UREA METABOLISM BY THE MARINE CRYPTOMONAD, *RHODOMONAS LENS*

by

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ABSTRACT

The effect of urea on a marine cryptomonad, Rhodomonas lens, was investigated. Cells from a nitrate enriched, natural sea water medium were acclimated to synthetic sea water with either nitrate (control medium) or urea (experimental medium) as the sole nitrogen source. Cells acclimated readily to the control medium; acclimation to the experimental medium was erratic. Control and experimental cells were taken from media on which good growth habitually occurred. Tested optimal growth conditions occurred at 22°C and a 16:8 hour, light:dark cycle with fluorescent light at approximately 150 foot-candles.

Growth was followed turbidimetrically and by direct cell count. Control cultures exhibited the typical lag, logarithmic and stationary phases. The stationary phase merged into a gradual decline phase. Culture colour proceeded from a red-pink during early phases to a red-orange, then green during stationary-decline phase. Initially, ultrastructure was typical of cryptomonads: cells exhibited a bi-lobed chloroplast having thylakoids, often arranged in bands of two, with a loculus size of 110-260 Å. Occasional lipid droplets were present within the cytoplasm. Stationary-decline phase showed an accumulation of a lipid-like material. Thylakoids were arranged singly; loculus size being decreased to 70-150 Å.

Experimental cultures at concentrations less than 1.25 mM urea exhibited similar growth to control cultures. At urea concentrations equal to or greater than 1.25 mM urea, cells during lag and early logarithmic phases showed a lipid-like accumulation; during later logarithmic phase, lipid droplets were observed. Stationary phase was short and cells proceeded directly into a rapid decline phase. Concomitantly, an increase in pH occurred and cultures became a creamy white in colour.

To test electrolytic and pH effects, NaOH and NH_4OH were added to a healthy control culture. Increased pH with NaOH had no gross effect on the cultures: increased pH with NH_4OH caused a lytic effect similar to lysis observed during decline phase of experimental cultures.

Observations on control cells during stationary-decline phases suggest a lack of available nitrogen, probably due to depletion: observations on experimental cells during lag and early logarithmic phases are similar, thus suggesting a lack of nitrogen.

As sufficient urea is present, it is suggested that the uptake and/or metabolism of urea in experimental cells is inefficient, causing nitrogen stress. Increase in pH prior to decline phase, and the results of electrolytic experiments suggest that urea is converted into ammonia and excess ammonia is released into the medium. The increasing ammonia concentration becomes toxic and accelerating cell lysis thus results in a decline phase.

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INTRODUCTION

Nitrogen is an essential element for plant growth. It is often the concentration of this nutrient which limits phytoplankton growth (Spoehr and Milner, 1949; Holmes, Williams and Eppley, 1967; Dugdale and Goering, 1970; Thomas and Owen, 1971; Ryther and Dunstan, 1971). Nitrogen occurs in a variety of combined forms in sea water. Of these, ammonium and nitrate are the most abundant and are the commonest sources of nitrogen for plant nutrition--in many situations ammonium is known to be utilized before nitrate (Syrett, 1962; Grant, Madgwick and DalPont, 1967; Strickland, Holm-Hansen, Eppley and Linn, 1969; Goering, Wallen, and Nauman, 1970; Naylor, 1970). Of the organic nitrogen compounds, amino acids and urea are found at concentrations in sea water which suggest that they may constitute a biologically significant source of nitrogen. Extensive analysis of marine surface waters by Hobbie, Crawford, and Webb (1964), Degens (1968), and Litchfield and Prescott (1970) show concentrations of free amino acids ranging from 38 to 70 μg liter^{-1}. Of these, aspartic acid is found in varying concentrations in all waters sampled; glycine, serine, and ornithine are found in the greatest concentration and are present frequently. The presence of these amino acids may be significant, not only as nutrients for certain phytoplankters (Lewin, 1962; Guillard, 1963; Shihira and Krauss, 1965; Cain, 1965; Hellebust and Guillard, 1967;

North and Stephens, 1969, 1971) but also as compounds directly or indirectly connected via the "urea cycle."

The distribution and concentration of urea found in surface waters is variable, and probably dependent on the nature of the biological conditions in the immediate environment. Observed concentrations range between 0.54 to 1.00 μg-atom urea-N liter^{-1} off LaJolla, California (McCarthy, 1970) and off the continental shelf between Panama and Callao (Remsen, 1971), 0.25 to 11.2 μg-atom urea-N liter^{-1} between Cape Cod and Cape May (inclusive of New York Harbour waters) (Remsen, 1971), 0.00 to 0.67 μg-atom urea-N liter^{-1} along the Peruvian coast (McCarthy, 1970), and 3.07 μg-atom urea-N liter^{-1} in the English Channel (Newell, 1967). The high figure for the area inclusive of New York Harbour suggests that much of this urea is terrigenous. The source becomes obvious from ecological studies. Cronin (1967) observed a sharp increase of sewage outfalls into coastal waters in 'the past fifteen years'. In the Potomac River effluent loads vary seasonally from 5 to 40% of river volume (Shapiro and Ribeiro, 1965); 16% of the summer flow of the Hudson River at Yonkers, before effluents from New York City enter, is municipal sewage effluent (Howells, Kneipe and Eisenbud, 1970). Man is a generous contributor to coastal organic nitrogen. Coastal birds may also contribute in a lesser quantity. Uric acid comprises approximately 70 to 80% of the nitrogen excreted by birds (Needham, 1931); this is rapidly broken down to urea

in sea water (McCarthy, 1971). In the open ocean, marine mammals in high density would be contributors in isolated instances. During this period they may excrete significant quantities of urea (McCarthy, 1971). Fish and planktonic invertebrates are the largest off-shore contributors to urea regeneration (McCarthy, 1970), a regeneration furthered by microbial action (Remsen, 1971). Thus, urea is present in marine surface waters--its sources in open waters being relatively stable and in coastal waters being continually augmented.

Can marine phytoplankton utilize urea at the concentrations naturally available? Harvey (1940) suggested that marine phytoplankton probably were capable of utilizing urea as a nitrogen source. Although all phytoplankton do not utilize urea as a sole nitrogen source, many have been observed to do so in culture (Birdsey and Lynch, 1962; Eppley et al., 1971; McCarthy, 1971; Carpenter et al., 1972), sometimes in preference to nitrate (Grant et al., 1967) or even with complete rejection of nitrate (Antia and Chorney, 1968). Harvey (1940), using a phytoplanktonic community heavy in diatoms, observed good growth on urea and on uric acid. The rate of growth was slower compared to that on ammonium. Eppley et al. (1971), with shipboard cultures, and Carpenter et al. (1972), with Chaetoceros sp. and Skeletonema sp., observed no dramatic differences in growth rate or quality of growth between cells grown on nitrate, ammonium, or urea. In all published work with cultures, the

nitrogen concentration greatly exceeds that measured for natural sea water. Although oceanic phytoplankters possess mechanisms for maximizing the utilization of very low levels of nitrogenous nutrients (Eppley, Rogers and McCarthy, 1969), in culture they appear to need much higher levels.[1]

In unicellular algae the catabolism of urea may proceed via one of two enzyme systems. Urease (urea amido hydrolase, EC 3.5.1.5) catalyzes the hydrolytic cleavage of urea;

$$CO(NH_2)_2 + H_2O \longrightarrow CO_2 + 2NH_3.$$

Thought to be nearly ubiquitous, urease has been found in representatives of the Bacillariophyceae, Chrysophyceae, Cyanophyceae, Euglenophyceae, Prasinophyceae, and Xanthophyceae (Allison et al., 1954; Berns, Holohan and Scott, 1966; Lui and Roels, 1970; Roon and Levenberg, 1970, 1972; Pecora, 1972; Leftley and Syrett, 1973). Representatives of the Chlorophyceae and certain yeasts, although able to metabolize urea, contain no detectable urease activity (Walker, 1952; Hattori, 1957; Baker and Thompson, 1962; Domnas, 1962; Kating, 1962; Cook and Boulter, 1964). These organisms possess an energy-requiring system--ATP: urea amidolyase (UALase). Originally reported to be one enzyme (Roon and Levenberg, 1968), UALase is now considered to be either two distinct enzymes (Thompson and Muenster, 1971) or an enzyme complex (Whitney and Cooper, 1972, a, b). The two possible enzymes are urea carboxylase (urea: CO_2 ligase (ADP), EC 6.3.4.6) and allophanate amido

hydrolase (EC 3.5.1.13). They catalyze the reactions:

$$\text{urea} + \text{ATP} + HCO_3^- \longrightarrow \text{allophanate} + \text{ADP} + \text{Pi}$$

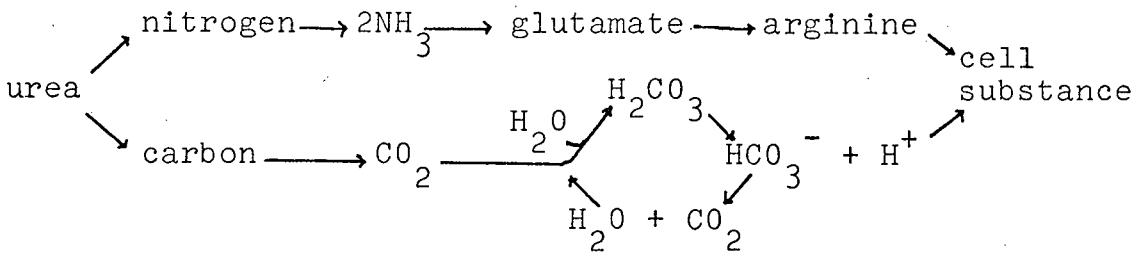
$$\text{allophanate} \longrightarrow 2NH_3 + 2CO_2.$$

The final product of both enzyme systems is ammonia. No trace of this product was observed in Chlorella cells cultured on urea by Hattori (1958) or Syrett (1953). More recently, however, Hodson and Thompson (1969) observed an accumulation of ammonia when Chlorella vulgaris was cultured on urea in the presence of cyanide. Cyanide inhibits the metabolism of ammonia to a much greater extent than it inhibits urea metabolism (Hattori, 1957). Their results suggest that both nitrogen atoms from urea form ammonia. The ammonia is probably very rapidly metabolized, accounting for its lack of detection in previous work. A further indication that ammonia is a product of urea metabolism is the excretion of ammonia by organisms grown on urea as the sole nitrogen source. Little and Mah (1970) found that when Chlorella ellipsoidea was cultured on urea plus limiting amounts of glucose, ammonia was excreted: recently Uchida (1975) observed the dinoflagellate Prorocentrum micans excreted ammonia when cultured in media with urea as the sole nitrogen source.

Although no unique pathway for urea nitrogen has been described (Thompson et al., 1966), the concentration of several amino acids is observed to increase concomitant with the assimilation of urea in nitrogen-poor cells.

After up to 30 minutes exposure to urea, there is a substantial increase in glutamic acid and a significant increase in glutamine. An initial decrease is followed by a slight increase in alanine (Baker and Thompson, 1962). The control for this work was exposed to NH_4Cl. These cells exhibited a substantial increase in alanine and a significant increase in glutamine. Hattori (1957, 1958) with Chlorella ellipsoidea observed an increase in arginine corresponding to the rate of urea catabolism. Thomas and Krauss (1955) with Scenedesmus obliquus and Walker (1952) with Chlorella pyrenoidosa also observed an increase in arginine production when these organisms were cultured on urea. Thus glutamate is probably an early product of urea metabolism, followed at a longer time period, by arginine.

The fate of the urea carbon is ambiguous. It was not found incorporated into arginine by Allison et al. (1954) or Hattori (1960), but Baker and Thompson (1962) observed some incorporation of the urea carbon into most amino acids, with the greatest concentrations in glutamate, aspartate and serine (in decreasing order). There is evidence that about 40% of the urea carbon enters the CO_2: carbonate cycle during metabolism (Hattori, 1960) and Baker and Thompson (1962) concluded that the direct product of urea catabolism is CO_2. A suggested mechanism is:



It has been shown: that urea is and will continue to be a significant component of marine surface waters; that the present concentration is sufficient for the nitrogenous nutrition of phytoplankton; and that a variety of phytoplankters contain systems which metabolize urea. Evidence has been presented on two modes of urea catabolism and the probable products of assimilation. Work on the relationship of urea and marine phytoplankton has provided a quantity of data. Unfortunately, the results of various workers have not been integrated and often the experimental organisms are dissimilar. (Metabolic data is largely based on the Chlorophyta, enzyme data on Chlorophyta and yeast, and growth data from a variety of phytoplankters but primarily in the Bacillariophyta.)

To augment present knowledge and to help integrate the diversity of existing data, it was proposed to study the cytological and physiological effects of urea on a member of a neglected group of marine phytoplankton.

Rhodomonas lens[2], a representative of the Cryptophyceae, was chosen as the experimental organism. The Cryptophyceae are "a small but well defined group of generally ovoid. . . organisms characterized by the two equal or subequal

homodynamic flagella and a furrow, groove or tubular gullet on one surface." (Butcher, 1967) A number of unique characteristics can be seen with the light microscope. Penard (1921) studied the nature of trichocysts in Cryptomonas ovata. These trichocysts, or ejectosomes, (differing from the trichocysts of other flagellates) are found lining the single anterior gullet. The periplast contains the cell and is occasionally striate (Butcher, 1967). Each cell contains one or two chloroplasts and generally one pyrenoid. A large variation in colour occurs, dependent upon culture conditions and the age of the culture (Butcher, 1967). Starch and lipid are produced as storage products.

Electron microscopic observations have augmented the knowledge obtained from use of the light microscope. Detailed fine structural descriptions are given by Dodge (1969), Lucas (1970), Hibbard et al. (1971), Taylor and Lee (1971), and Antia et al. (1973). The cell surface of Chroomonas sp. is covered with a fibrous 'fuzz' (Gantt, 1971); under this layer the cell is completely enclosed by a periplast. Recently, several workers have examined the Cryptomonad periplast in detail. It is composed of either two or three distinct layers, one of which is always the plasma membrane (Dodge, 1969; Gantt; 1971, Faust, 1974). Chroomonas mesostigmatica has a triple layered periplast with about 7 oblique grooves adjacent to which the small ejectosomes are located (Dodge, 1969); Chroomonas sp. has a

tripartite periplast with a thickness of about 250 Å in cross section (Gantt, 1971); Cryptomonas ovata var. palustris has a bipartite periplast composed of polygonal plates which are delineated by shallow ridges (Faust, 1974). The periplast, then, consists of the plasma membrane and further layers which are appressed to its outer and inner surfaces, except over the flagellar and gullet region where the plasma membrane is not covered (Gantt, 1971; Faust, 1974). The inner layer is composed of plates and it is the arrangement and possibly the shape of these plates and the associated ejectosomes which give the cell its distinct surface structure (Butcher, 1967; Dodge, 1969; Lucas, 1970; Gantt, 1971; Faust, 1974). The ejectosomes have been described by Dragesco (1951), Anderson (1962), Joyon (1963), Hovasse, Mignon and Joyon (1967), and Schuster (1970). They are bodies of two distinct sizes having a regular geometric shape and a complex internal structure (Anderson, 1962; Hovasse et al., 1967). Hovasse et al. (1967) suggested that they consist of two unequal components, each possibly curled in a tight spiral and enclosed by a thin membrane. The large ejectosomes are about 500 nm in diameter and 400 nm deep and are found within the anterior furrow (Butcher, 1967; Hovasse et al., 1967; Dodge, 1969; Lucas, 1970). The smaller ejectosomes, about 200 nm in diameter, are part of the periplast and appear to be on the exterior of the cell, separated from the cytoplasm by the plasma membrane (Lucas, 1970; Gantt, 1971). The single chloroplast is bilobed, the

two lobes being connected by a granular pyrenoid (Gibbs, 1962, a, b; Dodge, 1969; Lucas, 1970; Gantt, Edwards and Provasoli, 1971). The thylakoids are generally associated in bands of two or more (Cryptomonas cryophila has an atypical chloroplast in which the thylakoids are arranged in layers of variable numbers, Taylor and Lee, 1971.) separated by a space of 3-8 nm (Gibbs, 1962, a, b; Wehrmeyer, 1970; Gantt, Edwards and Provasoli, 1971; Bisalputra, 1974) and are often found penetrating or traversing the pyrenoid (Gibbs, 1962, a, b; Wehrmeyer, 1970; Gantt et al., 1971). The thylakoid loculus ranges from 10-50 nm, dependent on the genus and contains a fine granular, electron opaque matrix (Wehrmeyer, 1970; Gantt et al., 1971). Gantt et al. (1971) and Faust and Gantt (1973) suggest that this matrix contains the phycobilin proteins. They found no evidence of phycobilisome-like aggregates. The chloroplast and pyrenoid are enclosed by a double-membrane envelope and, outside of it, by a sac of endoplasmic reticulum which is continuous with the nuclear envelope (Gibbs, 1962 a; Wehrmeyer, 1970; Gantt et al., 1971). Starch grains are formed around the pyrenoid and within the perichloroplastic matrix (Gibbs, 1962 a, b; Wehrmeyer, 1970; Gantt et al., 1971; Bisalputra, 1974).

The interphase nucleus is located posteriorly and contains a prominent nucleolus and many chromatin areas (Dodge, 1969; Lucas, 1970; Taylor and Lee, 1971; Oakley, 1974). It is frequently transected by a narrow tongue of cytoplasm (Dodge, 1969; Taylor and Lee, 1971). Prior to

cell division the nucleus moves to a more central position (Oakley, 1974).

The mitochondria are large and of variable shape with uniform flattened cristae (Dodge, 1969; Lucas, 1970; Taylor and Lee, 1971; Taylor, 1971). The Corps de Maupas, described by Lucas (1970) as a roughly spherical body containing short stacks of fibrils, numerous small vesicles and short lengths of membrane enclosed in a single smooth membrane, is usually situated near the golgi apparatus (Dodge, 1969; Lucas, 1970; Taylor and Lee, 1971).

The two flagella are alike but may be equal or unequal in length (Butcher, 1967; Dodge, 1969; Lucas, 1970; Taylor and Lee, 1971). Mastigonemes are found over the entire flagellar length (Lucas, 1970; Taylor and Lee, 1971). The usual 9 + 2 axoneme arrangement is present: at a point just prior to flagellar insertion in the mouth of the gullet the sheath is constricted so that it touches the 9 outer doublets while at the same point the central 2 tubules pass through a disc or transverse septum (Dodge, 1969; Lucas, 1970). About 100 nm nearer to the cell the central tubules terminate at a second disc (Dodge, 1969; Lucas, 1970). Within the cell the 9 doublets become triplets. The two basal bodies are connected by a 'block of densely staining material' (Dodge, 1969) and lie close together and almost parallel (Dodge, 1969; Lucas, 1970). Small groups of microtubules run from the flagellar bases posteriorly toward the nucleus (Dodge, 1969; Oakley, 1974).

The cytoplasm also contains many ribosomes, rough endoplasmic reticulum, small vesicles, and occasional lipid globules (Dodge, 1969; Lucas, 1970; Taylor and Lee, 1971; Antia et al., 1973; Oakley, 1974).

METHODS AND MATERIALS

I. Cultures

The culture of Rhodomonas lens, Pascher and Ruttner, was obtained from Dr. N. Antia[3] of the Fisheries Research Board of Canada, Vancouver, B.C. One ml of this culture was aseptically transferred to experimental flasks containing enriched synthetic sea water. The contents per liter of this medium are given in Table 1. The medium is without combined nitrogen. Nitrogen was added as either potassium nitrate or urea. Media were sterilized by filtration[4]. Stock cultures were maintained on two types of media, one with 0.5 mM KNO_3 and one with 1.25 mM urea.

To test for bacterial contaminants, periodically two drops of the stock cultures were added aseptically to either sterility medium STP or ST_3 (see Table 2). These were kept in the dark at room temperature (22°C) and were observed after one week for turbidity in the liquid media as evidence of bacterial growth. Cultures were also examined microscopically at each transfer for contaminants.

II. General Methods

1. Growth Measurements

Growth of the cultures was followed by turbidimetric determination at 600 nm on a Sargent-Welch SM spectrophotometer in 13 mm precalibrated tubes and/or by direct cell count using a Spencer Bright Line improved

Table 1. Enriched synthetic sea water medium.[5]

$NaH_2PO_4.H_2O$	6.9	mg	Trace metals (chelated)		
$Na_2SiO_3.9H_2O$	84.0	mg	$Na_2EDTA.2H_2O$	16.2	mg
			$FeCl_3.6H_2O$	5.4	mg
			$MnSO_4.4H_2O$	2.25	mg
			$ZnSO_4.7H_2O$	1.15	mg
Vitamins:					
Thiamin.HCl	1.25	mg	$Na_2MoO_4.2H_2O$	0.586	mg
Biotin	2.50	mg	$CuSO_4.5H_2O$	0.050	mg
B_{12}	5.00	mg	$CoSO_4.5H_2O$	0.028	mg
Buffer: TRIS.HCl	200	mls	(41.3 mM, pH 6.8)		
Synthetic sea water*	(to one liter)				

*Synthetic sea water (Salinity = 28 parts /oo.)

NaCl	23.48 gms	KBr	96 mg
$MgCl_2.6H_2O$	10.13 gms	H_3BO_3	26 mg
Na_2SO_4	3.92 gms	$SrCl_2.6H_2O$	40 mg
$CaCl_2.6H_2O$	2.17 gms	NaF	3 mg
KCl	0.66 gms	distilled water	950 mls
$NaHCO_3$	0.19 gms		

Table 2. Sterility media. (Tatewaki and Provasoli, 1964)

	STP	ST_3
sea water	80 ml	70 ml
H_2O	15 ml	25 ml
soil extract	5 ml	5 ml
$NaNO_3$	20 mg	5 mg
K_2HPO_4	1 mg	
Na_2glycerophosphate		1 mg
Hy-Case (Sheffield Chemical)		2 mg
Yeast autolysate (N.B.C.)	20 mg	
Yeast extract (Difco)		1 mg
Liver oxoid L-25 (Oxo, Ltd.)		2 mg
B_{12}		0.01 µg
Vitamin mix 8A[1]	0.1 ml	0.10 ml
Sucrose	100 mg	
C-source Mix II[2]		2 ml
NaH-glutamate	50 mg	
DL-alanine	10 mg	
Trypticase (BBL)	20 mg	
Glycine	10 mg	
Glycylglycine		40 mg
pH	7.5-7.6	7.9

[1]One ml of Vitamin mix 8A contains: thiamine.HCl 0.2 mg; nicotinic acid 0.1 mg; putrescine.2HCl 0.04 mg; cobalt pantothenate 0.1 mg; riboflavin 5 µg; pyridoxine.2HCl 0.04 mg;

Table 2. (continued)

pyridoxamine.2HCl 0.02 mg; p-aminobenzoic acid 0.01 mg; biotin 0.05 µg; cholinehydrogencitrate 0.5 mg; inositol 1.0 mg; thymine 0.8 mg; orotic acid 0.26 mg; B_{12} 0.05 µg; folic acid 2.5 µg; folinic acid 0.2 µg.

^{2}C-sources Mix II contains: glycine 100 mg; DL-alanine 100 mg; L-asparagine 100 mg; sodium acetate.$3H_2O$ 200 mg; glucose 200 mg; L-glutamic acid 200 mg; H_2O 100 ml.

Neubauer haemocytometer. The cells were immobilized for direct cell counts by adding one drop of 0.5% glutaraldehyde to a one ml aliquot of culture. The generation time was calculated from the equations[6]:

$$n = \frac{\log_{10} y - \log_{10} x}{.301} ,$$

$$\text{and } g = \frac{t}{n} ,$$

where n is the number of generations, y is the larger number of organisms at the end of a time period t, x is the smaller number of organisms at the beginning of a time period t, and g is the generation time.

2. Urea Analysis

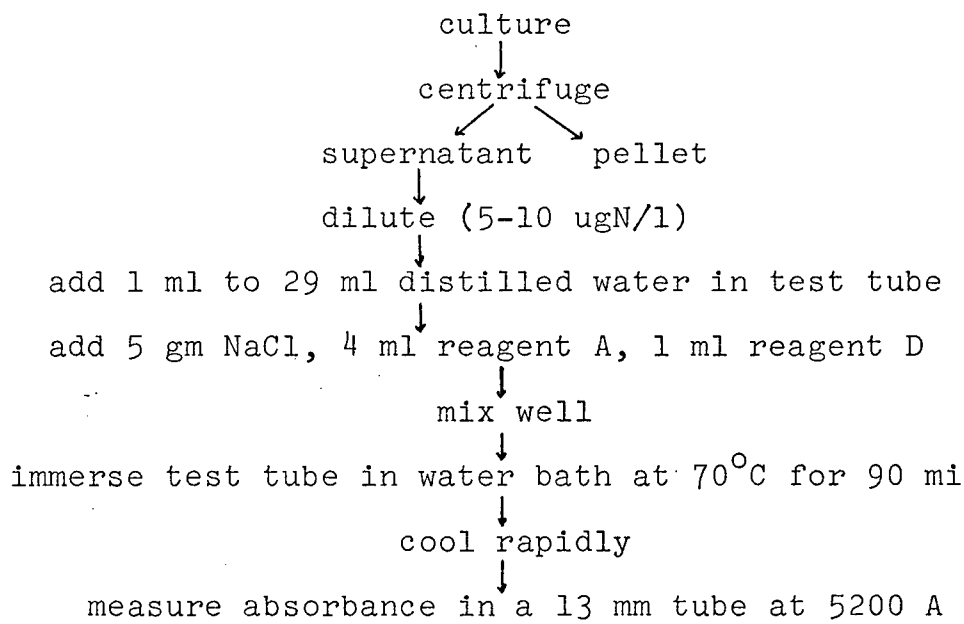
The amount of urea in the media was determined by the method of Newell et al. (see Table 3). A five ml aliquot of the culture to be analysed was centrifuged at 6500 x g in an International HN centrifuge for five minutes.[7] The supernatant was reserved and frozen. When several samples had accumulated the samples were thawed and the analysis immediately conducted.

3. Microscopy

Material for light microscopy was prepared by adding one drop of 0.5% glutaraldehyde to one ml of culture. Micrographs of the freshly fixed culture were obtained using a Zeiss Photomicroscope with phase contrast or Nomarski

Table 3. The determination of urea in sea water (Newell, Morgan, and Cundy, 1967)

Reagents: A) Dissolve 85 gm $NaH_2PO_4.2H_2O$ in 1 liter of concentrated H_2SO_4. B) Dissolve 5 gm di-acetyl monoxime in 100 ml water. Warm to assist solution. Cool, and add 0.06 gm semi-carbazide HCl. C) Dissolve 200 gm $MnCl_2.4H_2O$ and 4 gm KNO_3 in 500 ml water. D) Mix equal quantities of B) and C).



A calibration factor is determined in 20% NaCl solution from a stock urea solution containing 500 ug N per ml and diluted 100 times just before use. Reagent blanks are also determined in 20% NaCl solution.

interference illumination system.

Material for thin sectioning was concentrated by centrifugation at 3600 x g for five minutes in an International HN centrifuge. The fixation and dehydration schedules are shown in Tables 4 and 5. After dehydration the cells were infiltrated with increasing concentrations of Spurr's embedding medium (Spurr, 1969) in ethanol and cured in 100% resin. Sections were cut on either a Reichert OMU 3 ultramicrotome or a PorterBlum MT 3 ultramicrotome using a duPont diamond knife and were post-stained for 20 minutes with 5% uranyl acetate in 50% ethanol and then for 10 minutes with Reynold's lead citrate (Reynolds, 1963). Sections were viewed with either a Zeiss EM 9S electron microscope or an AEI-6B electron microscope.

III. Experimental Methods

1. Acclimation of Cultures to Media

The organisms were adapted from a nitrate enriched, natural sea water medium to enriched synthetic sea water plus 0.5 mM KNO_3 and then to 1.25 mM urea in enriched synthetic sea water. This was achieved by aseptically transferring a one ml aliquot of the original culture to duplicate 50 ml Erlenmeyer flasks containing 20 mls of enriched synthetic sea water plus 0.5 mM KNO_3. When good growth was observed, a one ml aliquot from these flasks was transferred to a duplicate set of flasks and similarly a third transfer was made. When three transfers resulting

Table 4. Fixation (Gantt, Edwards, and Provasoli, 1971) and dehydration schedule I

Medium	Time
2% glutaraldehyde added directly to liquid culture in 1 : 1 proportion	1 hour
0.1M sodium cacodylate buffer with 2.5% sucrose (pH 7.4)	15 minutes
0.1M sodium cacodylate buffer with 1.0% sucrose	15 minutes
0.1M sodium cacodylate buffer	15 minutes
1.0% OsO_4, buffer	1 hour
buffer	10 minutes
buffer	10 minutes
20% methanol	15 minutes
50% methanol	15 minutes
70% methanol	15 minutes
90% methanol	15 minutes
100% methanol	30 minutes
100% methanol	30 minutes

Table 5. Fixation and dehydration schedule II (Oakley, personal communication)

Medium	Time
1% glutaraldehyde, 0.125 M phosphate buffer, 0.3 M sucrose, pH 7.4	1 hour
buffer, 0.15 M sucrose	15 minutes
buffer	10 minutes
1% OsO_4, buffer	1 hour
buffer	10 minutes
distilled water	10 minutes
25% ethanol	30 minutes
50% ethanol	30 minutes
75% ethanol	30 minutes
absolute ethanol	30 minutes
absolute ethanol	30 minutes
absolute ethanol	30 minutes

in good growth were completed, the culture was considered to be acclimated to KNO_3 in enriched synthetic sea water. These are termed the "control" cultures and media. This culture was then acclimated to 1.25 and 12.5 mM urea in enriched synthetic sea water using a similar procedure. During the acclimation to the use of urea, transfers were made at one week intervals even if good growth was not visible. When good growth was observed following three or more transfers, the cultures were considered to be acclimated to the urea media. These are termed the "experimental" cultures and media.

2. Light and Temperature

To determine the optimal temperature and light conditions for good growth, a one ml innoculum from control cultures was aseptically transferred to 50 ml Erlenmeyer flasks containing 20 mls of control medium. Duplicate flasks were incubated at 7°, 10°, 15°, and $22^{\circ}C$ and a 16:8 hour, light:dark cycle, and at $22^{\circ}C$ with continuous light. Illumination was with fluorescent light[8] at approximately 150 foot-candles. Subjective comparisons were made between the growth and pigmentation of the cells in each pair of flasks after one and two weeks of incubation.

3. Urea Effects

Urea was added to synthetic sea water to give final concentrations of 0.10, 0.25, 0.50, 1.25, 6.25, and 12.50

mM urea. A one ml innoculum from the experimental stock culture was aseptically transferred to 125 ml Erlenmeyer flasks containing 50 mls of the experimental medium. Duplicate flasks were prepared for each concentration: these were incubated at 22°C and a 16:8 hour, light:dark cycle. Turbidimetric readings and direct cell counts were made at approximately 24 hour intervals. The pH change during growth was followed using a Radiometer PHM 63 pH meter and the amount of urea present in the cultures at 1.25 and 12.50 mM urea was determined at each time interval (see Table 3, p.18). Cultures at 1.25 and 12.50 mM urea were sampled at 48 hour intervals and the material prepared for electron microscopy (see Tables 4 and 5, pp.20, 21.). Similar determinations were made on control cultures at 0.50 and 2.50 mM KNO_3 in enriched synthetic sea water.

4. pH Effects

The enzymatic breakdown of one mole of urea yields one mole of CO_2 and two moles of NH_3. Excessive catabolism of urea could lead to ammonia poisoning and/or a lethal change of pH. Therefore, these effects were simulated in a healthy control culture. The effects of a high pH were observed by artificially adjusting the pH between 8.2 and 9.2 at intervals of 0.2 pH units with NaOH. The effects of an increased pH and an increased concentration of ammonia were observed by adjusting the pH to the same pH intervals with

NH_4OH. Periodically an aliquot from each was removed and gross structural changes were observed on the Zeiss Photomicroscope. The time required at each pH interval for a change in pigmentation and for culture lysis to occur was measured.

RESULTS

1. Acclimation of Cultures to Media

The results for the acclimation of the original culture to the experimental media are summarized in Table 6. The categories used are broad as there was variation in the M concentration (the total population of cells per ml at the end of stationary phase) and in the culture pigmentation among individual cultures which were under apparently identical culture conditions. These categories are as follows: (i) +++, the culture was turbid when shaken and appeared reddish-pink; (ii) ++, the culture was turbid when shaken and appeared yellow-orange; (iii) +, the culture was visible before shaking as tiny pin-prick spots on the bottom of the flask and after shaking produced very little turbidity and appeared colourless; (iv) MV, viable (Motile cells only were arbitrarily taken as a visible sign of viability during microscopic examination.) cells were visible only with microscopic examination and the culture appeared clear and colourless; (v) -, no viable cells were observed. While acclimation of the original culture to control media presented no difficulties and was easily duplicated (Good growth was observed in each of three different experimental runs.), the acclimation of the control culture to experimental media was difficult and the duplication of the results was erratic (see Table 6).

Table 6. Acclimation of the control culture to enriched synthetic sea water plus 1.25 mM urea. The results of several different experimental runs are compiled under each experiment. (Experiment 3 is a synthesis of 50 different testtubes, the other two experiments were run with duplicate testtubes.) After an incubation period of one week the type of growth was subjectively determined* and an innoculum transferred to fresh media. The culture was considered acclimated when good growth (+++) resulted after three transfers.

Experiment	Growth in control media	Growth in experimental media		
		Transfer 1	Transfer 2	Transfer 3
1	+++	++	MV	MV
2	+++	++	**	--
3	+++	++	**	+++

*Legend: +++, the culture is turbid when shaken and appears reddish-pink; ++, the culture is turbid when shaken and appears yellow-orange; +, the culture is visible before shaking as tiny pin-prick spots on the bottom of the flask and after shaking produces very little turbidity and appears colourless; MV, viable cells are visible only with light microscopic examination and the culture appears colourless; -, no viable cells are observed.

**Culture intermediate between + and ++ in that yellow-orange pin-prick colonies are observed.

2. Light and Temperature

The shortest lag period and the best culture colour were observed at 22°C with a 16:8 hour, light:dark cycle (see Table 7). As seen from Table 7, an increased lag period occurred with incubation at the lower temperatures and poor culture pigmentation was observed with continuous illumination.

3. Growth

(i) Measurements

A growth pattern typical of the control cultures incubated at 22°C with a 16:8 hour, light:dark cycle is shown in Figure 1. The short lag phase was followed by an extended period of logarithmic growth, then by a stationary phase. During the stationary phase very little cell division occurred and the culture colour changed from a clear reddish-pink to a duller reddish-orange. Many cells appeared to be non-motile but no other gross structural abnormalities were observed. Gradually the death or decline phase began[9] during which the culture pigmentation occasionally turned green and evidence of cell lysis such as starch grains and membrane fragments could be seen with the light microscope.

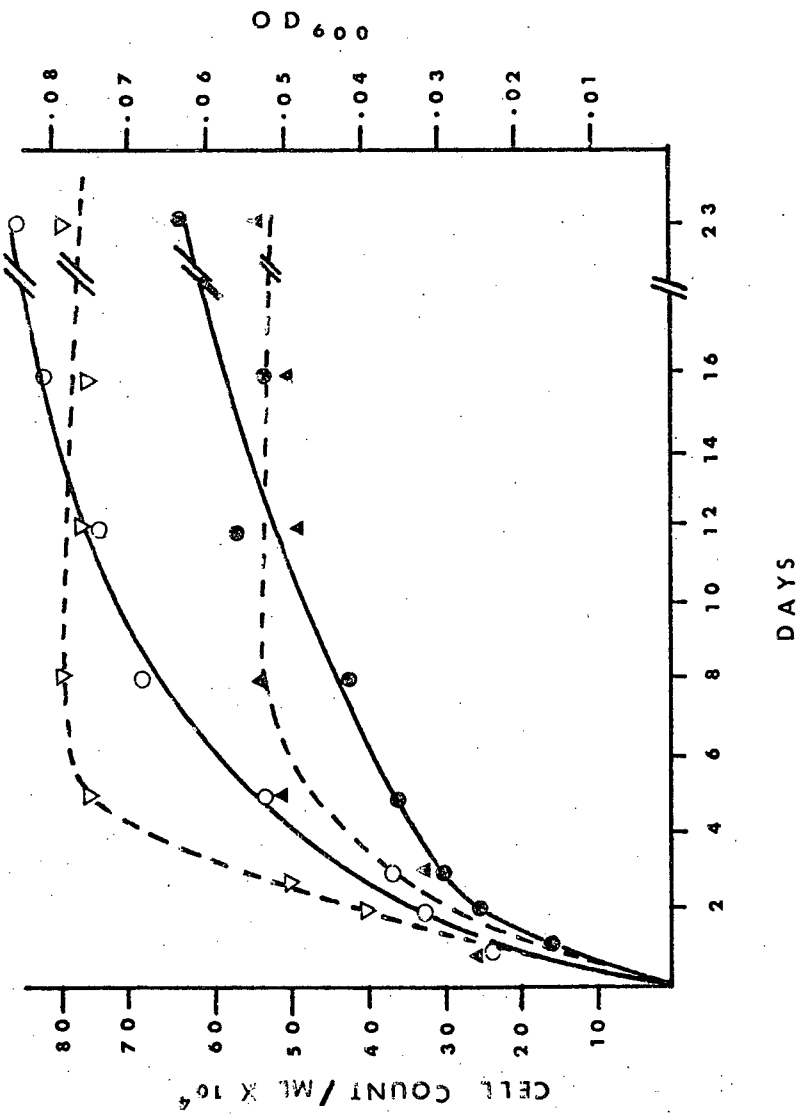
(ii) Ultrastructure

The ultrastructure of control cells during the lag and logarithmic phases was similar to that described for the

Table 7. A comparison of growth in control media at various temperatures and light periods. Parameters used are lag period and pigmentation of the culture, during logarithmic phase. A dash indicates no growth.

Growth conditions		Lag period	Pigmentation during log phase
Temp. °C	Illumination		
7	16:$\overline{8}$, light: dark	--	--
10	"	--	--
15	"	3 weeks	clear red-pink
22	"	3 days	clear red-pink
22	continuous	2 days	yellow-orange

Figure 1. Growth pattern of culture incubated in control media. Circles indicate 0.5 mM KNO_3; triangles indicate 1.0 mM KNO_3. Solid symbols are turbidimetric determinations, open symbols indicate cell count.


OD 600
.08
.07
.06
.05
.04
.03
.02
.01
CELL COUNT / ML X 10⁴
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
23
DAYS

Cryptophyta (see page7). The cells of Rhodomonas lens are smaller than those described by Butcher (1967) and average 8-10 μ during interphase. Plates 1-3 illustrate the asymmetric cell shape, the Corps de Maupas (CM), the trichocysts (T), and the two unequal flagella (F) inserting near the anterior gullet (G) which are typical of this organism (Butcher, 1967). The flagella are covered with regularly arranged mastigonemes (M, Plate 2). A bilobed chloroplast (CHL, Plate 3) follows the cell shape and is close to but not appressed to the periplast (P). The thylakoids, usually associated in bands of two (arrows, Plates 1 and 3), are often arranged in parallel stacks. They do not traverse the pyrenoid (Py, Plate 1). Starch grains (S, Plate 1) are found around the pyrenoid and occasionally within the perichloroplastic matrix. When present the small lipid bodies are located posteriorly. Near the chloroplast and often between the chloroplast and the periplast are numerous mitochondria (M, Plates 1 and 3). The periplast appears bipartite (Plates 1 and 3, white arrows), consisting of the plasma membrane (PM) and another inner layer (IL). The plasma membrane is continuous over the entire cell: the inner layer is absent over the flagella and the gullet region.

During stationary and decline phases in the control cultures some cell division occurs, and cells which are used as innocula from these phases readily produce good growth in fresh media. The ultrastructure of such cells shows

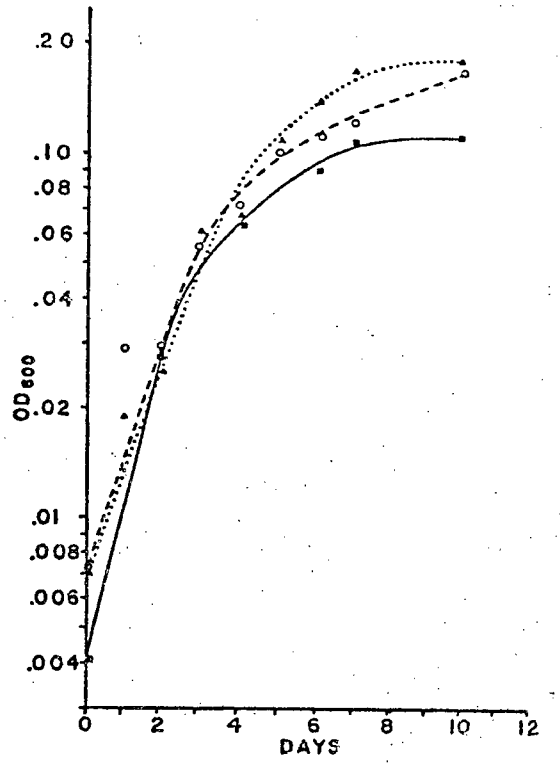
distinct differences from that of earlier phases. Plate 4 illustrates these differences. The chloroplast occupies a lesser area of the cytoplasm with the thylakoids occurring singly (arrows) rather than in associated pairs. The stromal area (SA) has increased significantly and large starch grains are found within the perichloroplastic matrix. Mitochondria appear only near the base of the flagella. Large bodies of lipid-like (LL) material occupy much of the cytoplasm; it is this material which appears to be extruded first when lysis occurs.

4. Urea Effects

(i) Growth Measurements

Preliminary experiments, in which turbidimetric readings were used as a measurement of culture growth, indicated little variance in growth among the cultures at the several concentrations of urea (Figure 11). A long stationary phase and a very gradual decline phase was observed in cultures grown in media containing 0.25, 0.50, 1.25 and 2.50 mM urea. Table 8 shows the comparative changes in pigmentation and M-concentration at varying levels of urea. The results from cultures grown in media containing 6.25, and 12.50 mM urea show a very short stationary phase followed by a sharp decline phase. In later experiments both turbidimetric readings and direct cell counts were used as measurements of growth, the two methods acting as a check, one for the other. Figure 12

Figure 11. Growth pattern of cultures at several concentrations of urea based on turbidimetric determinations. Closed squares = 0.25 mM urea; open circles = 0.50 mM urea; closed triangles = 1.25 mM urea; open triangles = 2.50 mM urea; closed circles = 6.25 mM urea; open squares = 12.50 mM urea.


.20
.10
.08
.06
.04
OD_{600}
.02
.01
.008
.006
.004
0
2
4
6
8
10
12
DAYS

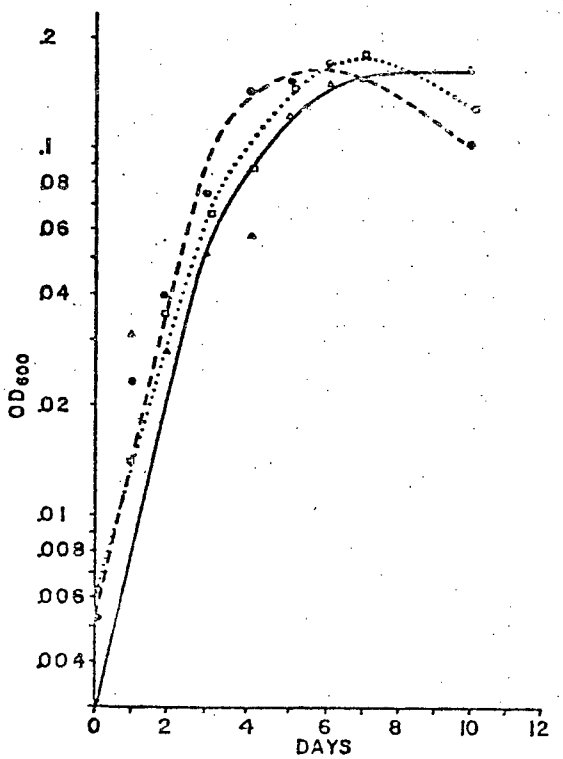

.2
.1
.08
.06
.04
OD_{600}
.02
.01
.008
.006
.004
0
2
4
6
8
10
12
DAYS

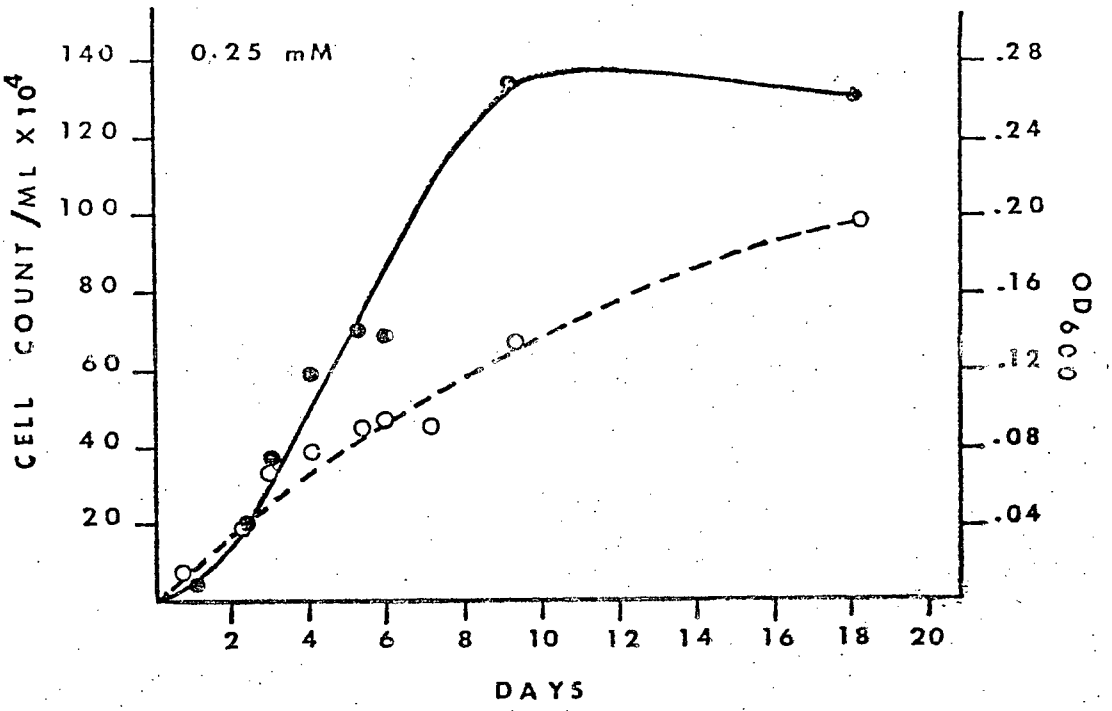
Table 8. Comparison of culture colour and M-concentration* during growth of experimental cultures at varying concentrations of urea.

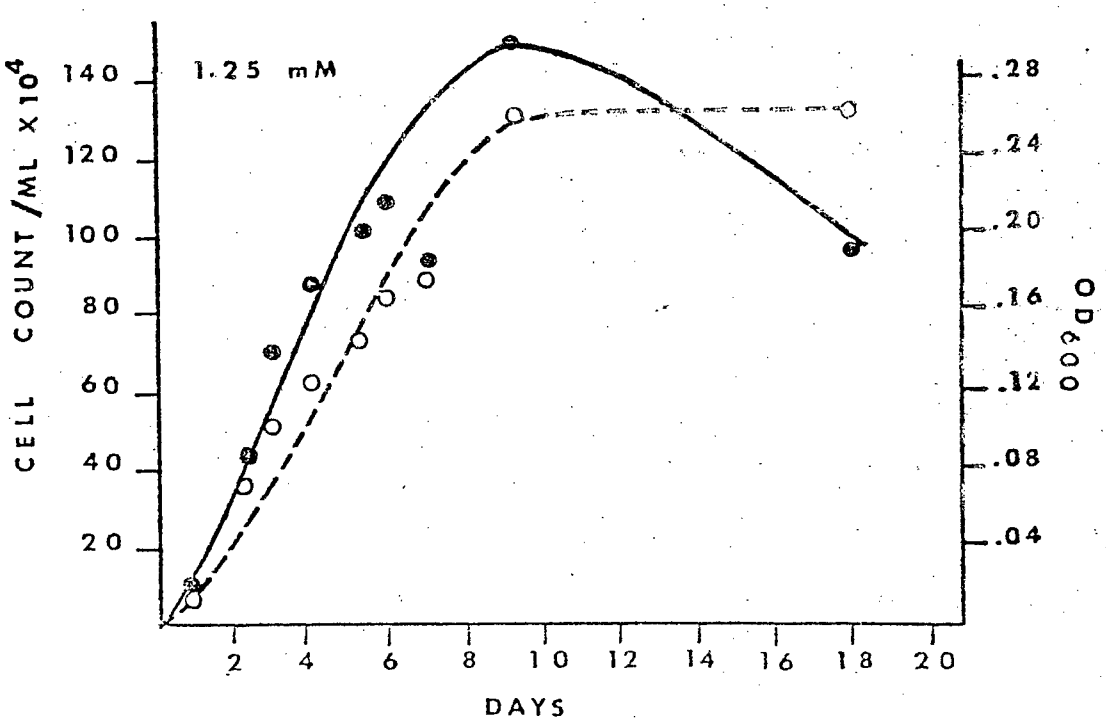
Concentration of urea (mM)	Pigmentation during logarithmic phase	Pigmentation during stationary phase	M-Concentration X 10^4
.25	yellow-orange	yellow-green	135
.50	yellow-orange	yellow-green	--
1.25	clear red-pink	orange-red	150
2.50	clear red-pink	orange-red	115
6.25	clear red-pink	**	58
12.50	clear red-pink	**	52
control (.5 mM NO_3)	clear red-pink	orange-red	80

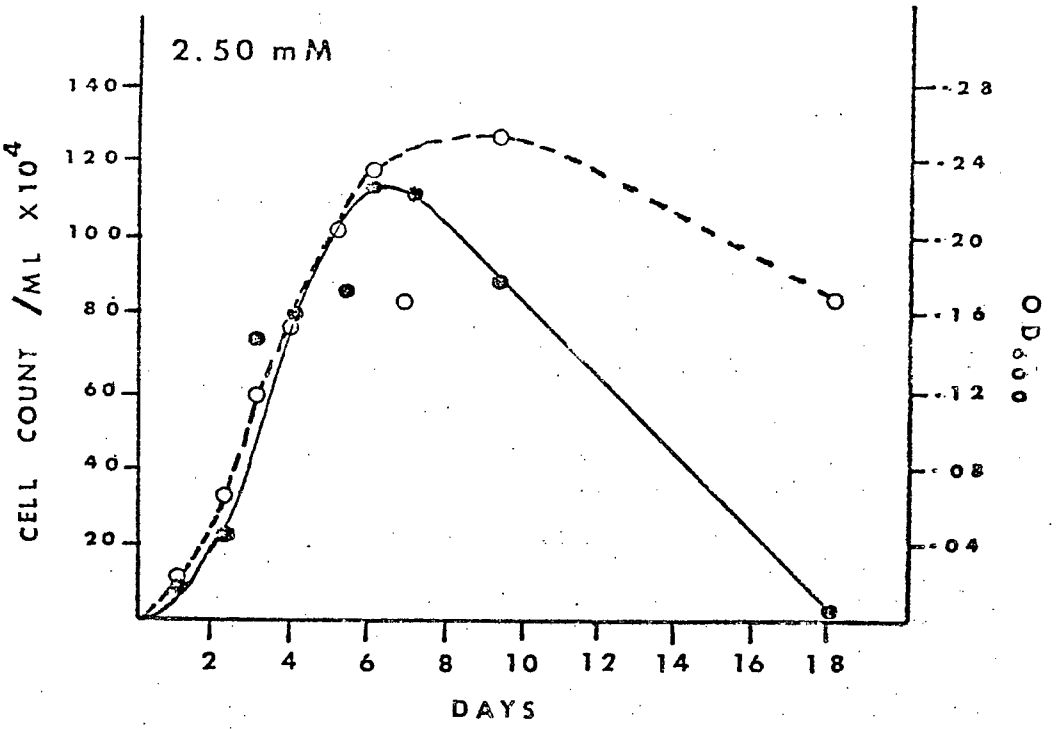
* M-concentration is the total population of cells per ml at the end of stationary phase.

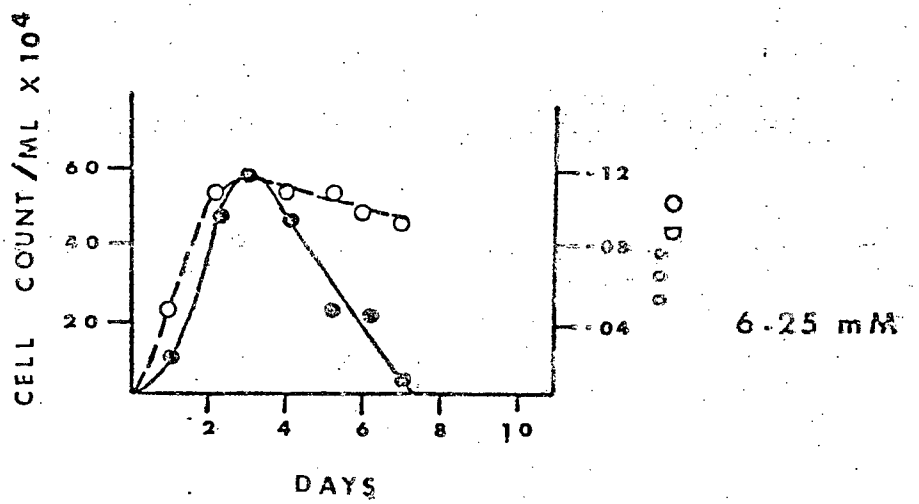
** No stationary phase observed.

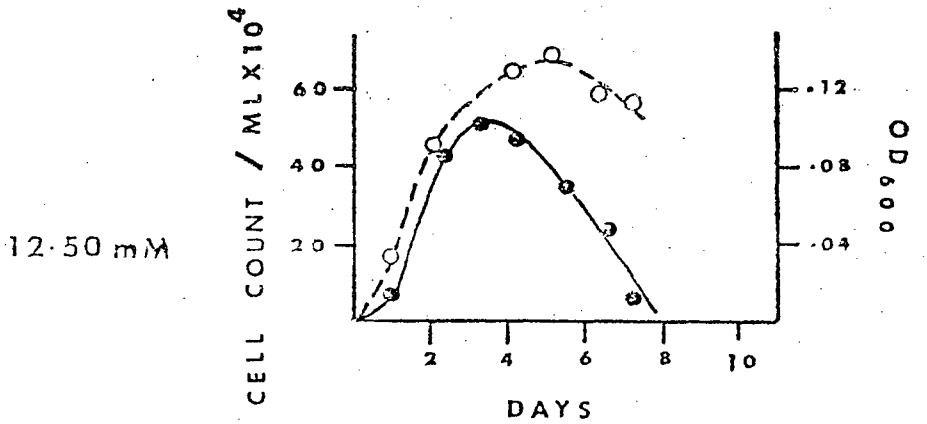
Figure 12. Growth pattern of experimental cells at several concentrations of urea. Closed circles show growth as determined by direct cell count; open circles, by turbidimetric determinations.


0.25 mM
CELL COUNT /ML X 10^4
140
120
100
80
60
40
20
.28
.24
.20
.16
.12
.08
.04
OD600
2
4
6
8
10
12
14
16
18
20
DAYS


1.25 mM
CELL COUNT /ML X 10^4
140
120
100
80
60
40
20
.28
.24
.20
.16
.12
.08
.04
OD600
2
4
6
8
10
12
14
16
18
20
DAYS


2.50 mM
CELL COUNT /ML X10^4
140
120
100
80
60
40
20
.28
.24
.20
.16
.12
.08
.04
OD600
2
4
6
8
10
12
14
16
18
DAYS


CELL COUNT /ML X 10^4
60
40
20
.12
.08
.04
OD600
2
4
6
8
10
DAYS
6·25 mM


12·50 mM
CELL COUNT / ML X10^4
60
40
20
.12
.08
.04
OD600
2
4
6
8
10
DAYS

illustrates growth measured in this way. During the lag and logarithmic phases of cultures grown at concentrations equal to or greater than 1.25 mM urea the growth as measured by either method was roughly equivalent. However, once stationary phase began, the turbidimetric readings indicated a substantially greater degree of growth than was observed by direct cell counts. Data from these two types of measurement were not similar for cultures grown in 0.25 mM urea. The calculated generation times for cultures at each concentration of urea are given in Table 9. Figure 13 shows the logarithmic decrease in generation time which occurs with increasing urea concentration.

(ii) Ultrastructure

The ultrastructure of experimental cells at all levels of urea during lag and logarithmic phases is similar to that of the control cells during these phases (Plates 1-3). Increased lipid accumulation (L) was observed in all experimental cells during lag and early logarithmic phases (Plate 5) but most of this disappeared at the onset of rapid division. Some lipid was usually observed in discrete drops near the cell periphery (Plate 6).

(iii) Electrolytic Effects

The pH recorded during growth varied considerably between individual cultures (see Table 10). In Run 1 the lysis of cells shortly after the onset of logarithmic

Figure 13. The relationship between the generation time of a culture and the concentration of urea in which it has been incubated.

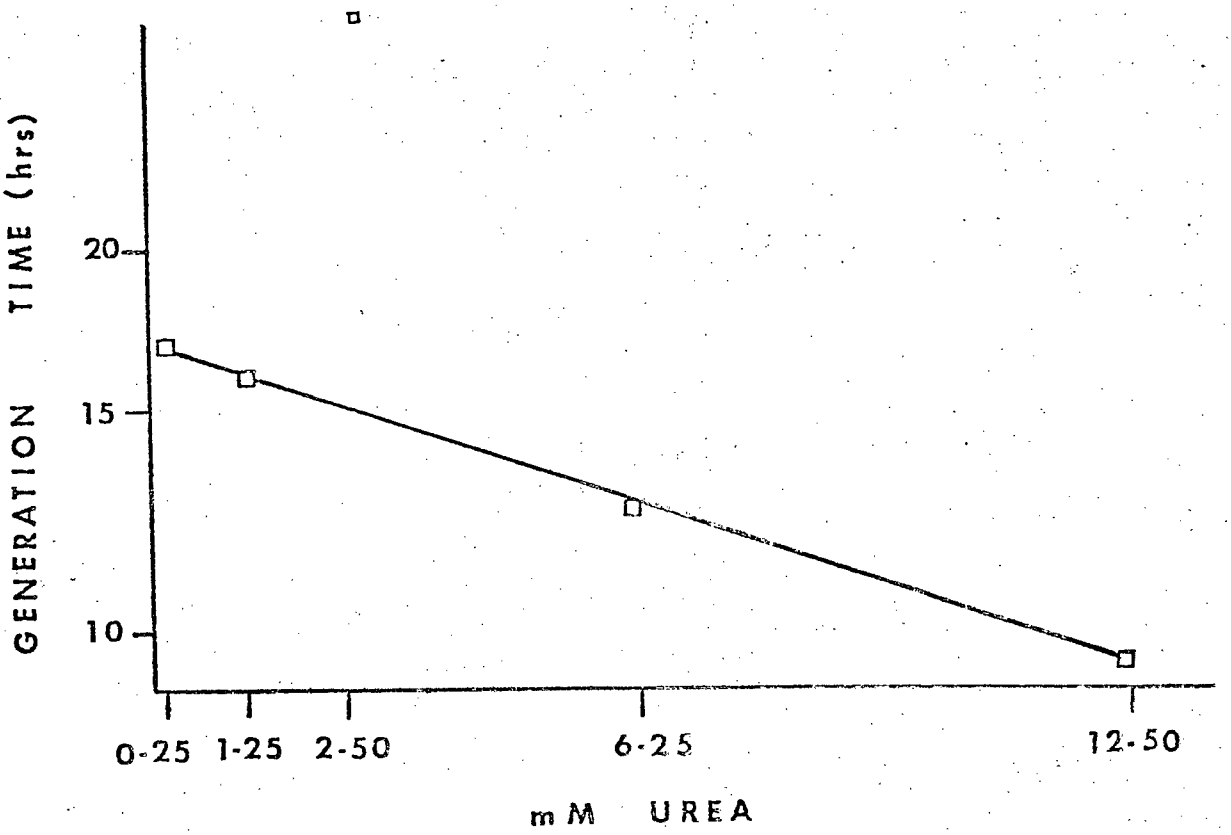

GENERATION TIME (hrs)
20
15
10
0·25
1·25
2·50
6·25
12·50
m M UREA

Table 9. A comparison of growth by division of cultures at increasing concentrations of urea.

Concentration of urea (mM)	No. of generations, n[a]	Time (hrs)	Generation time g[b]
control[c] (0.5 mM NO_3)	1.74	24	13.8
0.25	1.42	24	17.0
1.25	1.51	24	15.9
2.50	0.79	24	30.4
6.25	1.92	24	12.5
12.50	2.50	24	9.6

[a]This figure was calculated from the equation $n = \frac{\log_{10}y - \log_{10}x}{.301}$, where n is the number of generations, y is the larger number of organisms at the end of a time period t, and x is the smaller number of organisms at the beginning of the time period t.

[b]This figure is calculated from the equation $g = \frac{t}{n}$, where g is the generation time, t is the time period, and n is the number of generations.

[c]The control was grown in media without urea, and with 0.5 mM KNO_3 added.

Table 10. Variation in pH and urea catabolism among individual cultures.

Culture	Time (days)	Cell no. per ml, x 10^4	pH	µg-atom urea-N per ml media
Control (0.5 mM NO_3)	6	54	7.6	--
	11	70	7.6	--
1.25 mM urea Run 1	0	estimated[a]	7.4	80[b]
	2	3.5	8.0	--
	6	14.8	8.5	83
	11	lysed	8.7	17
1.25 mM urea Run 2	0	estimated	7.4	80
	2	1.6	7.4	72
	6	29.6	7.4	79
	8	78.0	7.6	82
	--[c]	--	--	--
12.5 mM urea Run 1	0	estimated	7.4	750
	2	10.4	8.3	--
	6	23.0	9.1	338
	11	lysed	8.7	14
12.5 mM urea Run 2	0	estimated	7.4	750
	2	1.3	7.4	--
	6	45.9	7.5	749
	8	79.0	7.6	756
	--[c]	--	--	--

[a] A one ml innoculum containing approximately 15.4×10^4 cells ml^{-1} was added to each 20 mls of media.

[b] This figure is calculated from the known amount of urea added to the media.

[c] Bacterial contamination was observed at 10 days, therefore the run was terminated.

phase coincided with an increased pH and a disappearance of urea in the media. In the second (and identical) run a normal increase (as per Figure 12) in cell number per ml was observed and no significant increase in pH or decrease in urea occurred. In order to clarify this discrepancy in results the pH experiments were initiated. Table 11 summarizes these experiments. The artificial increase in pH with NaOH did not induce any gross structural changes in the cells and culture growth continued. When the pH of the culture was adjusted by the addition of NH_4OH, culture death was observed with pH levels equal to or greater than 8.4 (Table 11). Plate 7 illustrates the sequential changes from normal cell structure at the point of pH change to culture death. At pH levels between 8.4 and 8.65, cells initially lose their characteristic shape and become rotund (Figure 18, 19); the cup-shaped chloroplast becomes flattened towards the posterior of the cell (Figures 20, 21); then protrusion of the cell contents and cytoplasmic vacuolation occurs (Figures 22 to 24). Finally, one or more of the protrusions bursts, and the cell contents are released (Figure 25). At pH levels greater than 8.65 culture death occurs very rapidly. The cell contents protrude, ejected trichocyst-like threads are released externally, and one or more of the protrusions bursts.

Table 11. The effects of pH on a healthy control culture. (The pH was adjusted with either NaOH or NH_4OH.)

pH	NH_3 conc. (mM)	Time required for culture lysis with: NH_4OH	NaOH
7.4		no lysis	no lysis
8.0	0.8	no lysis	↓
8.2	2.4	no lysis	
8.4	4.0	24 hours	
8.65	5.6	8 hours	
8.8	8.0	4-6 hours	
9.0	10.30	1 hour	
9.2	13.50	1 hour	

DISCUSSION

Rhodomonas lens is a photoautotroph. Such organisms usually possess a latent capacity to use various nutrient sources via the induction of the enzyme(s) appropriate to metabolize the compound. The uptake of nitrate in many species of marine phytoplankton is mediated by a membrane-bound (NO_3^-, Cl^-)-activated adenosine triphosphatase (Falkowski, 1975 a, b) and the metabolism of nitrate requires the enzyme nitrate reductase which catalyzes the reduction of nitrate to nitrite and the enzyme nitrite reductase, which catalyzes the reduction of nitrite to ammonia. Ammonia is utilized via transamination reactions. Nitrate reductase has been shown to be inducible in the presence of nitrate and molybdenum (Schrader et al., 1967). Nitrite reductase is also known to be inducible (Schrader et al., 1967). The metabolism of urea is catalyzed by either urease or UALase. Both of these enzymes have been shown to be induced in the presence of urea (Jeffries, 1964; Berns, Holohan and Scott, 1966; Roon, and Levenberg, 1970). These enzymes serve to convert alternative sources of nitrogen to ammonia, which can then be metabolized in the central metabolic pathway.

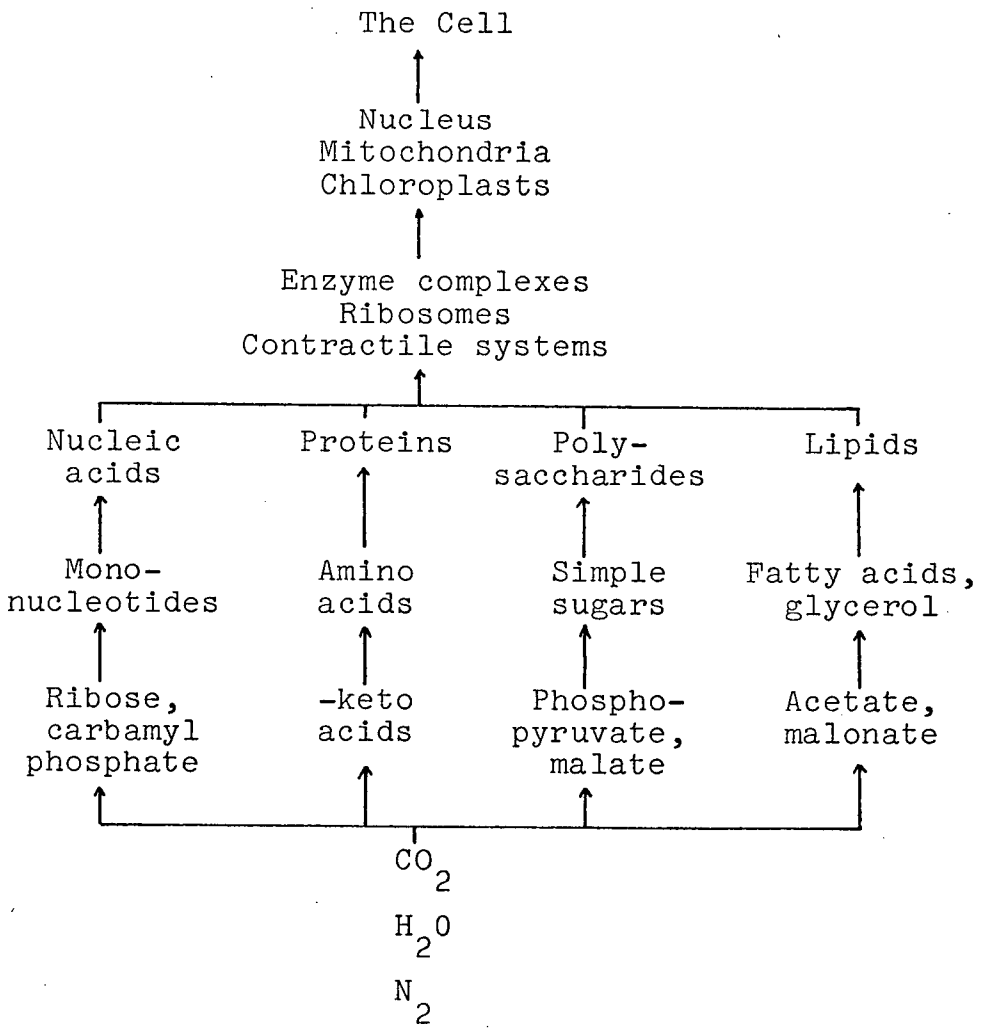
The results indicate that Rhodomonas lens readily acclimated to the nitrate medium. However, the acclimation of Rhodomonas lens to urea medium was very erratic although experimental conditions were apparently identical. It was

assumed that when growth occurred the enzyme[10] had been induced, since experimental results indicated the improbability of acclimation resulting from change or mutation: (Table 6). The cause of the erratic behaviour of this organism in urea is unknown. Antia (personal communication) observed some erratic growth with Rhodomonas lens in a urea enriched, natural medium. As the culture was originally isolated from the Gulf Stream, it is possible that this organism requires a higher salinity[11] than that provided by the culture medium: if the salinity of the medium was at the edge of its minimum requirement, erratic growth would be expected. This could be tested by inoculating control cells into experimental media adjusted to several levels of salinity. (Temperature and light optima were checked.)

Another possible variable is the urea media. The media was not always prepared directly before use and especially in cases where it was allowed to sit for several days at room temperature, the presence of breakdown products inhibitory to cell growth cannot be discounted. This will be discussed in detail later.

The role of nitrogen in a balanced metabolism is illustrated in Figure 26 (left side). The rate of synthesis of each type of macromolecule is geared so that one does not overgrow another. Generally, if an imbalance leading to underproduction of a metabolite occurs, either lysis or culture stasis would be expected. However, dependent on

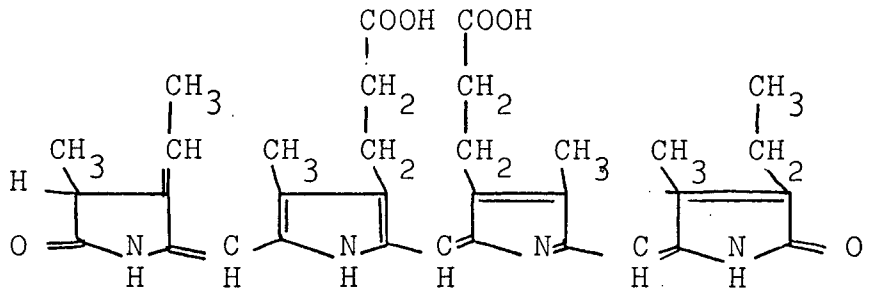
Figure 26. Metabolic interrelationships. (Lehninger, p. 19.)



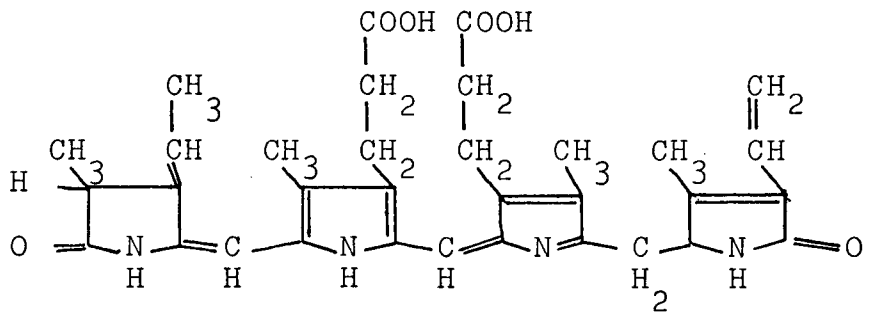
the nutrient, this would occur in varying degrees. If nitrogen was depleted from the medium, the synthesis of amino acids, nucleotides and hexosamines would stop as would all polymers derived from them. Referring to Figure 26, it will be noted that only the polysaccharides and non-protein lipids would continue to be synthesized. If nitrogen was not depleted but the culture was subjected to some degree of nitrogen stress[12], again lipids and polysaccharides would continue to be synthesized normally but the synthesis of all molecules containing nitrogen would proceed at the rate at which nitrogen was made available. This would result in an unbalanced metabolism, the most obvious symptoms being an accumulation of storage products, a lack of any non-vital molecules containing nitrogen, and a rate of division directly related to the rate at which nitrogen was made available.

Rhodomonas lens produces dispensible, nitrogen-containing products, the phycobilin pigments, which can be used as a subjective indication of the nutritional well-being of the cells. Three classes of pigments are found in the Cryptophyta: the chlorophylls, the carotenoids, and the phycobilins. Chlorophyll is the primary light-trapping molecule; the carotenoids and the phycobilins probably serve as supplementary light receptors for that portion of the visible spectrum not completely covered by chlorophyll. The carotenoids probably also function to protect chlorophyll from degradative attack by molecular

oxygen. The phycobilins are open-chain tetrapyrroles which are conjugated to specific proteins:



Phycocyanobilin



Phycoerythrobilin

The phycobilins are degraded during conditions of nutritional deficiency with their components, specifically nitrogen, being utilized for more vital cell processes (Antia et al., 1973). Since each class of pigment gives the culture a specific colouration[13], when one class is degraded the culture changes colour. The colour change has always been observed in the following order: clear red-pink to reddish-orange to green to cream.

As the only variable between the control and experimental media was the nitrogen source, the differences which occurred are attributable to the source of this

nutrient.

The control cultures during lag and logarithmic phases exhibited the clear red-pink colour characteristic of the phycobilin pigment(s) and deductively, indicative of sufficient nitrogen. Microscopy also pointed to healthy, balanced metabolism: light microscopic observation showed a normal degree of motility, the typical asymmetric cell shape, and some evidence of various stages of cell division. The amount of rough endoplasmic reticulum, and numerous ribosomes and golgi vesicles suggest active protein synthesis, while a balanced metabolism was indicated by the lack of lipid or other storage products. (The chloroplast was actively producing starch, but this product was found only within the perichloroplastic matrix and not accumulated in the cytoplasm.) Shortly after the onset of stationary phase the culture colour changed to an orange-red. This apparent degradation of the phycobilins suggests that nitrogen had become limiting; the cells' response to a lack of nitrogen in the medium being the mobilization of the nitrogen components from the phycobilins[1A]. Cytological evidence corroborates this.

The loculus contains lipids, proteins and soluble pigments. A depletion of any of these could conceivably cause a change in loculus size. In the control cells during logarithmic phase the loculus measures about 110-260 Å in diameter; during stationary phase, when the thylakoids are arranged singly, the loculus ranges between 70 and 150 Å.

It can be argued that one or more 'filler' substance(s) which helped to maintain the original loculus size has been depleted somewhat: the obvious substance would be the phycobilin pigments. Thus the change in loculus size may be an indication that the phycobilins have been depleted. Electron microscopy also shows copious amounts of a lipid-like material and numerous starch granules. While Antia et al. (1973), suggest that these may be symptoms of senescence, both Beijerinck (1904) in diatoms and Spoehr and Milner (1949) in Chlorella noted an increase in lipid content with an exiguous supply of nitrogen. It is probable that the 'aging' is directly attributable to the depletion of nitrogen from the media. Eventually, during a prolonged stationary-decline phase, the culture may turn green and generally shortly thereafter, a creamy-white. The breakdown of much of the carotenoids indicates that the culture is in serious trouble nutritionally and this is corroborated by the subsequent colour-change observed as the cells lyse.

The fine structure of experimental cells during late lag and early logarithmic phases approximates that of the control cells during stationary phase when they exhibit signs of nitrogen stress. A slightly lesser accumulation of lipid is found in the experimental cells during these phases. The other two parameters, pigmentation and rate of division, are difficult to apply at this stage.

A trend toward nitrogen stress is noted during

logarithmic phase: small lipid droplets are often seen within the cytoplasm and a division rate somewhat lower[15] than that of the control is observed. The direct increase of division rate with increasing concentrations of urea suggests that it is the uptake and/or metabolism of this compound which directly affects the division rate. Therefore, although it is very unlikely that the culture is actually metabolizing all the urea in the media, the experimental cultures appear to be undergoing nitrogen stress during lag and early logarithmic phases and to a lesser degree during mid- and late logarithmic phase. As previously discussed, this would account for an accumulation of storage products, especially during the extended period of time when the cells are replenishing their internal supply of intermediates and manufacturing essential metabolites and enzymes preparatory to multiplication. To a lesser extent this accumulation would occur during logarithmic growth, nitrogen stress being reflected more indirectly in the division rate.

Decline phase is marked by a complete loss of pigmentation and a definite series of morphological changes which terminate in culture autolysis. These changes are characteristic of cells suffering from osmotic imbalance. The increase in pH levels prior to the onset of decline phase suggests that the change is caused by or associated with a basic ion. The changes are not precipitated by a simple pH effect; rather the cells are tolerant of high pH

levels. When the pH of the culture medium was raised artificially with NaOH, the cells exhibited no structural abnormalities and continued to divide, even at a pH of 9.2. However, when pH levels were adjusted with NH_4OH, morphological changes similar to those observed during the decline phase of the experimental cultures occurred. Shilo and Shilo (1962) report the induction of similar morphological changes in Prymnesium using ammonia and acetic acid as weak electrolytes associated with pH levels between 8.0 and 9.0.

A consideration of the experimental culture media suggests that the most likely source of basic ions is urea. Urea is enzymatically catabolized giving 2 moles of ammonia and one mole of carbon dioxide. The decomposition of urea in media is unknown but in distilled water 8.0 M urea decomposes slightly, giving 20 mM ammonium cyanate. That the decline phase occurs only above a certain concentration of urea (1.25 mM) and that the onset of the decline phase is more rapid at increasing concentrations of urea, is a further indication that the factor which induces this phase is a product of urea decomposition, probably ammonia.

Two questions arise from these observations:

1. Why do cells capable of metabolizing urea as a nitrogen source show signs of nitrogen stress in media containing excess urea?
2. Why do the same cells during logarithmic phase develop signs of ammonia poisoning and lyse?

The μg-atom N liter^{-1} concentration is 2 to 50 times greater in the urea media than in the nitrate media. Nitrogen stress must, therefore, either be the result of an inefficient assimilation of urea or an inefficient metabolism of urea by the cell. As the results indicate a later accumulation of a basic ion, probably ammonia, it is suggested that the urea-N pathway is inhibited such that the ammonia produced is not efficiently assimilated but released into the media.

Uchida (1975) found that the dinoflagellate Prorocentrum micans took up urea when grown in a medium with urea as the sole nitrogen source, and then released ammonia into the medium. This occurred during a prolonged lag phase. When a 'critical' level of ammonia in the medium was attained, the organism entered logarithmic phase and apparently maintained this phase until much of the ammonia was assimilated. The organism then repeated the process, the second 'critical' level of ammonia being lower than the first. The growth curve thus appears typical of diauxic growth.

In two experimental runs with Rhodomonas lens, urea was depleted rapidly with a concomitant increase in pH (indicating an increase in basic ions--probably NH_4^+) (Table 10): thus Figure 12, at concentrations of 0.25-2.50 mM, could be drawn to include all points (rather than drawing the best line among all points). This curve would then be indicative of diauxic growth. Therefore, it is

possible that Rhodomonas lens is exhibiting growth on urea similar to that shown with Prorocentrum micans. Speculatively, if Rhodomonas lens is behaving in this manner and if the amount of ammonia excreted is directly proportional to the concentration of urea in the medium it is probable that at higher levels of urea (6.25 and 12.50 mM) the amount of ammonia excreted would reach toxic levels which, with the increased pH, would cause lysis of the culture.

A possible alternative answer to the questions posed is that breakdown products of urea in solution are inhibitive to some part of the nitrogen metabolic pathway.

Hattori (1957), with Chlorella, found that cyanide in a concentration of 4.0 mM almost completely halted ammonia assimilation, while it was almost completely without effect on urea assimilation. Cyanide at a concentration of 0.4 mM inhibited 78% of ammonia assimilation (Hattori, 1957). Hodson and Thompson (1969) also used cyanide at a concentration (10 mM) which inhibited ammonia uptake completely but urea uptake only slightly in Chlorella; under these conditions they found an accumulation of ammonia in the medium. Another substance causing similar results is arsenite (Hattori, 1957; Hodson and Thompson, 1969).

A survey of the literature revealed that urea decomposes in aqueous solution. Fawsitt (1902) and Walker and Hambly (1895) both demonstrated that an intermediate

cyanate stage formed when aqueous solutions of urea are heated above 70°C. Werner (1918) showed that the 'hydrolysis' of urea under all conditions involved the simple dissociation of the urea molecule into ammonia and free cyanic acid:

$$CON_2H_4 \longrightarrow NH_3 + HNCO.$$

Fearon (1923) detected the presence of cyanic acid in his urea/urease system although he wrongly attributed it to an enzymatic breakdown product of urea. More recently, Antia and Landymore (1974) found that uric acid is chemically unstable in sea water phytoplankton culture medium. Uric acid showed a slow degradation in darkness: this was increased several-fold by illumination. A major factor in the acceleration of uric acid breakdown was found to be free trace-metal ions, whose importance has been attested to by many organic chemists. Unfortunately, the breakdown products were not identified.

If urea decomposes to cyanate and ammonium in aqueous solution, it is probable that it also does so to a greater extent under illumination and in the presence of free trace metal ions.

The effect of cyanate on nitrogen metabolism is unknown. The amount of ammonia produced by such a decomposition is unlikely to produce the toxic effects noted in the experimental cultures. If, however, cyanate reacted similarly to cyanide in inhibiting ammonia assimilation, a

very low level of cyanate in the media could cause a degree of nitrogen stress concomitant to an increasing accumulation of ammonia. At a certain point, dependent upon the concentration of urea, the level of ammonia would become toxic and the electrolytic effects noted would occur.

The presence of cyanate in urea culture media could be evaluated by removing aliquots of a sterile sample of such media over a period of time and treating these aliquots with urease. This would give a quantitative indication of the amount of urea not decomposed. The urease could then be removed by means of aluminum hydroxide and a silver nitrate test for cyanate employed (Fearon, 1923).

The effect of cyanate on nitrogen metabolism should also be evaluated. This could be determined by the addition of varying concentrations of cyanate to cultures grown in urea, nitrate and ammonia media. The rate of uptake of the nitrogen compound and the amount of ammonia in the media over a period of time should be ascertained. The ammonia media would give a direct indication of the effect of cyanate on ammonia assimilation. The urea media would give a direct indication of the effect of cyanate on urea assimilation and inhibition of the subsequent metabolic step. The nitrate media would act as a check, as normally no ammonia would be observed in the media unless the metabolic pathway were inhibited.

CONCLUSION

One effect of urea on Rhodomonas lens under the imposed experimental conditions appears to be the induction of nitrogen stress during lag and logarithmic phases. This stress could be caused by an inefficient uptake mechanism regulated perhaps by the salinity of the media or by an inefficient metabolism of urea. A decline phase during growth of the experimental organism occurs when concentrations of urea are equal to or greater than 0.50 mM. The appearance of cells during this phase is similar to that of cells exhibiting ammonia toxicity. It is suggested that the pathway of urea metabolism is inadequate, such that the ammonia produced by the breakdown of urea is inefficiently assimilated, with the excess ammonia being released into the medium. The increasing accumulation of ammonia in the medium from the higher urea concentration accelerates cell lysis, resulting in a decline phase due to ammonia toxicity. A possible inhibitory factor in the urea utilization pathway is a breakdown product of urea: urea $\longrightarrow NH_4^+$ cyanate.

PLATE 1.

Figure 2. An optical micrograph of control cells in culture using Nomarski interference illumination. Note the asymmetric cell shape and the insertion of the flagella (F) near the anterior gullet (G). X 2920.

Figure 3. A slightly oblique transverse section from a control culture during logarithmic phase. Organelles which can be seen are the nucleus (N), mitochondria (M), golgi body (Go), large trichocysts (T) which line the gullet (G), small trichocysts (t) near the periplast (P), and the chloroplast (CHL). Within the chloroplast part of the pyrenoid (Py) and associated starch grains are visible. The thylakoids are associated in bands of two (arrows). Note the bipartite nature of the periplast (open arrows). X 12,000.

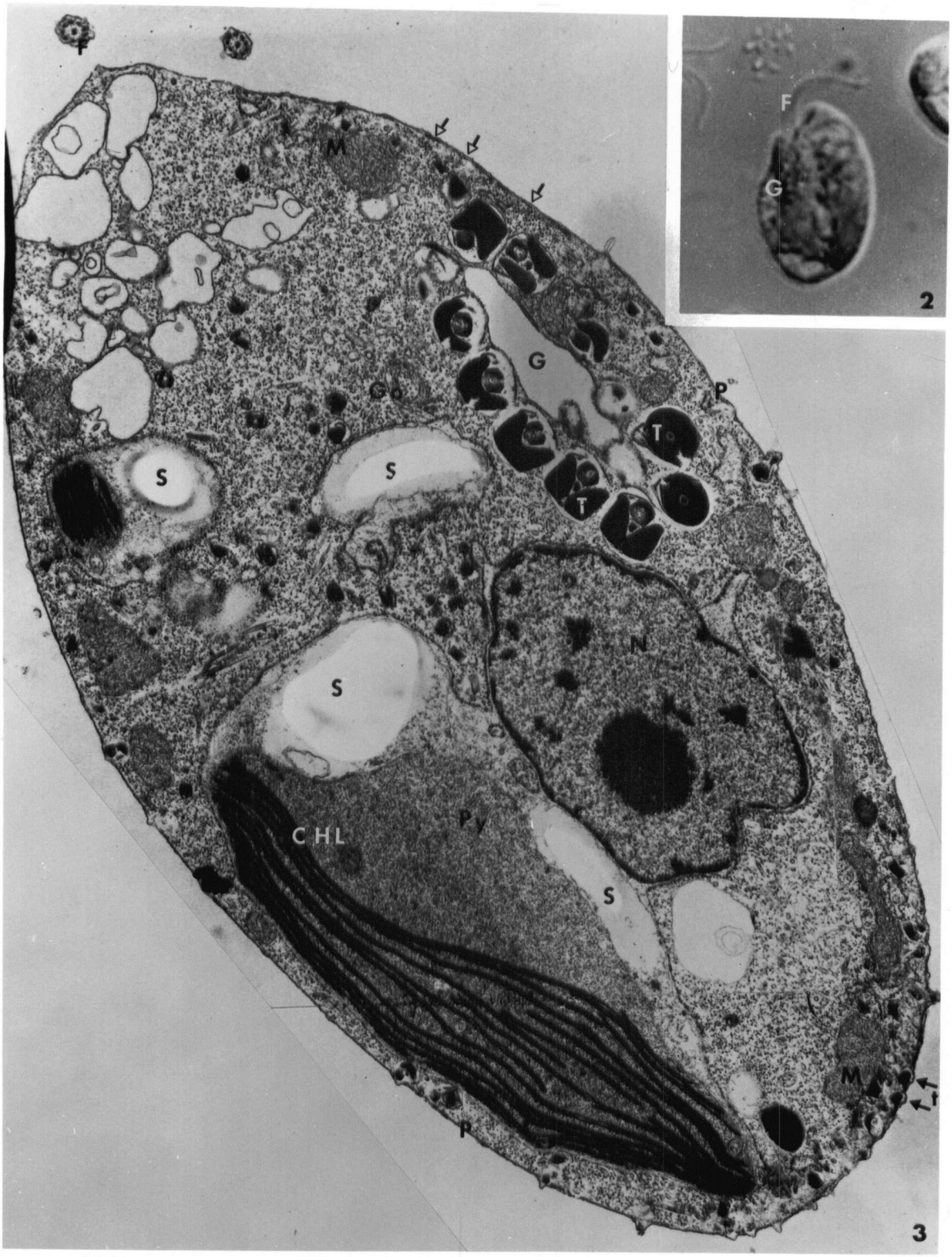
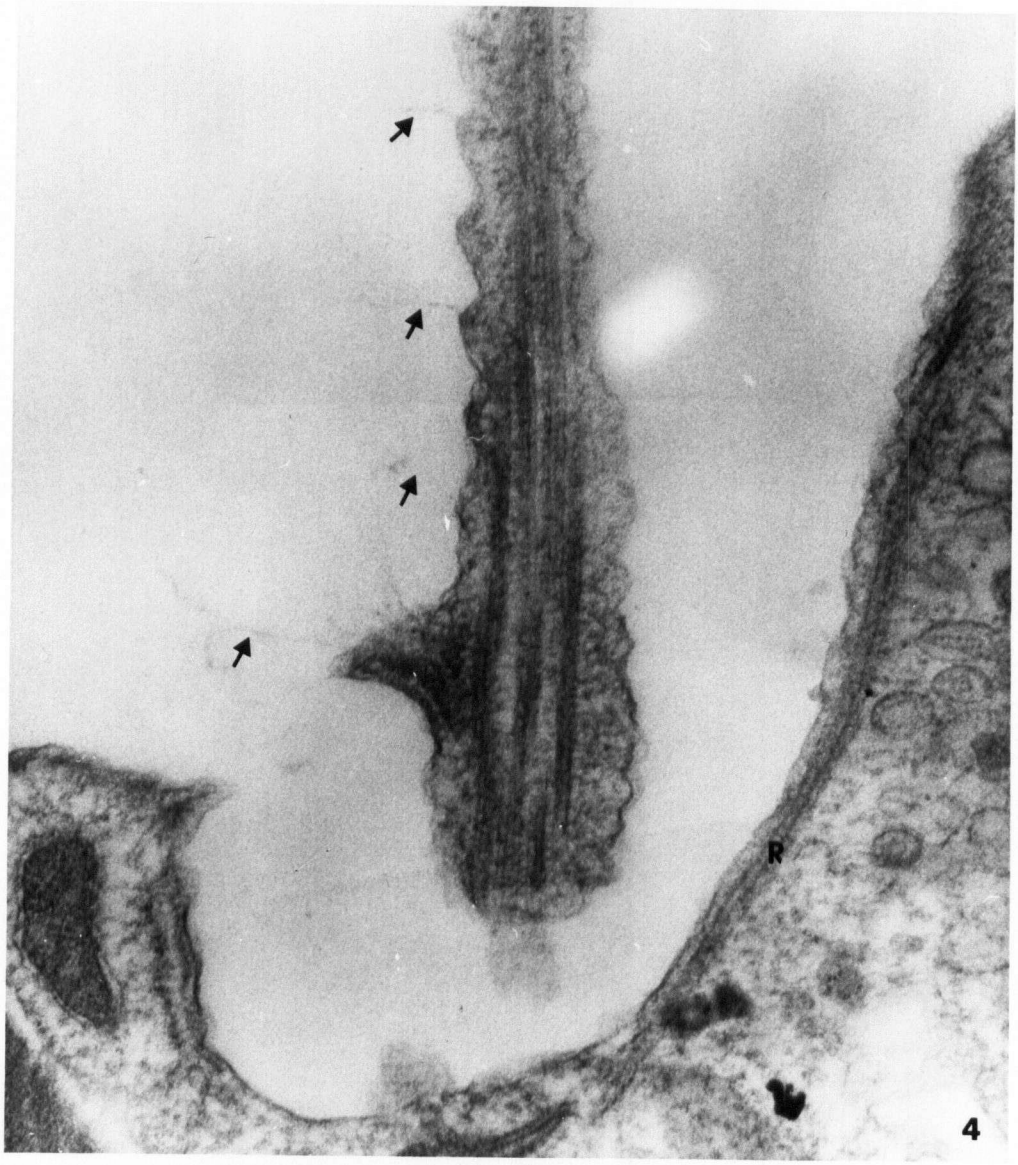

F
G
2
F
M
G
Go
P
T
S
S
T
N
S
CHL
Py
S
M
P
3

PLATE 2. Control cells.

Figure 4. A longitudinal section of a flagellum showing the regular arrangement of the fibrous mastigonemes (arrows). These are similar to those observed in Ochromonas danica (Bouck, 1971) and measure about 50-70 Å in diameter. Note also the lateral rootlet of the flagellum (R). X 59,000.

Figure 5. An oblique section of the flagella (F) showing the tubular mastigonemes (M), about 200 Å in diameter, in longitudinal section. X 47,000.


R
4

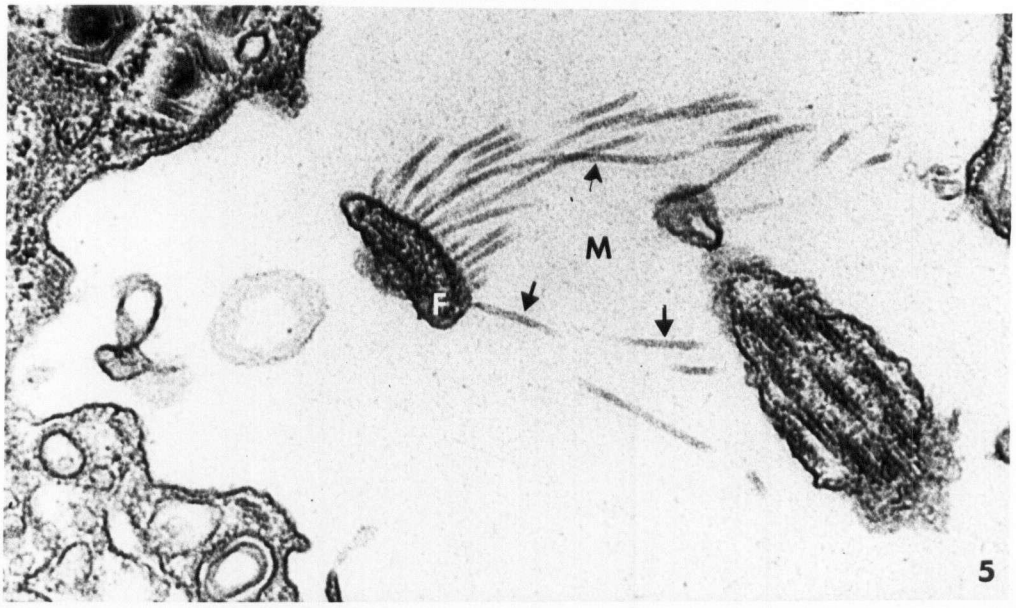
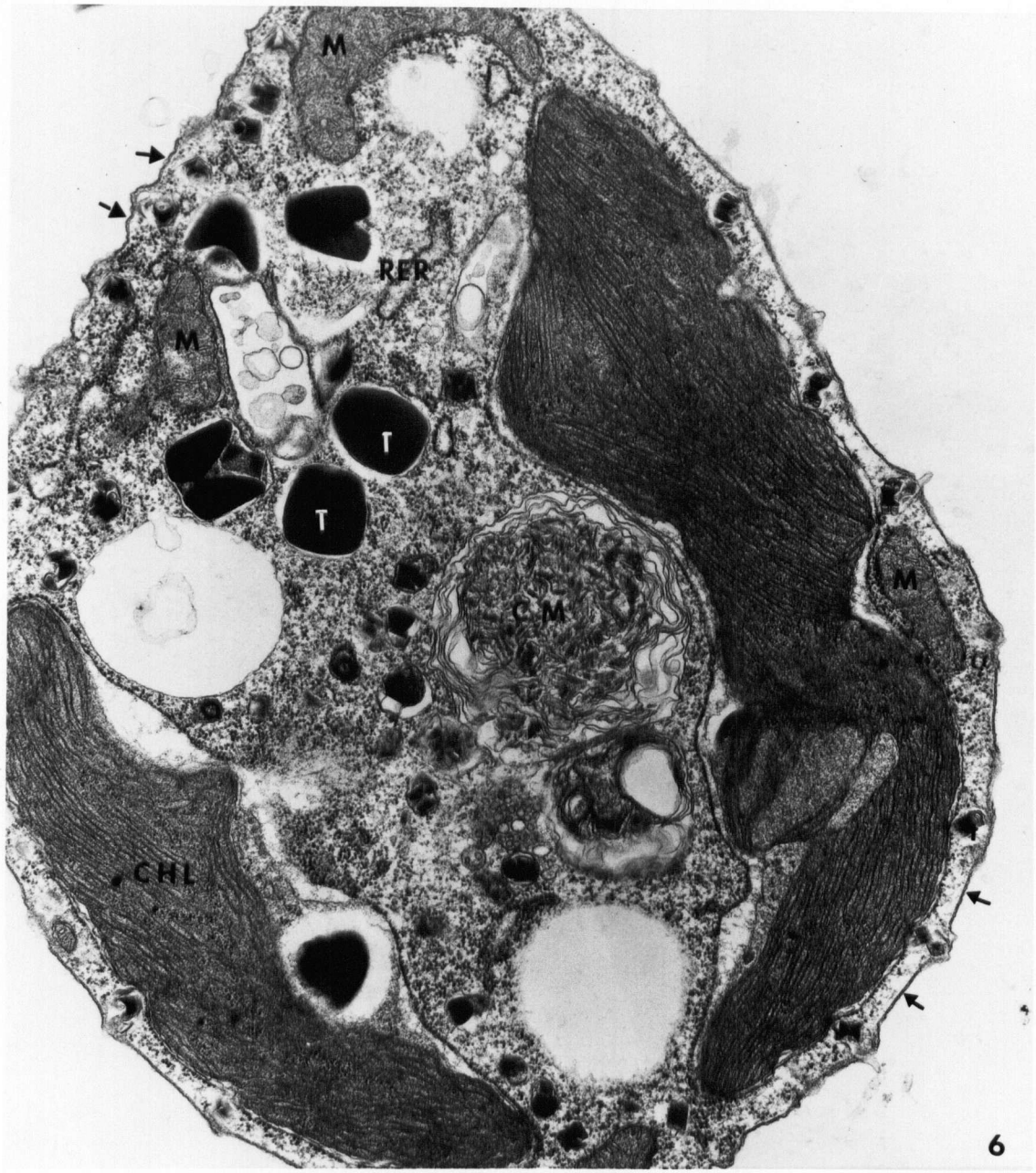

M
F
5

PLATE 3. Control cells.

Figure 6. An oblique section showing the arrangement of the chloroplast within the cell. Organelles visible are the Corps de Maupas (CM), mitochondria (M), rough endoplasmic reticulum (RER), and the large and small trichocysts (T & t). Note the bipartite periplast (arrows). X 22,000.

Figure 7. A section of the peripheral region of the cell showing the arrangement of the thylakoids (arrows) within the chloroplast. Note the greater electron opacity of the loculus compared to that of the stromal area (SA). The plasma membrane (PM) and the inner layer (IL) of the periplast are visible. X 27,000.



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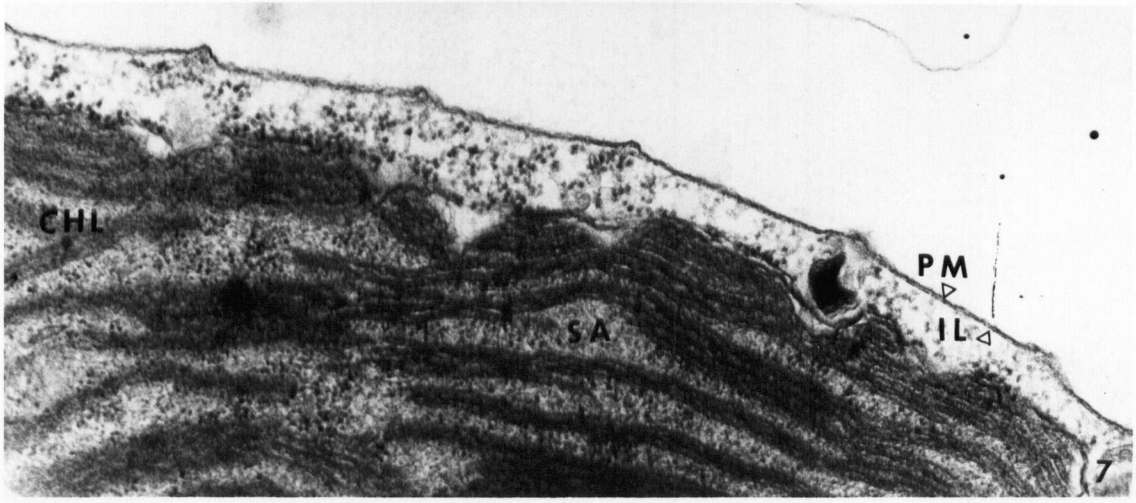

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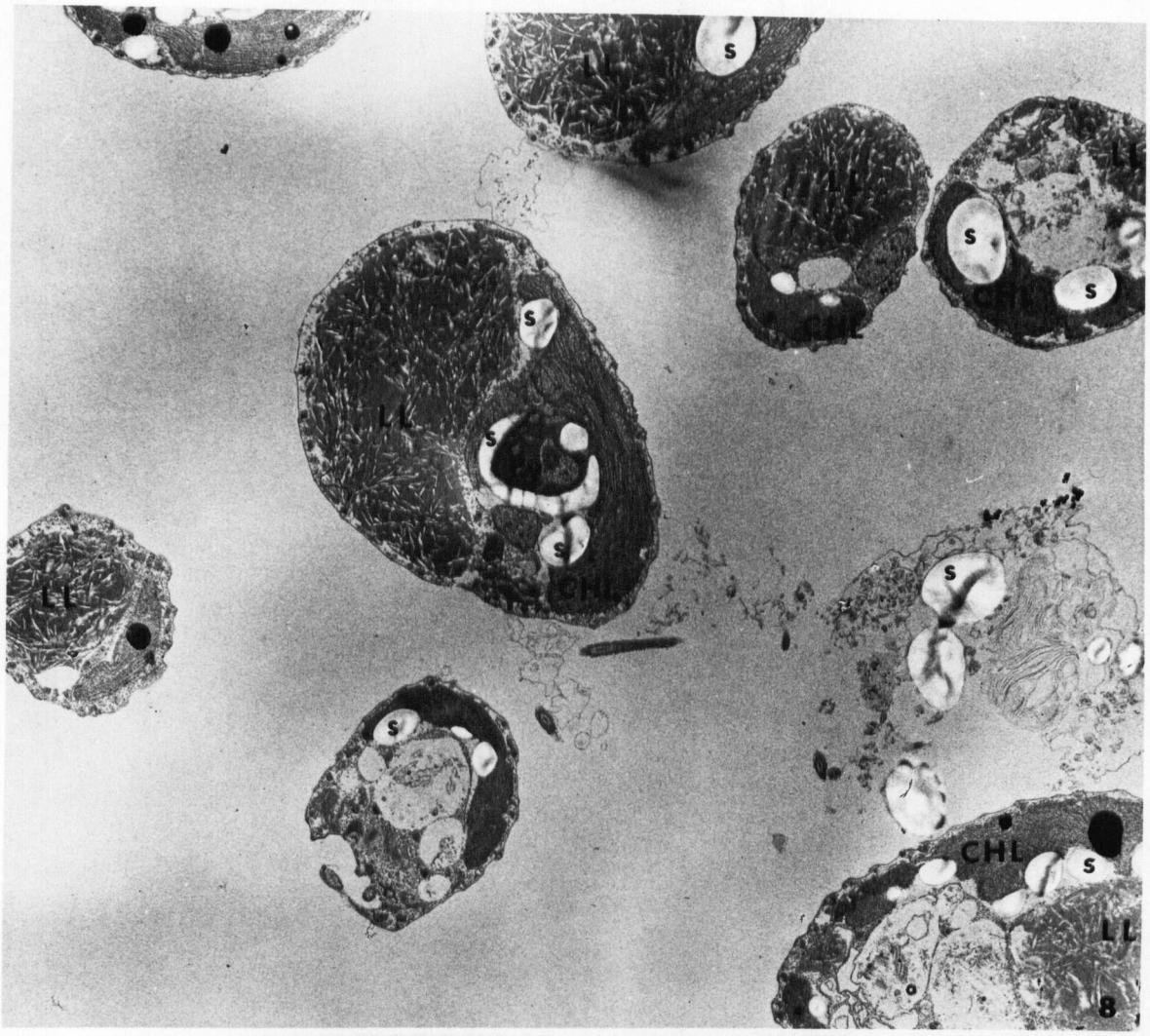
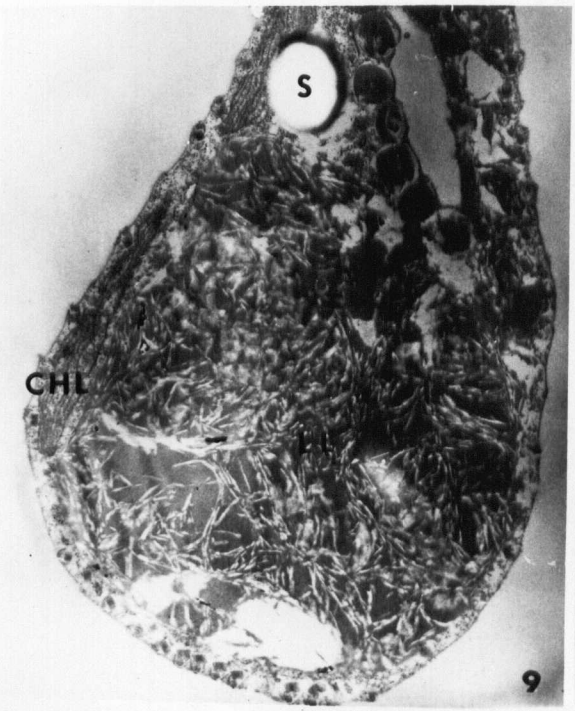
PLATE 4.

Figure 8. Control cells during late stationary phase. Note the relative positions of the chloroplast (CHL) and the lipid-like material (LL). Large starch grains (S) can be seen within the perichloroplastic matrix and near the pyrenoid (Py). X 7310.

Figure 9. A longitudinal section during late stationary phase showing the amount of lipid-like material within the cell. X 10,200.

Figure 10. A slightly oblique longitudinal section near the cell periphery showing the thylakoidal arrangement (arrows). Note the starch grains (S), small trichocysts (t), and the Corps de Maupas (CM). X 29,400.


S
LL
S
S
S
LL
S
S
S
LL
S
CHL
S
S
CHL
S
LL
8


S
CHL
LL
9

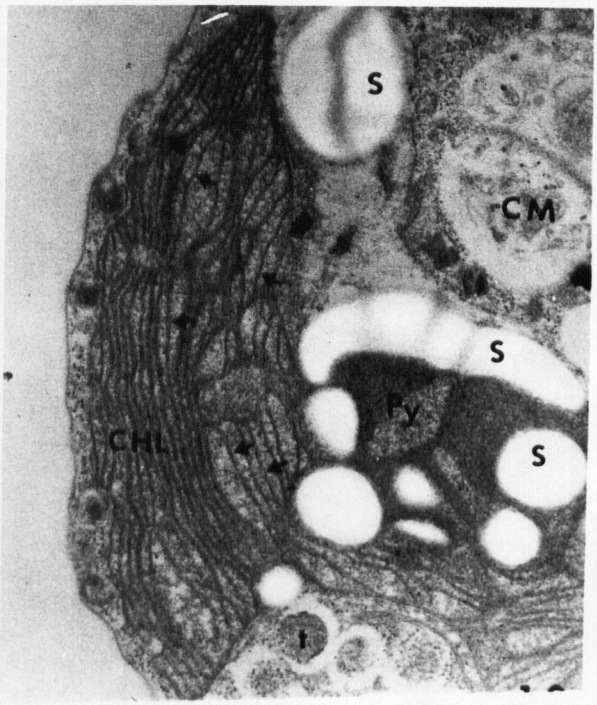
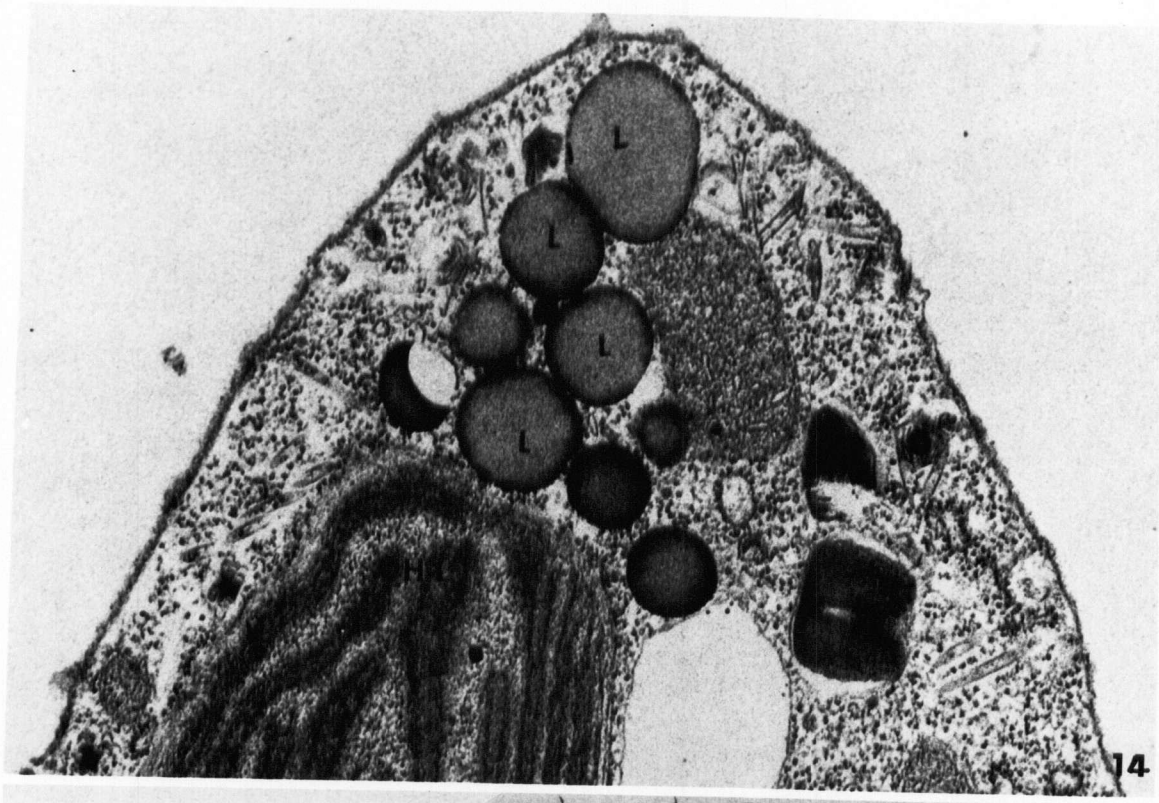

S
CM
S
Py
CHL
S

PLATE 5.

Figure 14. Experimental cell during logarithmic phase. Lipid droplets (L) are seen between the periplast and the chloroplast (CHL). X 33,000.

Figure 15. An oblique section near the posterior of the cell showing some lipid (L) accumulation and the chloroplast (CHL) with plastoglobuli (Pl). X 19,200.


L
L
L
L
14

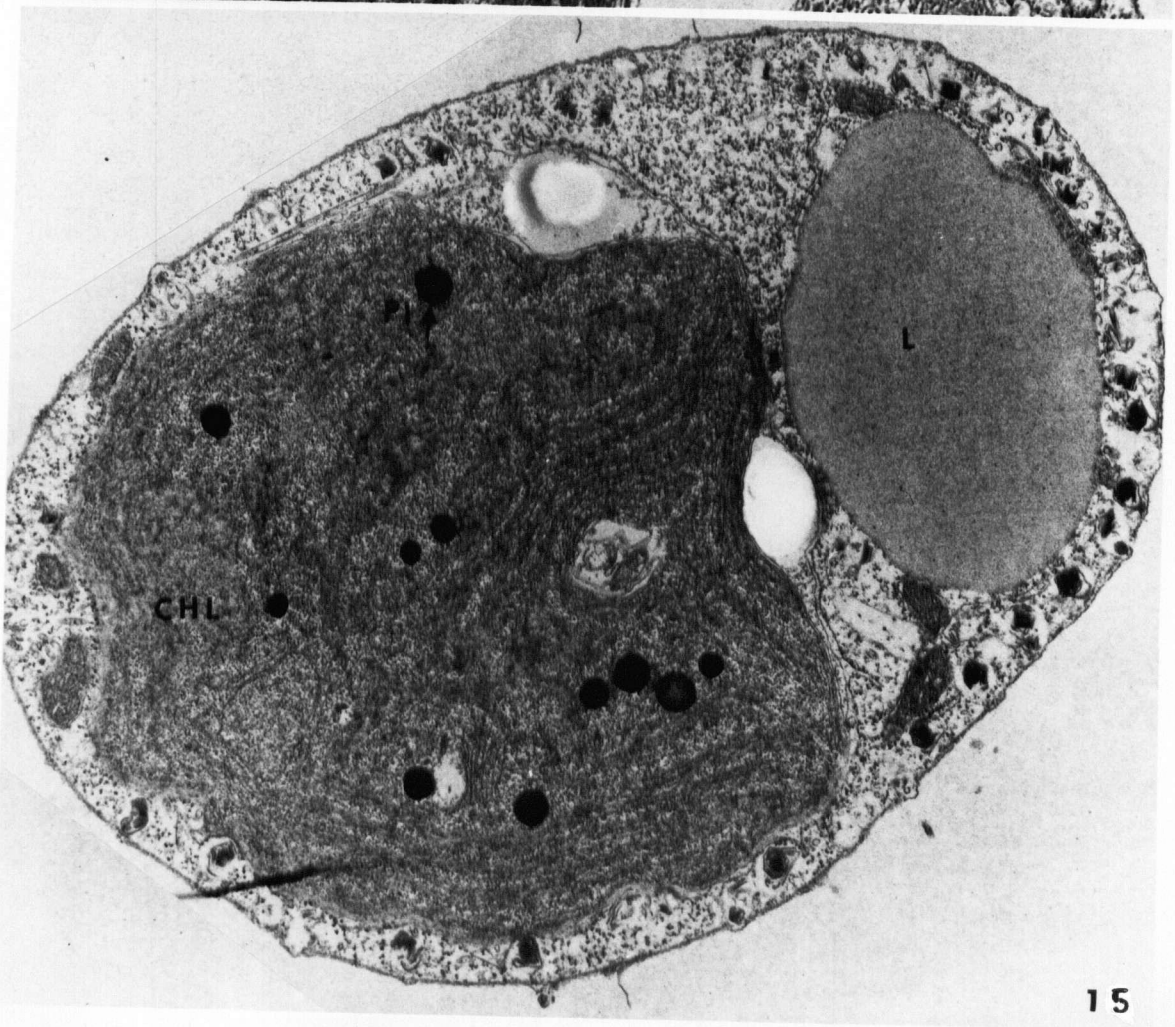
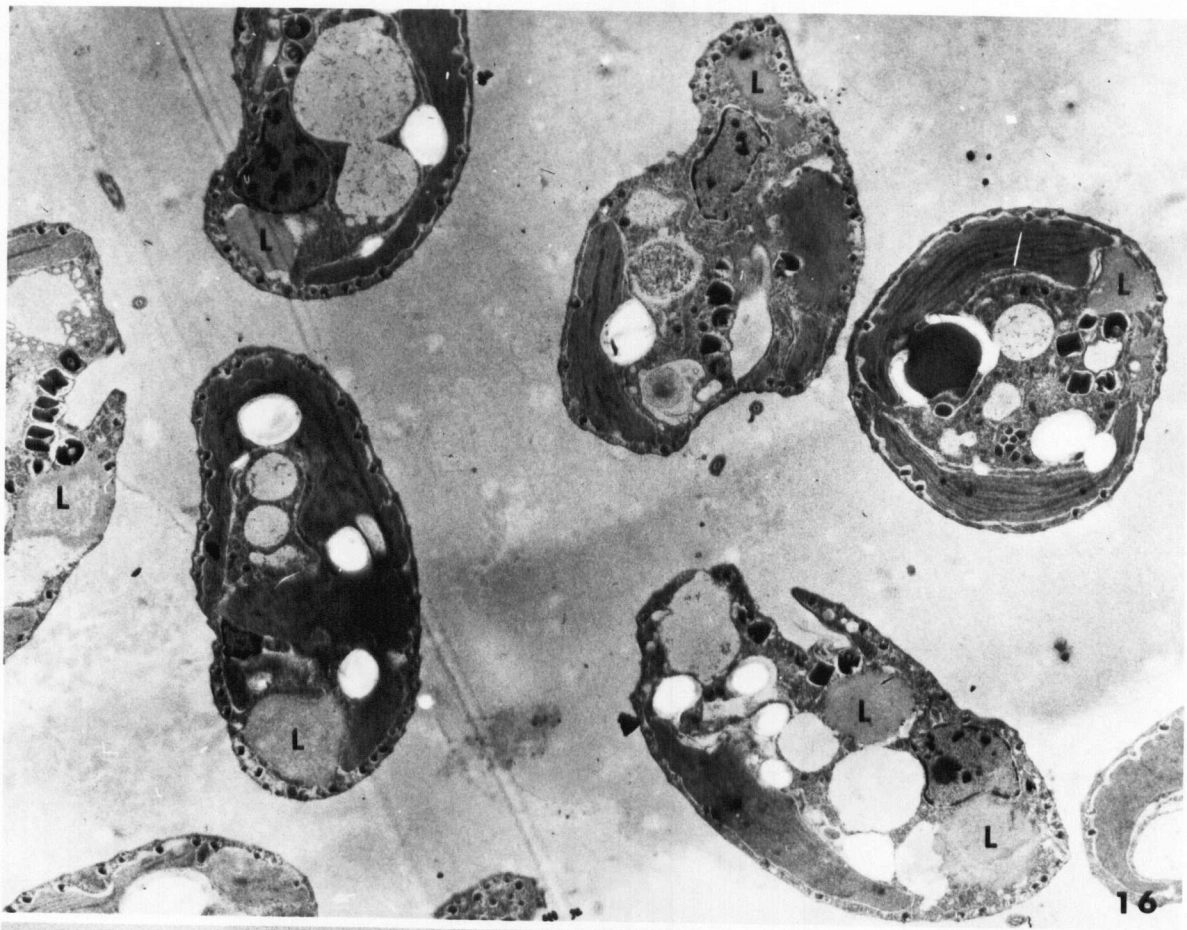

L
CHL
15

PLATE 6.

Figure 16. A section showing several experimental cells fixed during early logarithmic phase. Note the accumulation of lipid (L) in each cell. X 6800.

Figure 17. A longitudinal section of an experimental cell fixed in early logarithmic phase showing the large lipid droplet (L) and the arrangement of thylakoids in the chloroplast (CHL). X 22,800.


L
L
L
L
L
L
L
16

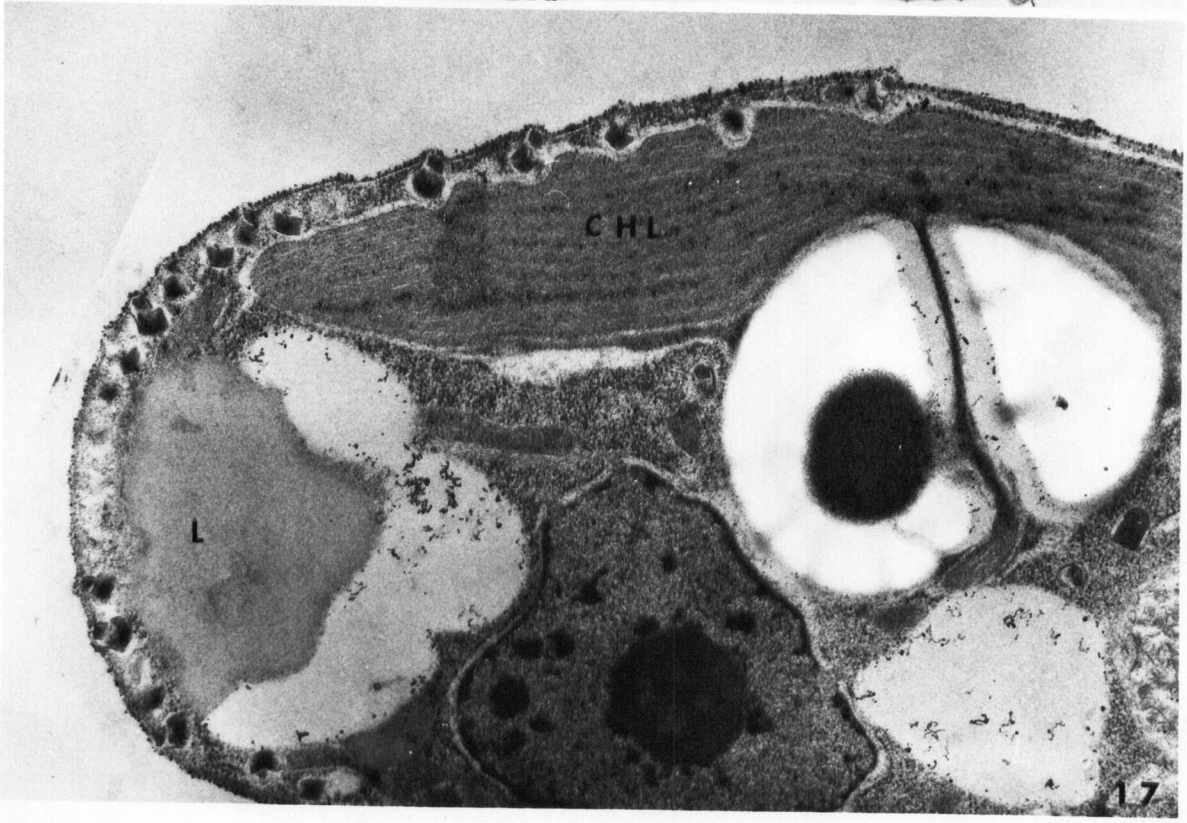

CHL
L
17

PLATE 7.

Figures 18 to 25. Optical micrographs of control cells under toxic ammonia conditions at increased pH using Nomarski interference illumination. X 2830.

Figure 18. A normal cell at pH 7.6 and zero time.

Figure 19. pH 8.65, 1½ hours. Cell has begun to round.

Figure 20. pH 8.65, 3 hours. Cell is rotund: chloroplast is slightly flattened posteriorly.

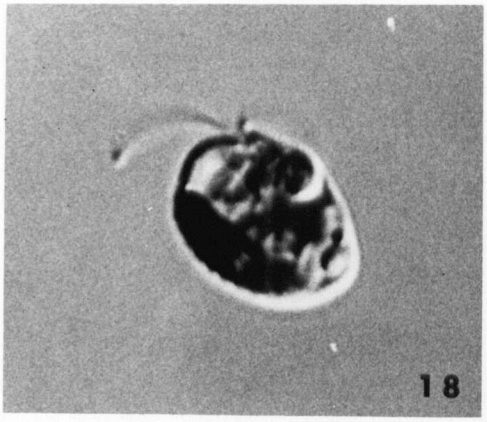
Figure 21. pH 8.65, 4½ hours. Cell is rotund: chloroplast is much more flattened posteriorly.

Figure 22. pH 8.65, 5½ hours. Cell contents begin to protrude: flagella are still present.

Figure 23. pH 8.4, 8 hours. Protrusions very pronounced.

Figure 24. pH 8.5, 8 hours. Cell swelling is noticeable, accompanying vacuolation and increasing protrusions.

Figure 25. pH 8.5, 8 hours. The cell has lysed and the contents are extruded into the media.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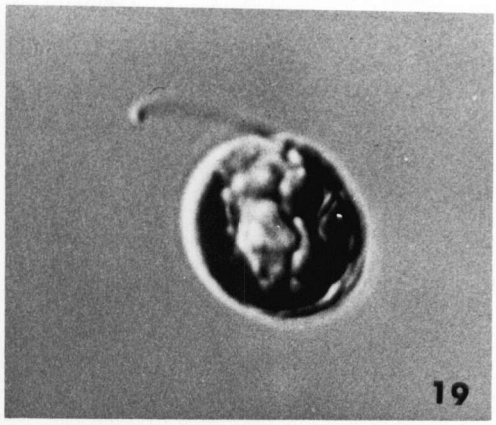
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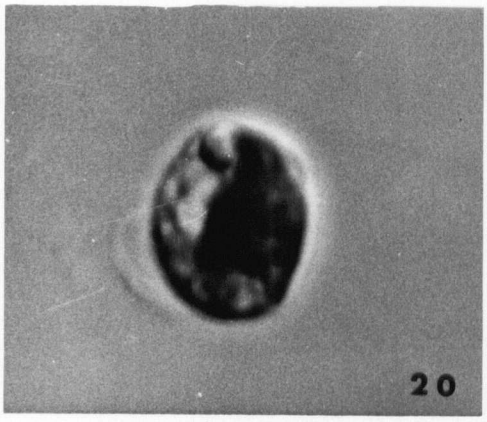
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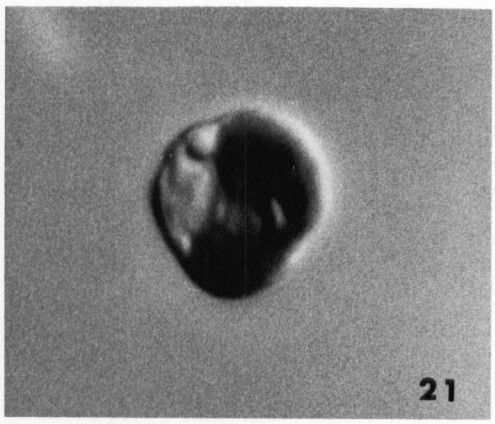
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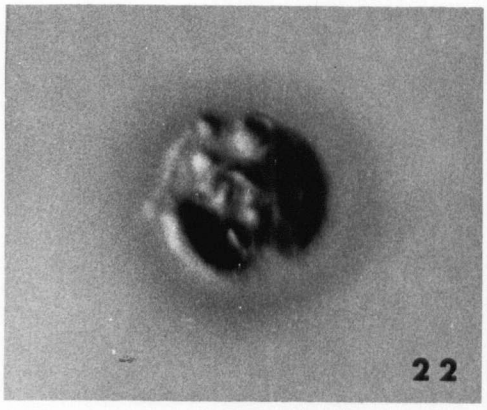
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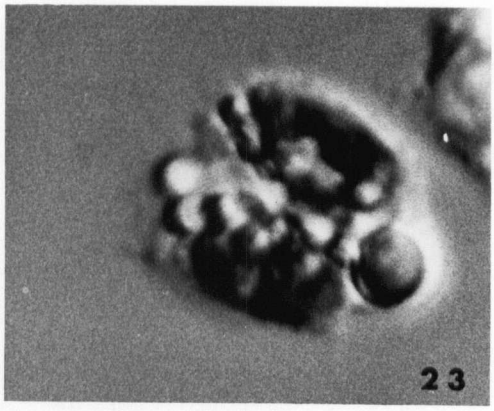
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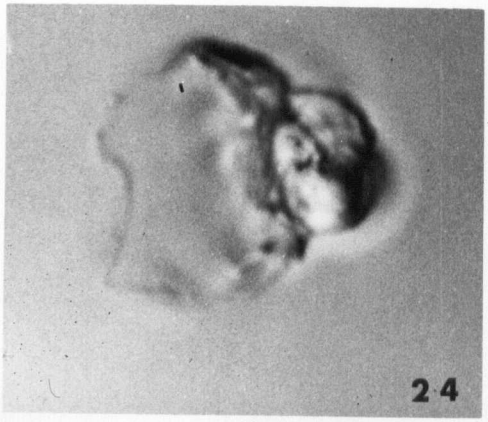
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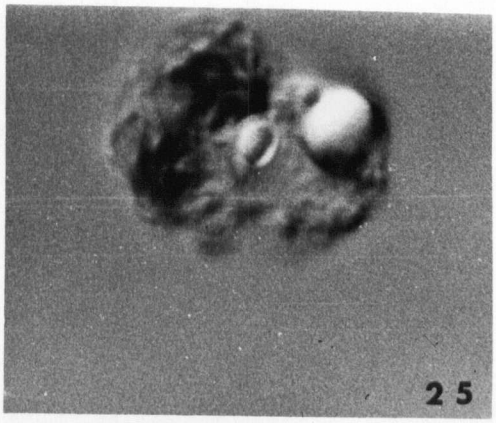

25

FOOTNOTES

[1]Oceanic phytoplankton probably compete for the use of urea: blue-green algae (Berns, Holohan and Scott, 1966) and many types of bacteria (Jeffries, 1964) are known to contain urease and grow well in urea media.

[2]Butcher (1967) does not recognize Rhodomonas as a genus, nor does his description of Rhodomonas lens accurately describe the organism used in this work. However, published work (Gibbs, 1962 a, b; Cheng and Antia, 1970; Gantt, Edwards and Provasoli, 1971; Oakley, 1974) with this organism used this classification. Therefore, pending further clarification, in this thesis the organism will be called Rhodomonas lens.

[3]Rhodomonas lens was originally isolated from the Gulf Stream by R. Lasker. Dr. N. Antia obtained it from Haskins Labouratories, New York.

[4]Two millipore sizes were used consecutively: Millipore Type HA followed by Millipore GSWP 047 00. (Pores: .45μ,.22μ).

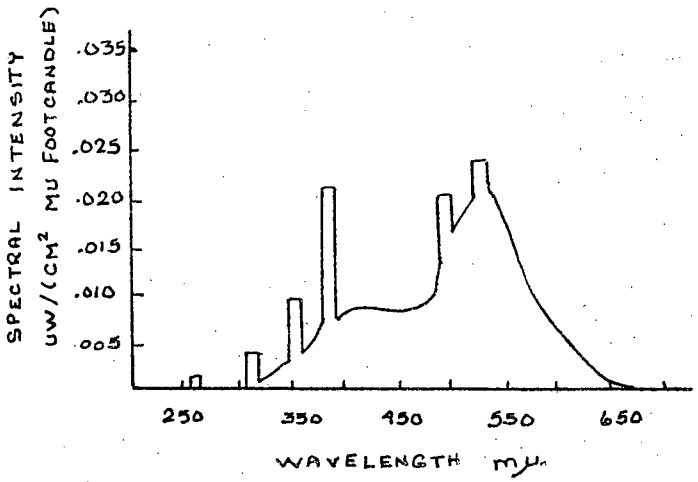
[5]Compounds for synthetic sea water medium were elucidated by Lyman and Fleming: the medium was published by Antia and Cheng (1970). Enrichment media was published by Antia and Chorney (1968).

[6]Formulae are taken from Eagon's Advanced General Microbiology, page 27.

[7]Some cell lysis may occur with this method. A small amount of cell lysis may have occurred during centrifugation

with an accompnaying release of intracellular urea and/or urease.

[8]Sylvania "cool white" fluorescent lights (#F48T12/CW) were used.



[9]This was not always observed. Often no decline phase was noted and the stationary phase was still present after several weeks.

[10]The enzyme utilized to catalyze the breakdown of urea in Rhodomonas lens is probably urease (Leftley, personal communication).

[11]The salinity of the media was 28‰. Salinity of the Gulf Stream surface waters is approximately 36‰.

[12]Nitrogen stress, as used in this thesis, is not used in a strict physiological sense, but is based on one parameter, the cytological evidence of lipid accumulation (Spoer and Milner, 1949; Van Baalen, 1963).

[13]Chlorophyll plus carotenoids gives a green or a red-orange colouration; chlorophyll, carotenoids and phycobilins, a clear red-pink colour (Cheng, Don-Paul

and Antia, 1974).

[14]Certain accessory pigments may be non-essential; therefore, during a nutritional crisis their components can be utilized in the synthesis of compounds vital to the cell.

[15]Comparatively, a 10-fold increase in ug-atom N-liter^{-1} in the experimental media is required to attain an equal division rate to that of control cultures.

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ADDENDUM

Information in the following papers has relevance to this thesis:

I. Antia et al. (1975), from a survey of 26 phytoplankton species, noted that utilization of organic nitrogen appeared to be a species-specific trait; an important factor influencing initial growth on organic nitrogen being the concentration of the nitrogen source. They suggest, due to the efficiency and facility with which 85% of the tested species utilized urea, that "urea may be the most important source of organic nitrogen available for growth by marine phytoplankton in the oceans."

The medium used was enriched sea water, the sea water being procured from a Mediterranean coastal inlet, with a final salinity of 31‰ and a nitrogen level of 500 µg-atom $liter^{-1}$.

II. Zgurovskaya and Kustenko (1968) observed a similar concentration of ammonia nitrogen toxicity for the tested organisms to that noted in this thesis.

109. Zgurovskaya, L.N. and N.G. Kustenko. 1968. The effect of ammonia nitrogen on cell division, photosynthesis and pigment accumulation in Sceletonema costatum (Grev) Cl., Chaetoceros sp. and Prorocentrum micans Ehr. Oceanol. 8: 90-98.