and Pavlova lutheri in hypoxanthine also produces ultrastructural changes (i.e. increases in E.R. and mitochondria) directly related to the occurrence of a mechanism for nitrogen utilization through the catabolic degradation of purines and their derivatives. The cytochemical demonstration of both uricase and catalase activities suggests that in the absence of microbodies, the key step of the oxidation of urate to allantoin and the disposal of the H_2O_2 produced in this reaction was transferred to mitochondria.

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APPENDIXFIGURE EXPLANATION

Symbols: CH (or Ch)= chloroplast, ER = endoplasmic reticulum
L = lipid inclusion, M = mitochondrion,
m = microbodies, N = nucleus, n = nucleolus,
Pu = pusule, Py = pyrenoid, V = vacuole

Figure 1 - Growth curves of Hymenomonas elongata on urea (1 mM) without citrate addition (Δ), with 5 mM citrate (\blacktriangle) followed by the addition of 25 μM Ni^{2+} at day 16 (\downarrow), and also on allantoic acid (0.5 mM) without citrate addition (\square), with 5 mM citrate (\blacksquare) followed by the addition of 25 μM Ni^{2+} at day 16 (\downarrow). Corresponding control growth on nitrate (2 mM) with or without citrate addition is shown (\times).

Figure 2 - Growth curves of Amphidinium carterae on urea (1 mM) without citrate addition (Δ), with 5 mM citrate (\blacktriangle) followed by the addition of 25 μM Ni^{2+} at day 14 (\uparrow), and also on hypoxanthine (0.5 mM) without citrate addition (\bullet), with 5 mM citrate (\circ) followed by the addition of 25 μM Ni^{2+} at day 10 (\uparrow). Corresponding control growth on nitrate (2 mM) with or without citrate addition is shown (\times).

Figure 3 - Detection of xanthine dehydrogenase activity in cell - free extracts of Amphidinium carterae previously grown on 0.5 mM hypoxanthine in the presence of $1 \mu\text{M Ni}^{2+}$. Absorbance measurements at 340 nm for the enzyme or extract (\square), enzyme + allopurinol (Δ), enzyme + hypoxanthine (\dagger), and enzyme + hypoxanthine + allopurinol (\bullet).

Figure 4a - Longitudinal section through a cell grown in 2 mM nitrate and no nickel. Arrow points to ER in the vicinity of chloroplast. Figure 4b shows part of the pusule region.

Figure 5 - Longitudinal section through a cell grown in 1 mM urea and $1 \mu\text{M}$ nickel. Arrow points to ER in the vicinity of the chloroplast.

Figure 6 - Longitudinal section through a cell grown in 0.5 mM hypoxanthine and $1 \mu\text{M}$ nickel. Arrowheads point to microbodies, while the arrow shows a microbody developing from ER - like elements.

Figure 7 - Section through a hypoxanthine/nickel - grown cell shows numerous mitochondria undergoing division. Arrows point to ER elements.

Figure 8 - Nucleus of a hypoxanthine/nickel - grown cell.

Figure 9 - Microbody - ER association in a hypoxanthine/nickel - grown cell.

Figure 10 - Microbodies connected by a narrow tubular structure (arrowhead) in an hypoxanthine/nickel - grown cell.

Figure 11 - Associations between dictyosome - ER in a nitrate- grown cell. Transition vesicles are indicated by arrowheads.

Figure 12 - ER - dictyosome associations in a urea/nickel - grown cell. Transition vesicles are indicated by arrowheads.

Figure 13 - ER - dictyosome associations in a hypoxanthine/nickel - grown cell. Arrowhead points to transition vesicles.

Figure 14 - Deposition of reaction product (arrowheads) indicative of uricase activity in microbodies of a hypoxanthine/nickel - grown cell.

Figure 15 - Deposition of reaction product indicative of catalase activity in a microbody of a hypoxanthine/nickel - grown cell.

Figures 16 to 19 - Longitudinal sections through cells of Dunaliella tertiolecta grown in 2 mM nitrate (Figure 16), 1 mM urea (Figure 17), 0.5 mM allantoinic acid (Figure 18) and 0.5 mM hypoxanthine

(Figure 19). Arrowheads point to elements of the endoplasmic reticulum.

Figures 20 to 23 - Longitudinal sections through cells of Pavlova lutheri grown in 2 mM nitrate (Figure 20), 1 mM urea (Figure 21), 0.5 mM allantoic acid (Figure 22) and 0.5 mM hypoxanthine (Figure 23). Arrowheads point to elements of the endoplasmic reticulum.

Figures 24 and 28 - Cytochemical localization of uricase activity in mitochondria of Dunaliella tertiolecta (Figure 24) and Pavlova lutheri (Figure 28) grown on hypoxanthine (0.5 mM) as sole source of nitrogen.

Figures 25 and 29 - Cytochemical inhibition of uricase-dependent reaction product deposition in mitochondria of Dunaliella tertiolecta (Figure 25) and Pavlova lutheri (Figure 29) treated with 2 mM trichloropurine.

Figures 26 and 30 - Cytochemical localization of catalase activity in mitochondria of Dunaliella tertiolecta (Figure 26) and Pavlova lutheri (Figure 30) grown on hypoxanthine (0.5 mM) as sole source of nitrogen.

Figures 27 and 31 - Cytochemical inhibition of catalase-dependent reaction product deposition in mitochondria of Dunaliella tertiolecta (Figure 27) and Pavlova lutheri (Figure 31) treated with 3-3' amino-1,2,4,-triazole.

Figure 1 - Hymenomonas elongata
Nickel chelation with Urea/Allantoic acid as N-source

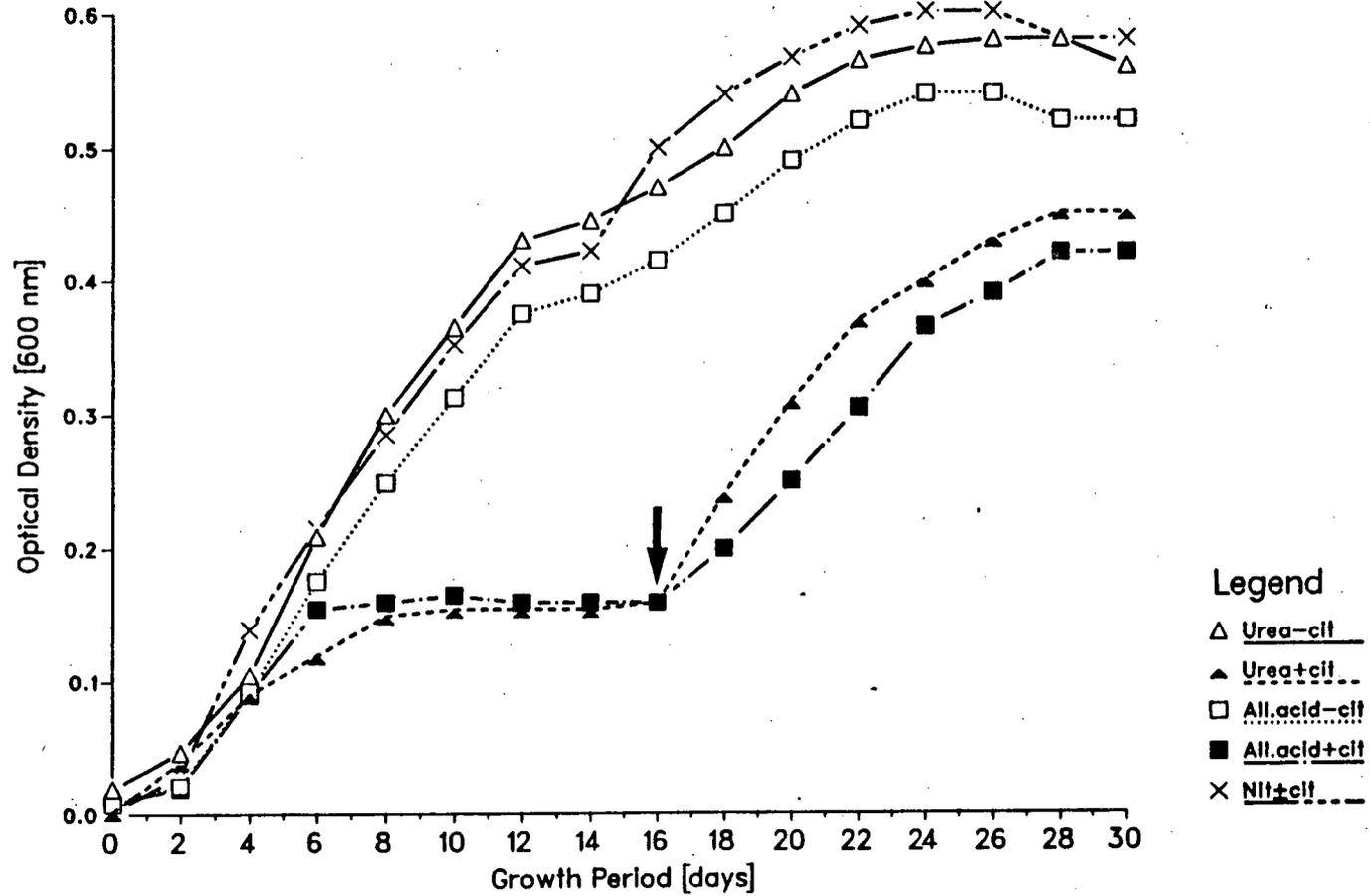


Figure 2 - *Amphidinium carterae*
Nickel chelation with Urea/Hypoxanthine as N-sources

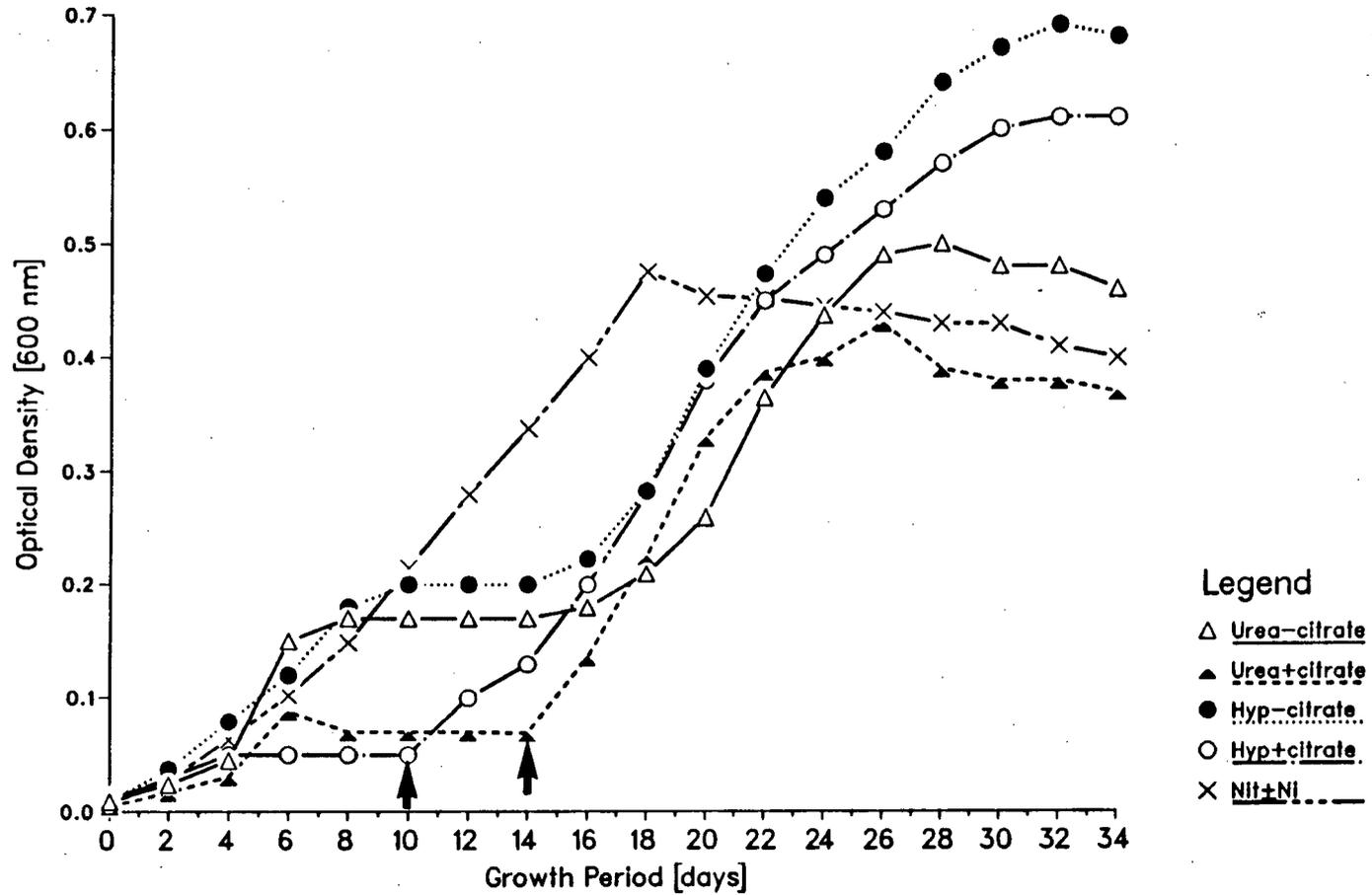
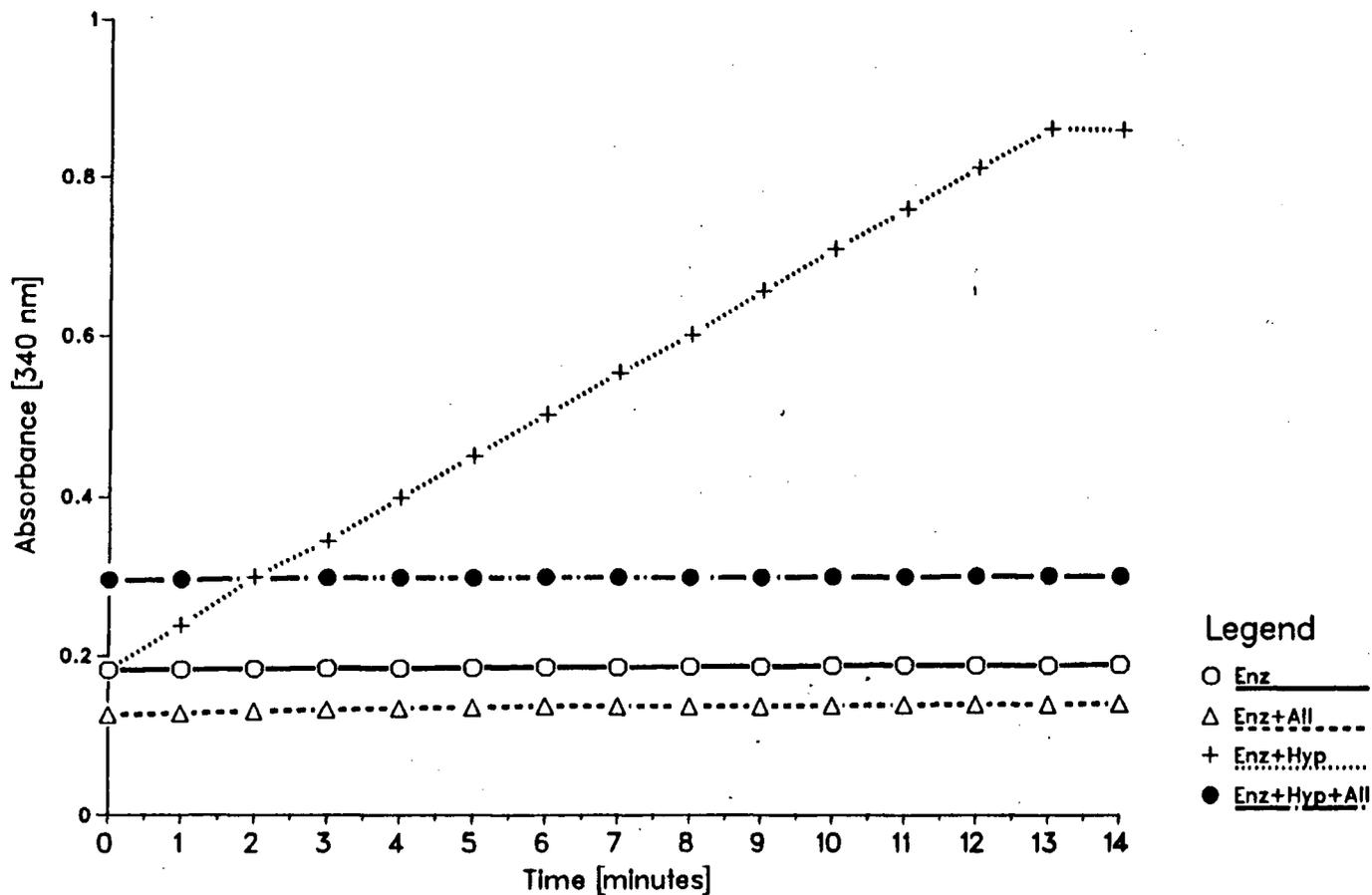
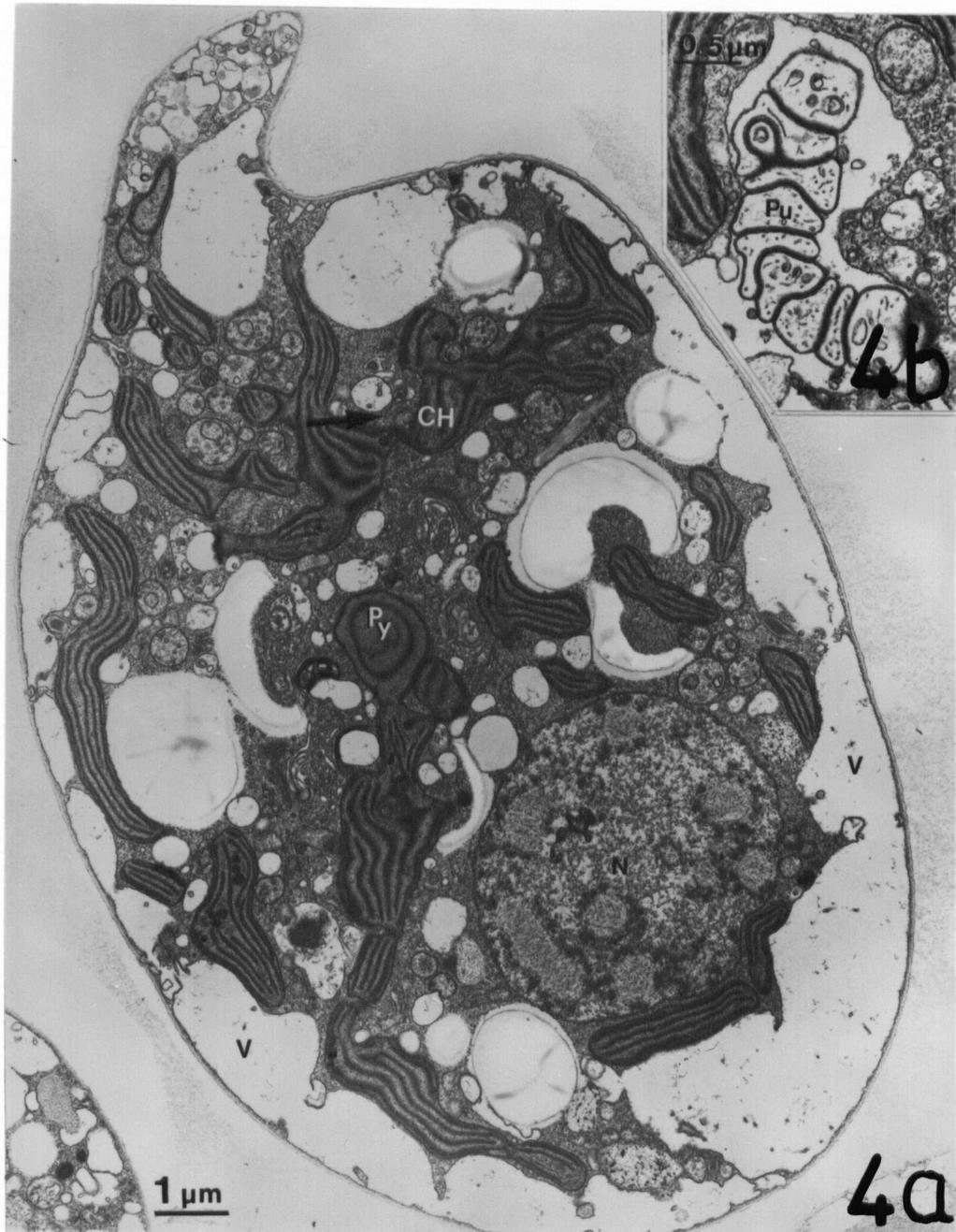
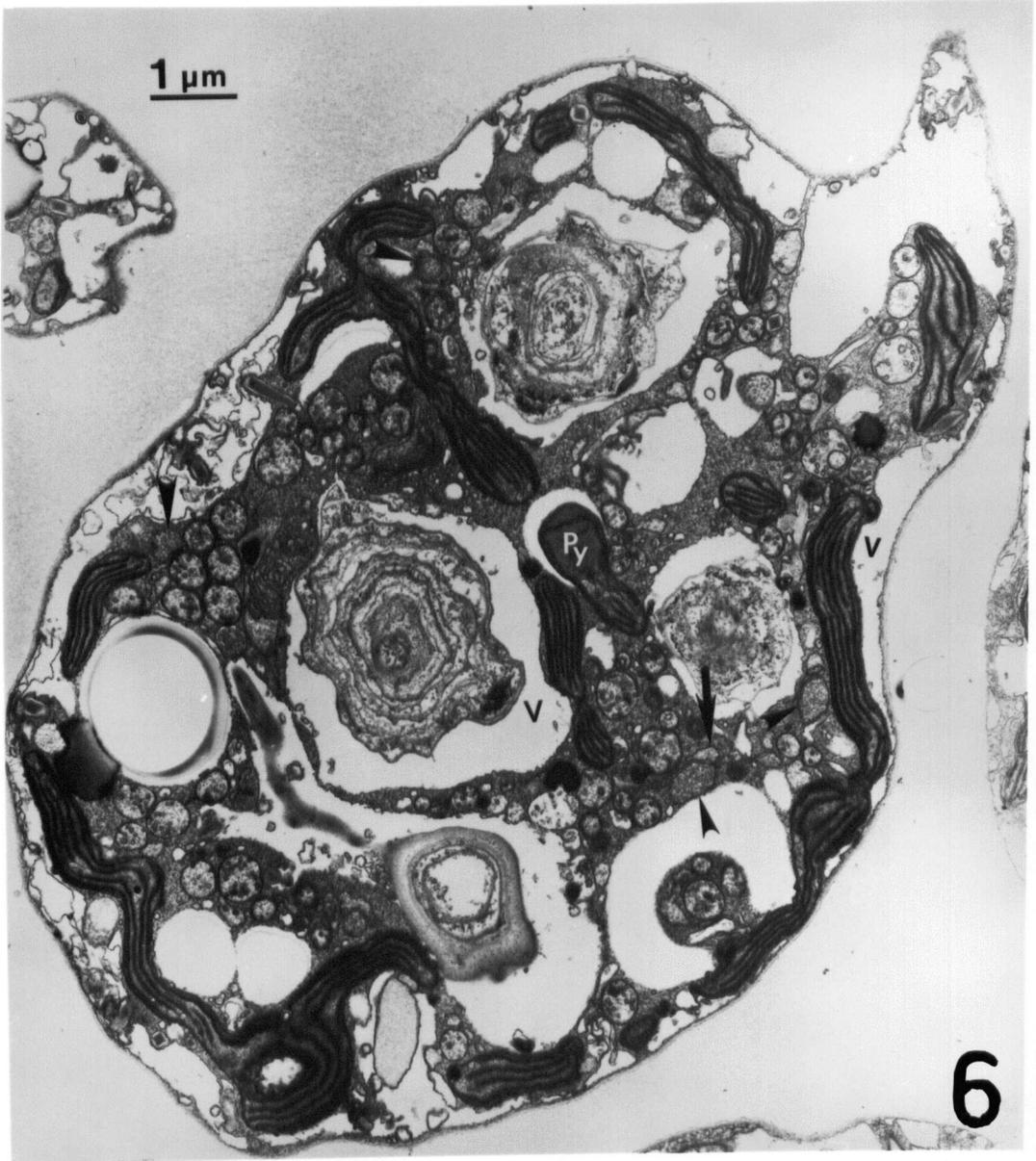


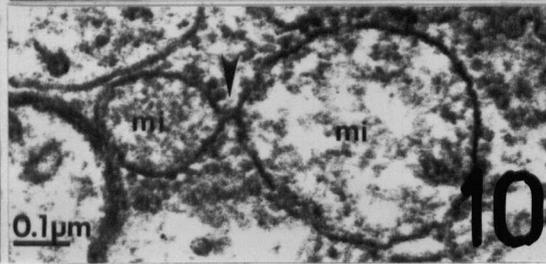
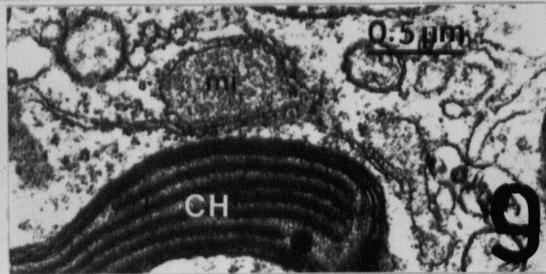
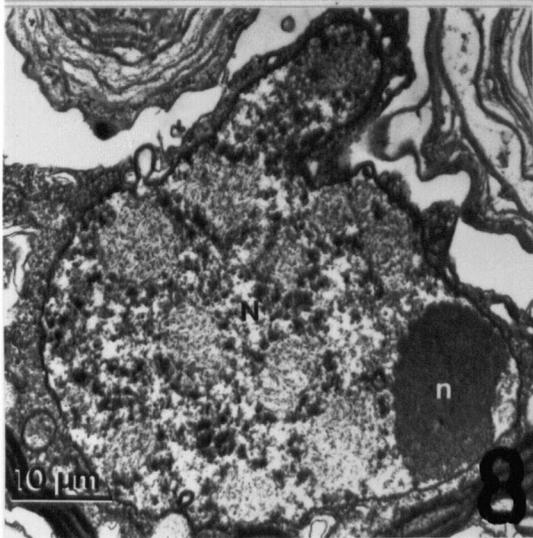
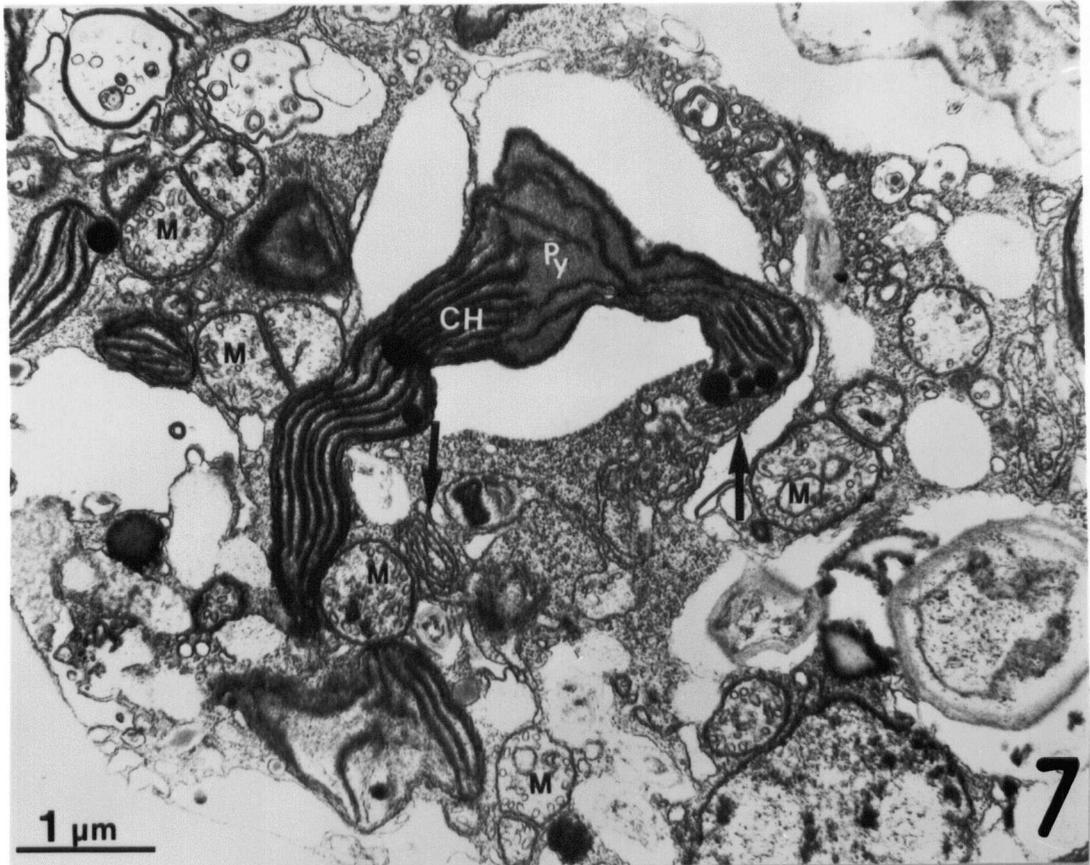
Figure 3 - *Amphidinium carterae*
Xanthine Dehydrogenase Activity

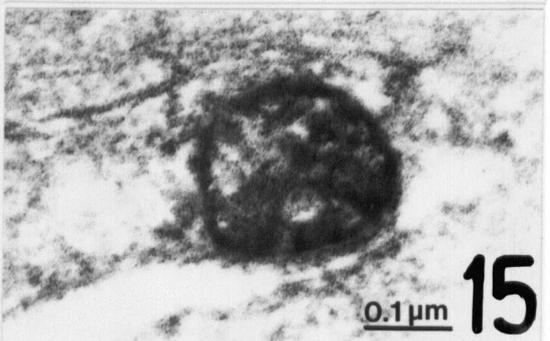
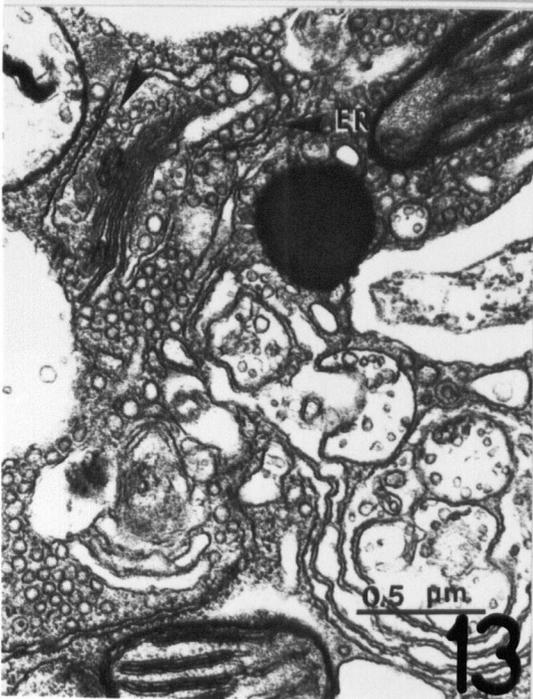
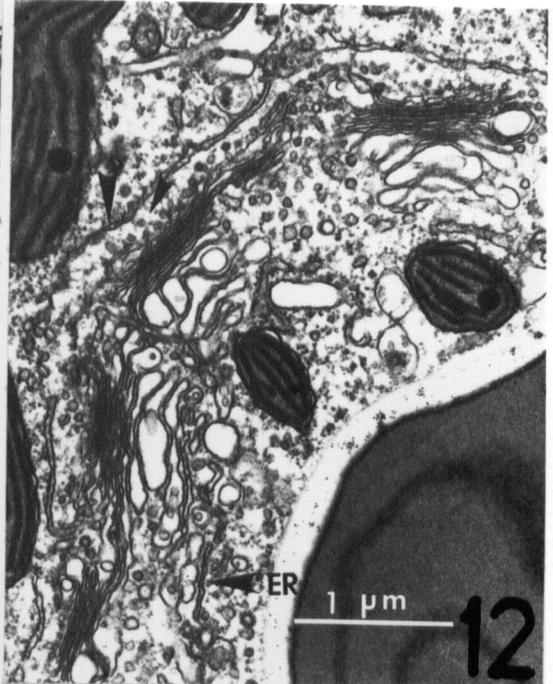


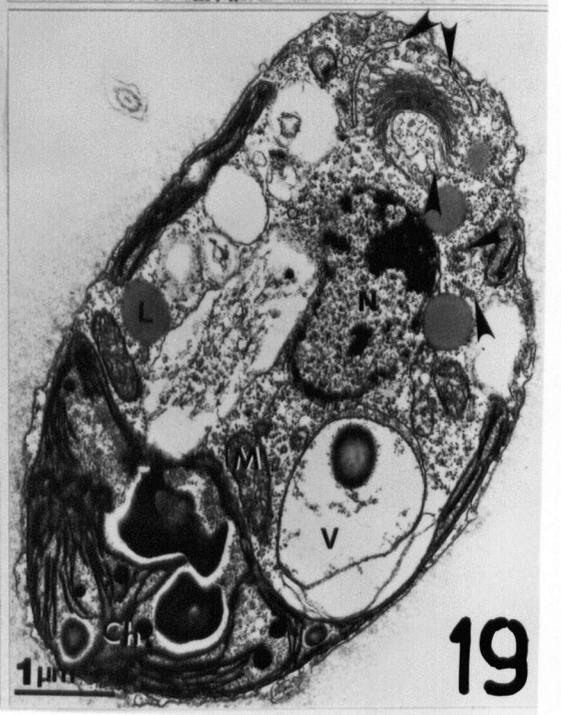
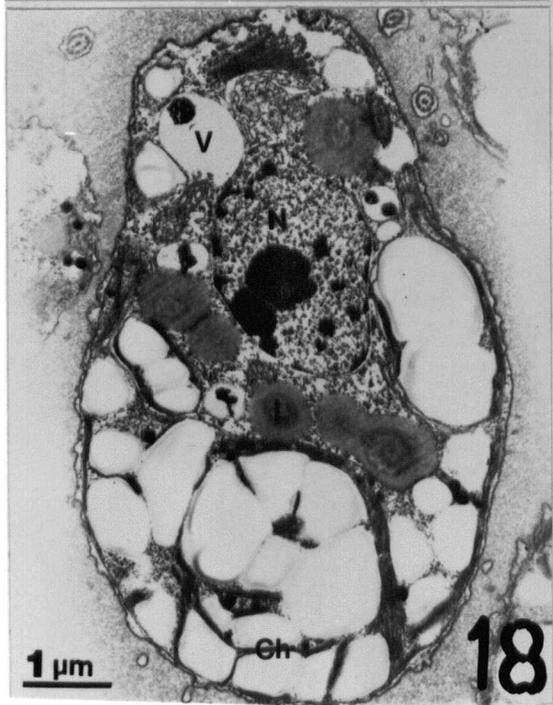
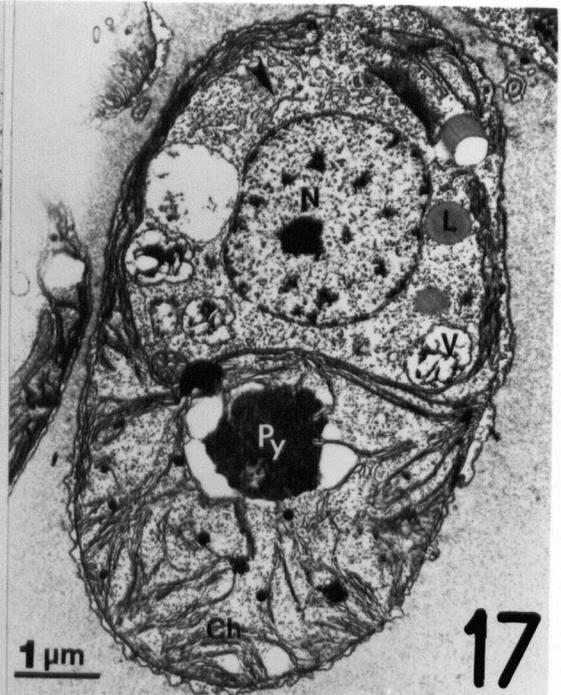


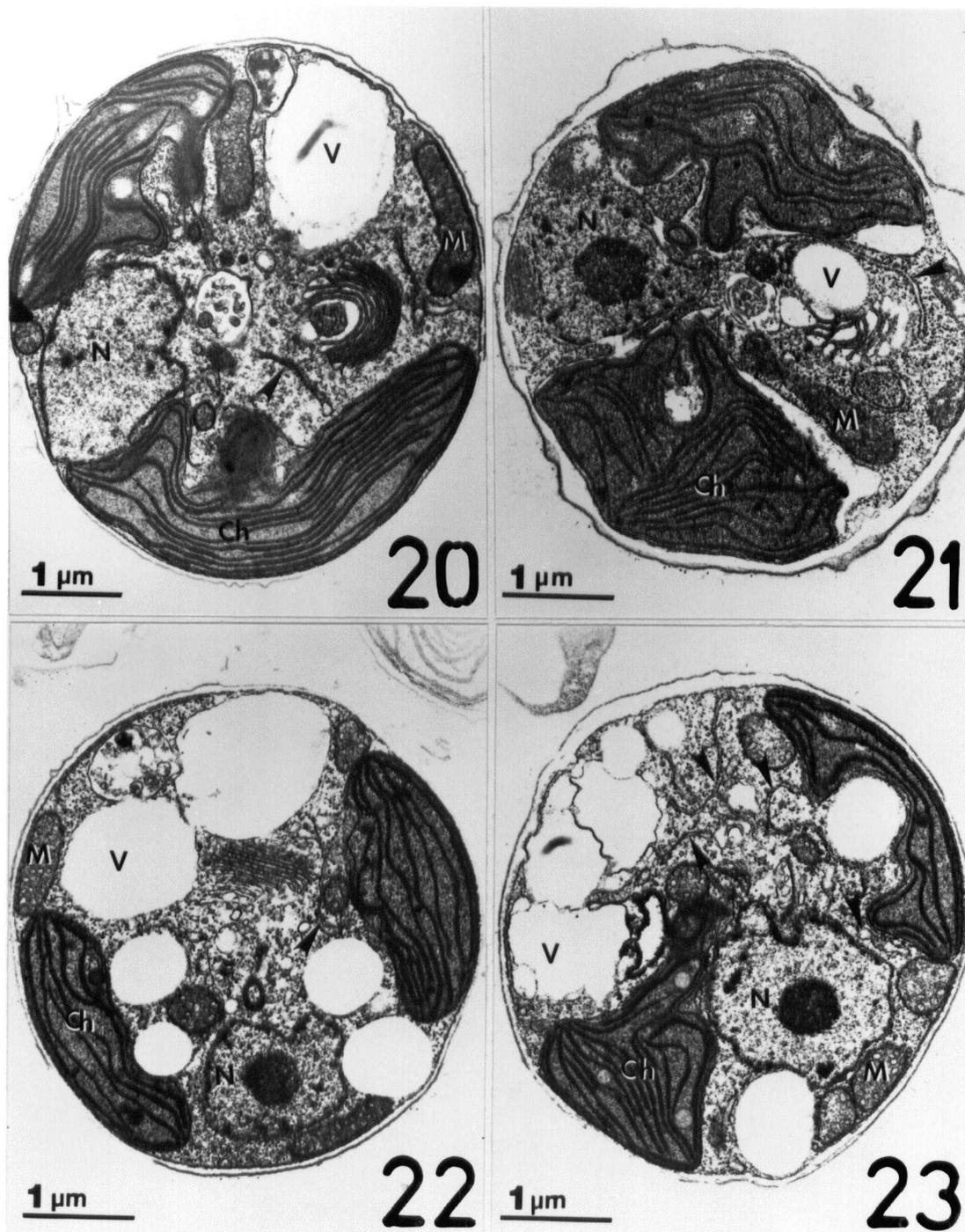












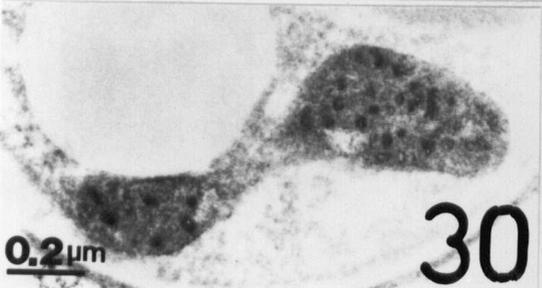
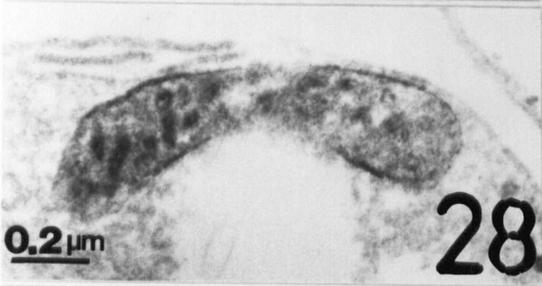
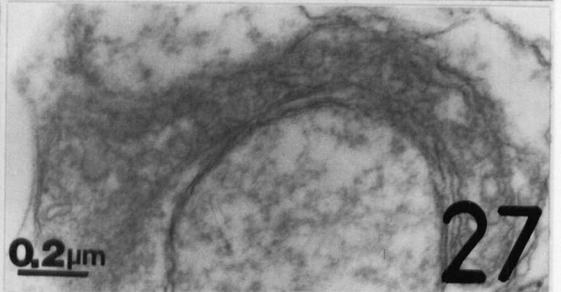
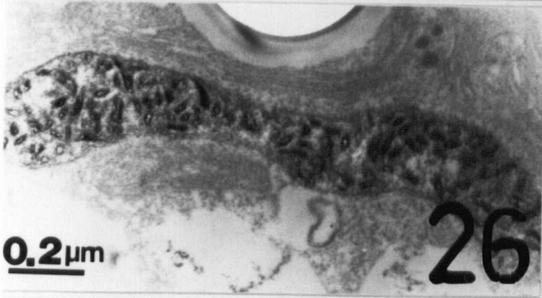
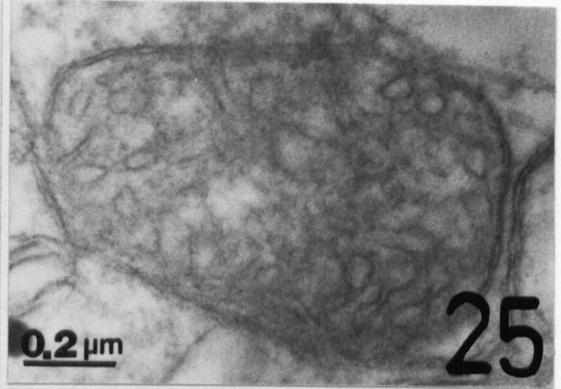
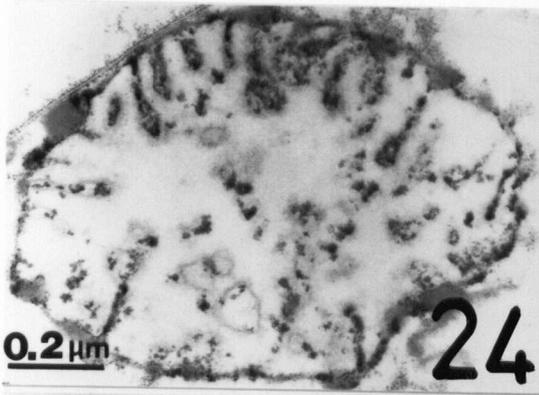


Table 1 - Microalgae used in the present study

ALGA	STRAIN/CLONE (isolator/supplier/source)
PRYMNESIOPHYCEAE	
<u>Isochrysis galbana</u>	Leftley, SMBA No. 58
<u>Hymenomonas elongata</u>	Leftley, SMBA No. 62
<u>Prymnesium parvum</u> Carter	Droop, SMBA No. 65
<u>Pavlova lutheri</u>	Leftley, SMBA No. 261
BACILLARIOPHYCEAE	
<u>Thalassiosira nordenskioldii</u> Cleve	Rao, Bedford Institute, Dartmouth, N.S.
<u>Thalassiosira pseudonana</u>	Neil Price, U.B.C.
CHLOROPHYCEAE	
<u>Dunaliella tertiolecta</u> Butcher	Wood Hole Oceanographic Institution
CYANOPHYCEAE	
<u>Agmanellum quadruplicatum</u>	Van Baalen, ATCC No. 27264
DINOPHYCEAE	
<u>Amphidinium carterae</u> Hulburt	J. McLachlan (Halifax NRC)
CHRYSOPHYCEAE	
<u>Olisthodiscus luteus</u> Carter	R.A. Cattolico, Seattle
EUSTIGMATOPHYCEAE	
<u>Nannochloropsis oculata</u>	Leftley, SMBA No. 66

Table 2 - Growth of microalgae on urea (1 mM) with nickel supplementation.
 (a) adaptation period (days); (b) exponential growth rate (%); (c) maximum yield (%)
 Growth parameters are expressed as percentage of those in media containing nitrate
 (2 mM) without nickel.
 (--) = no growth

ALGA	NICKEL SUPPLEMENTATION (μM)																				
	0			4×10^{-3}			1×10^{-2}			1×10^{-1}			1			5			10		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
<i>Isochrysis galbana</i>	4	109	91	5	110	91	5	106	89	5	105	90	5	110	91	5	103	88	5	102	89
<i>Hymenomonas elongata</i>	3	53	83	2	55	80	2	60	82	2	66	85	3	80	98	4	76	90	6	70	82
<i>Prymnesium parvum</i>	10	23	34	10	21	25	10	20	24	10	20	22	10	16	18	10	16	18	10	17	19
<i>Pavlova lutheri</i>	4	96	103	4	96	104	4	92	97	4	89	90	4	96	103	4	90	93	4	91	92
<i>Thalassiosira nordenskioldii</i>	2	73	79	2	80	85	2	84	86	2	105	103	2	133	117	2	120	110	2	118	110
<i>Thalassiosira pseudonana</i>	2	76	46	2	72	50	2	69	48	2	70	52	2	76	69	2	70	68	2	65	60
<i>Dunaliella tertiolecta</i>	2	122	110	4	92	110	4	88	106	4	89	108	4	77	110	4	80	103	4	81	105
<i>Agmanellum quadruplicatum</i>	2	102	103	5	112	110	5	107	103	5	109	107	5	112	110	7	106	101	7	108	103
<i>Amphidinium carterae</i>	--	--	--	--	--	--	--	--	--	9	48	65	6	69	95	6	68	80	6	50	68
<i>Olisthodiscus luteus</i>	--	--	--	--	--	--	--	--	--	4	42	40	2	63	66	2	72	76	2	65	64
<i>Nannochloropsis oculata</i>	2	107	94	4	98	91	4	95	88	4	90	89	4	99	90	4	97	89	4	91	90

Table 3 - Growth of microalgae on allantoic acid (0.5 mM) with nickel supplementation.

(a) adaptation period (days); (b) exponential growth rate (%); (c) maximum yield (%)
 Growth parameters are expressed as percentage of those in media containing nitrate (2 mM) without nickel.

(--) = no growth

ALGA	NICKEL SUPPLEMENTATION (μM)																							
	0			4×10^{-3}			1×10^{-2}			1×10^{-1}			1			5			10					
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c			
<i>Isochrysis galbana</i>	4	99	74	5	99	74	5	96	71	5	95	73	5	99	74	5	91	69	5	90	70			
<i>Hymenomonas elongata</i>	4	65	76	4	70	78	4	80	89	4	79	88	4	83	93	8	77	86	8	70	72			
<i>Prymnesium parvum</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--			
<i>Pavlova lutheri</i>	6	115	109	6	115	113	6	101	102	6	99	101	6	115	110	6	97	100	6	95	101			
<i>Thalassiosira nordenskioldii</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--			
<i>Thalassiosira pseudonana</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--			
<i>Dunaliella tertiolecta</i>	2	78	83	4	85	84	4	82	80	4	81	83	4	85	84	4	79	78	4	80	81			
<i>Agmanellum quadruplicatum</i>	2	98	55	5	110	55	6	102	52	6	101	51	5	109	55	10	98	51	10	99	52			
<i>Amphidinium carterae</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--			
<i>Olisthodiscus luteus</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--			
<i>Nannochloropsis oculata</i>	2	81	89	4	79	88	4	77	86	4	75	82	4	76	85	4	75	81	4	70	76			

Table 4 - Growth of microalgae on hypoxanthine (0.5 mM) with nickel supplementation.
 (a) adaptation period (days); (b) exponential growth rate (%); (c) maximum yield (%)
 Growth parameters are expressed as percentage of those in media containing nitrate
 (2 mM) without nickel
 (--) = no growth

ALGA	NICKEL SUPPLEMENTATION (μM)																				
	0			4×10^{-3}			1×10^{-2}			1×10^{-1}			1			5			10		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
<i>Isochrysis galbana</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>Hymenomonas elongata</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>Prymnesium parvum</i>	4	84	83	4	86	85	4	87	81	4	83	85	4	84	83	6	81	83	6	80	83
<i>Pavlova lutheri</i>	6	135	113	6	124	113	6	121	108	6	123	112	6	129	112	6	115	105	6	116	108
<i>Thalassiosira nordenskioldii</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>Thalassiosira pseudonana</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>Dunaliella tertiolecta</i>	2	79	110	4	79	112	4	82	110	4	76	105	4	79	112	4	78	109	4	83	104
<i>Agmanellum quadruplicatum</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>Amphidinium carterae</i>	--	--	--	--	--	--	--	--	--	7	71	58	4	95	90	4	89	72	4	72	54
<i>Olisthodiscus luteus</i>	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
<i>Nannochloropsis oculata</i>	2	91	86	4	89	88	4	85	82	4	81	84	4	87	82	4	80	76	4	81	79

Table 5 - Optimal concentrations of nickel (if required), urea, allantoinic acid and hypoxanthine (where applicable) that support maximum yields of the microalgae
(--) = no growth

ALGA	NICKEL (μ M)	UREA (mM)	ALLANTOIC ACID (mM)	HYPOXANTHINE (mM)
<u>Isochrysis galbana</u>	0 [*]	1	0.5	--
<u>Hymenomonas elongata</u>	1	1	0.5	--
<u>Prymnesium parvum</u>	0	4	--	2
<u>Pavlova lutheri</u>	0	1	0.5	0.5
<u>Dunaliella tertiolecta</u>	0	1	0.5	0.5
<u>Agmanellum quadruplicatum</u>	0	1	0.5	--
<u>Amphidinium carterae</u>	1	2	--	1
<u>Nannochloropsis oculata</u>	0	2	1	1

*external addition of nickel was not required

Table 6 - Concentrations (μM) of allopurinol (Al), 2,6,8 - trichloropurine (Tc) and hydroxyurea (Hu) required for 50% inhibition of microalgal growth in media supplemented with hypoxanthine, allantoic acid or urea as organic N-sources

Alga	Organic N - Source / Inhibitors				
	Urea	Allantoic acid	Hypoxanthine		
	Hu	Hu	Al	Tc	Hu
<u>Isochrysis galbana</u>	1	1	-	-	-
<u>Hymenomonas elongata</u>	10	10	-	-	-
<u>Prymnesium parvum</u>	10	-	1	5	10
<u>Pavlova lutheri</u>	5	500	5	5	500
<u>Thalassiosira nordenskioldii</u>	5	-	-	-	-
<u>Thalassiosira pseudonana</u>	1	-	-	-	-
<u>Dunaliella tertiolecta</u>	500	500	2	5	500
<u>Agmanellum quadruplicatum</u>	10	10	-	-	-
<u>Amphidinium carterae</u>	1	-	1	10	1
<u>Olisthodiscus luteus</u>	5	-	-	-	-
<u>Nannochloropsis oculata</u>	10	10	1	10	10

* symbol (-) indicates that the microalga does not grow on that particular N-source

Table 7 - Xanthine dehydrogenase (XD), allantoinase (ALN) and allantoicase (ALC) activities in cell - free extracts of microalgae grown in hypoxanthine

Alga	Crude Extract Activities ¹		
	XD	ALN	ALC
<u>Amphidinium carterae</u>	3.1 ± 0.2	1.9 ± 0.3	2.3 ± 0.1
<u>Dunaliella tertiolecta</u>	2.2 ± 0.3	3.0 ± 0.2	1.5 ± 0.3
<u>Pavlova lutheri</u>	2.9 ± 0.1	2.6 ± 0.2	1.7 ± 0.2

¹ Enzyme activities are expressed as nmol product min⁻¹ mg protein and represent averages of three separate assays

Table 8 - Summary of the occurrence and characteristics of the cytochemical reactions observed in mitochondria of *Dunaliella tertiolecta* and *Pavlova lutheri* grown on four different sources of nitrogen

Nitrogen Source	Cytochemical Parameters							Enzyme activity
	Requirements		Incubation		Pre - Fixation		Inhibitor	
	Substrate	H ₂ O ₂	pH	Temperature	C(%)	T(min)		
Nitrate	--	None	6.0	22-27 °C	1% Ga	30-60	KCN	Cytochrome system
Urea	--	None	6.0	22-27 °C	1% Ga	30-60	KCN	Cytochrome system
Allantoate	--	None	6.0	22-27 °C	1% Ga	30-60	KCN	Cytochrome system
Hypoxanthine	--	None	6.0	22-27 °C	1% Ga	30-60	KCN	Cytochrome system
Hypoxanthine	Uric acid	Yes	9.0	37 °C	0.25% Ga	5	TC/OX	Uricase
Hypoxanthine	--	Yes	9.0	37 °C	1-2% Ga	60-90	AT	Catalase

Symbols: H₂O₂ = hydrogen peroxide, C = concentration of prefixative, Ga = glutaraldehyde
T = time (duration) of prefixation, KCN = potassium cyanide, TC = trichloropurine
OX = oxypurines, AT = aminotriazole