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(NASA-CR-173264) [PROCHLORON RESEARCH] Final Report (Scripps Institution of Oceanography, La Jolla) 2 p HC A05/MF A01 CSCL 06C G3/51

N84-20113 THRU N84-20124 Unclas 12499

Final technical report for N.A.S.A. Grant No. NAGW-181.

Ralph A. Lewin and L.C. Cheng, S.I.O.

Prochloron is a unicellular alga with many cytological features like those of other prokaryotic algae, but with certain biochemical features like those of green algae and higher plants. It can be regarded as a possible relative of ancestral chloroplasts, if one accepts the Mereschkovsky theory of symbiogenesis. To test such theories, detailed information of its fine structure, biochemistry and physiology is needed. Ideally, one would like data obtained from cells grown in pure culture, but despite many attempts during the last few years we have not yet succeeded in growing them in the laboratory. Accordingly, we have had to depend on material collected from Nature. Prochloron is characteristically associated with certain didemnid ascidians which occur in tropical coral-reef areas. We found that Palau was perhaps the most suitable source for collecting such material, and Lissoclinum patella the most suitable host species from which to extract Prochloron cells in quantity. Lyophilized or frozen cells were analyzed in various ways, by various workers in various laboratories, using material supplied by us, collected during expeditions to Palau financially supported largely by N.A.S.A. A number of publications have resulted from research on such material; others are in the course of preparation. We still lack unequivocal proof for or against the symbiogenesis theory, but much of the accumulating evidence seems to support it. We shall know more in the next two or three years.

N.A.S.A. supported two expeditions to Palau (IPE-VII and IPE-VIII), in 1982 and 1983 respectively, and an international workshop (IPW) in La Jolla in 1983. We enclose reports from these expeditions and the workshop, a brief report of the work carried out on the samples collected, and a list of publications resulting therefrom.

More than 100 samples have been solicited from our collections and sent to more than 40 colleagues here and abroad. Included in the latter are scientists in the U.S.A. (22), Great Britain (5), Germany (8), France (2), Norway (2), the Netherlands (1), Switzerland (1), Australia (2), Canada (1), Japan (1) and China (1). Work on the material is presumably proceeding along with other projects in the various laboratories, and one may anticipate that in due course publishable results will appear in the literature. Each collaborator has been asked to acknowledge that the material had been collected on expeditions partly financed by NASA.

The following papers, accepted for publication or already published, are based largely or entirely on Prochloron research carried out, or on samples collected, at Palua, on expeditions financially supported in part by NASA Grant No. NAGW-181.

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Lanna Cheng and

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ABSTRACT

For the first time, Prochloron cells have been found associated with an animal other than a colonial ascidian - namely, a synaptid holothurian, Synaptula lamperti.

Green unicellular algae lacking an evident nucleus (i.e., they are prokaryotic) have elicited considerable interest in recent years because of their possible phylogenetic affinities to blue-green algae on the one hand and to the chloroplasts of higher plants on the other. They have been assigned to a new genus, Prochloron, in a new algal division, the Prochlorophyta (Lewin, 1976, 1977). They occur characteristically as symbionts of certain colonial ascidians (Protochordata) on tropical and sub-tropical shores around the world. So far, they have been reported only from members of the families Didemnidae (various species of Didemnum, Trididemnum, Diplosoma, Lissoclinum and Echinoclinum) and the closely related Polycytoridae (Cystidites sp.) (Kott, 1977, 1980; Lewin, 1979). They have never been found living free, and have not yet been grown in sustained laboratory culture, although Patterson and Withers (1982) have reported slow, non-sustained growth of Prochloron cells in a sea-water medium supplemented with tryptophan.

Prochloron cells occur in sporadic green patches on the surfaces of colonies of Didemnum candidum and other encrusting didemnids under rocks and on mangrove roots in Baja California, Mexico (Lewin and Cheng, 1975) and in

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Prochloron cells occur in sporadic green patches on the surfaces of colonies of Didemnum candidum and other encrusting didemnids under rocks and on mangrove roots in Baja California, Mexico (Lewin and Cheng, 1975) and in

similar habitats in Puerto Rico, the Galápagos Islands, Singapore, etc. (personal observations). In other didemnid species they occur as symbionts, and at least 18 spp. are never found without Prochloron cells, tightly packed in peripharyngeal spaces or in common cloacal ducts, less commonly embedded in the tunicin of the colonial tests. (For a review of such symbioses, see Kott, 1980). Many of these didemnid species occur in low-intertidal or subtidal reef areas in Palau, Western Caroline Islands, and it is there that we found Prochloron cells also in sporadic patches on the skin of a synaptid holothurian. This is the first report of their occurrence on any animal other than an ascidian, and it brings into question the supposedly "obligate" nature of the association of this problematic alga with didemnids and their allies.

Prochloron-bearing holothurians (Echinodermata) were found in a shallow fringing-reef area by the Kamori Channel, Koror, Palau, in February 1981 and again in February 1982, about 250 metres from the boat dock of the Nikko-Palau (Continental) Hotel. They were scattered over a relatively small area perhaps 100 m across, usually in small groups of 5–10 individuals, crawling in and over colonies of a purple-black sponge. They have been identified as Synaptula lamperti Heding by Dr. Frank Rowe of the Australian National Museum, Sydney. The animals were 3–5 mm wide and ranged in length from 5 cm (contracted) to 25 cm (extended). Around the mouth there were 10–11 tentacles. Their natural colour was mottled, lilac and white, but about 10–20% of the individuals (in surveys of 50–150) bore green patches on the posterior 1/3 of the body. These patches were attributable to green unicellular algae (Prochloron sp.) indistinguishable from those living symbiotically in nearby didemnid colonies (Diplosoma virens, D. similis, Lissoclinum patella, L. voeltzkowi, Didemnum molle and Trididemnum cyclops). They could have been transferred from the didemnids in the vicinity either as free cells (we detected by

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filtration some 10-20 Prochloron cells per litre of water in that area) or carried by free-swimming larvae of the symbiotic ascidians.

The algal cells were generally spherical, 12-16 µm in diameter; about 25% were in various stages of cell division (Fig. 1, 3 & 4). Although the available quantities were insufficient for an unequivocal demonstration of the presence of chlorophyll b as well as chlorophyll a, their bright green colour, and the presence of abundant cells of identical microscopic appearance in nearby symbiotic didemnids (which harbour authentic Prochloron), leave little reason to question that they are in fact Prochloron cells. Further evidence is provided by their fine structure as revealed by transmission electron-microscopy. As shown in Figs. 5 and 6 they exhibit features typical for this genus, notably the thickness and substructure of the cell walls, the form and distribution of the polyhedral bodies, and especially the paired or stacked thylakoids lacking phycobilisomes. They were associated with large numbers of phagocytic amoebocytes that had presumably emerged from the coelom of the host animal: some of these amoebocytes were found closely appressed to Prochloron cells (Fig. 2-4). The algae could be wiped off by stroking with a finger. Specimens of Synaptula bearing Prochloron were taken alive in sea water to the laboratories of the Micronesian Mariculture Demonstration Centre, where they lived for a few days in an aquarium; but during that time they tended to lose most of the algae, which were detached as the synaptulids squirmed and stroked one part of the body against another. We kept some in direct daylight and others in diffuse light, but noted no evident change in the size of algal patches in 5 days.

We have been able to find little information on the natural history of Synaptula spp., which have been reported from many coasts around the Indo-

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Pacific islands (Clarke, 1920), on the Great Barrier Reef (Clarke, 1938), and in the Caribbean (Pawson, 1976). They seem to be known to specialists mostly as specimens preserved in formalin or alcohol, in which symbiotic algae, if originally present, would soon be detached and discolored beyond recognition. We examined the normal yellow-green patches on larger species of Synapta in the same area, and the skins of other holothurians common in the vicinity, but found no Prochloron cells there. Their sporadic presence on Synaptula lamperti may be attributable to peculiar features of the holothurian skin, which has been shown in another apodous sea cucumber to accumulate amino acids (Townsley and Ahearn, 1973). Further investigations are needed to establish this point.

Specimens of the Synaptula have been deposited at the National Museum of Natural History, Washington, D.C., and the Australian Museum, Sydney.

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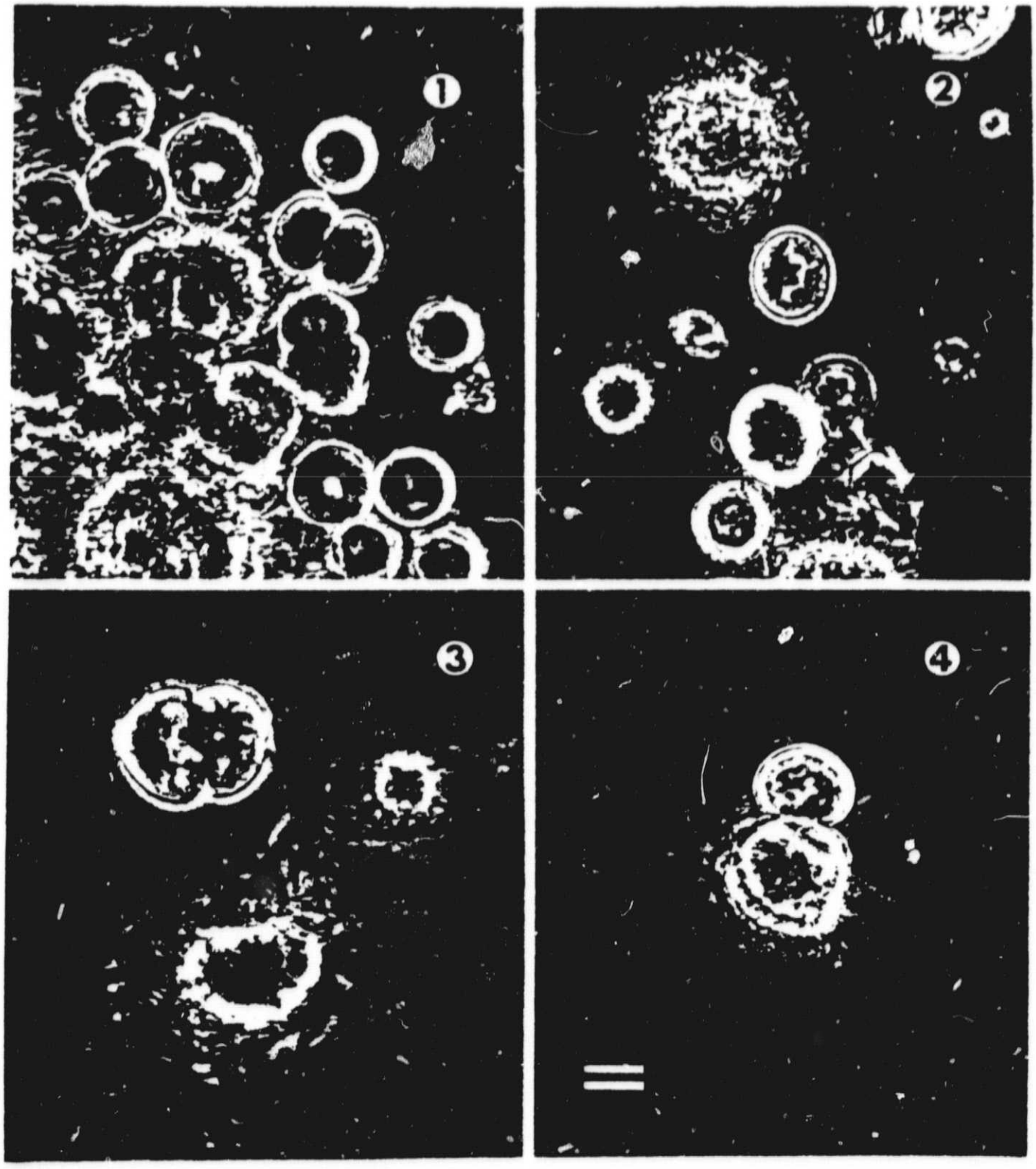

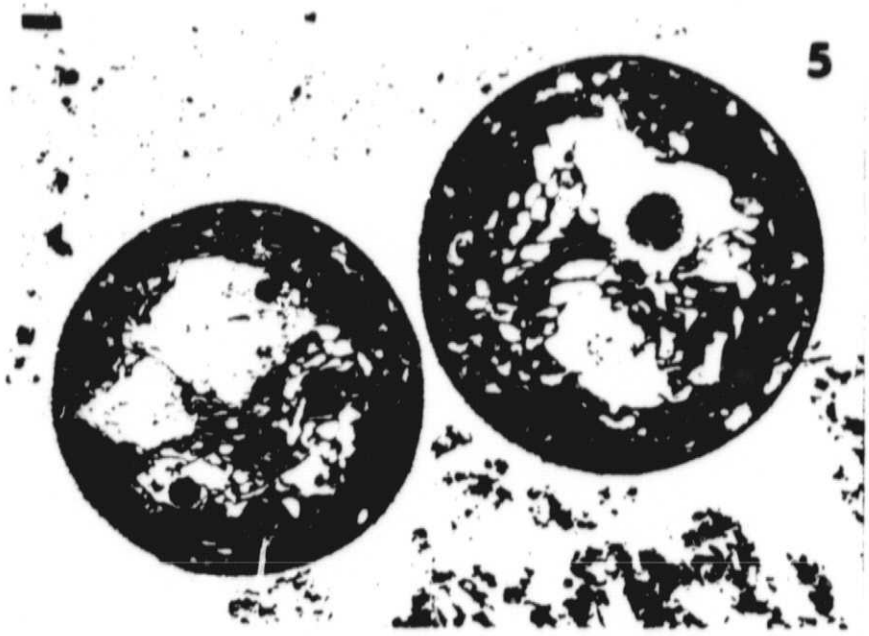

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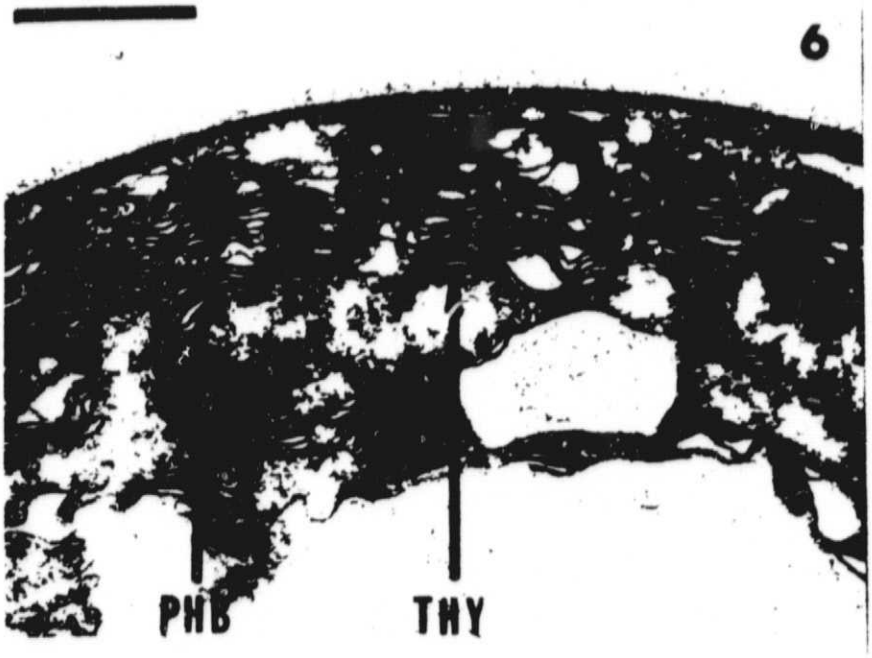

6

A COMPARATIVE STUDY OF THE FATTY ACID COMPOSITION OF PROCHLORON LIPIDS

Janette R. Kenrick, Elizabeth M. Deane and

David G. Bishop

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The procaryotic alga, Prochloron, has aroused considerable interest because it contains both chlorophylls a and b but lacks phycobilins (A). It occurs in symbiosis with a number of marine ascidians but as yet, little information is available concerning the relationships between the alga found in different hosts, and the organism has been successfully cultured in the laboratory, from only one host (B). The relationship between Prochloron and its hosts may be compared with that between the dinoflagellate, Gymnodinium microadriaticum and its diverse marine hosts. In this latter case, the existence of distinct strains (subspecies) of the dinoflagellate has been demonstrated by comparing biochemical and morphological properties (C,D,E) thylakoid lipids (F) and sterols (G). This paper reports analyses of the lipids of Prochloron isolated from several hosts, to determine whether differences in lipid composition could be used to characterize organisms from different sources.

MATERIALS AND METHODS

Prochloron cells were obtained as freeze dried samples harvested at Palau, Caroline Islands by L. Cheng and R.A. Lewin or as intact cells freshly expressed from host material collected on the Great Barrier Reef at Heron Island by A.W.D. Larkum or at Lizard Island by D.G. Bishop. Cells were extracted with chloroform:methanol, 2:1 (F) without addition of butylated hydroxytoluene. MGG, DGG and SL were quantitatively estimated (H) after thin-layer chromatography of total lipid samples on Silica gel HR plates in the solvent, acetone:benzene:water (I) or after thin-layer chromatography of samples freed of pigments by chromatography on Sep-pak® cartridges (J). Phosphatidylglycerol was estimated by phosphorus analysis (K) after thin-layer chromatography in the same system. Pure lipids for fatty acid analysis were isolated by preparative thin-layer chromatography, methylated and analysed as previously described (L,F).

RESULTS

The analyses of total lipids of Prochloron freshly isolated from Diplosoma similis showed four major lipid components MGG, (41 moles %), DGG (4 moles %), SL (47 moles %) and PG (8 moles %). A minor component, monoglucosyldiacylglycerol, detected in the lipids of Prochloron (M) and cyanobacteria (N,O) is included in the values for MGG. The values are similar to those recently obtained for the lipids of Prochloron isolated from Lissoclinum patella (M), the most striking feature being the high content of SL, which is normally a minor component of photosynthetic membranes (P) including those of cyanobacteria (Q). In addition the content of DGG is relatively low and the molar ratio MGG:DGG is unusually high for photosynthetic cells. Analysis of the MGG:DGG molar ratio in eight samples of Prochloron lipids (derived from five different hosts) gave a mean value of 7.2 (range 5.1 to 10.2), considerably higher than the mean value of 1.43 (range 1.00 to 2.06) found for 16 species of higher plants (P). Values reported for the MGG:DGG molar ratio in cyanobacteria are less than 4 (Q).

Analysis of the fatty acid composition of individual lipids of Prochloron from D. similis showed a distinctive distribution of fatty acids (Table I). Four fatty acids containing fourteen or sixteen carbon atoms were the major components of the total lipid. MGG and DGG were characterized by large amounts of the monounsaturated acids 14:1 and 16:1, while SL contained 75% saturated acids and PG contained about equal amounts of saturated and monounsaturated acids. No polyunsaturated fatty acids were detected. The data are similar to those of the lipids of Prochloron isolated from L. patella (M).

The fatty acid composition of the galactolipids of Prochloron isolated from different hosts and in different locations and times are shown in Table II. While the overall trends are similar, and the ratio of unsaturated to saturated acids is greater than 2 in all cases, some differences do occur.

Analysis of three samples of Prochloron isolated from L. patella in the Caroline Islands but over a three-year period shows that variations in fatty acid composition can occur, e.g. the content of 16:0 in DGG varies from 4.9% to 23.9%. Variations also occur in the fatty acids of galactolipids of Prochloron isolated from D. similis, samples of which have been obtained both from the Caroline Islands and the Great Barrier Reef (see also Table I). Analyses of the galactolipids of Prochloron from the remaining three hosts follow the general trends. Analyses of the SL fractions (results not shown) show the same high content of saturated fatty acids as was found from the alga from D. similis (Table I).

DISCUSSION

The object of this study has been to compare the fatty acid composition of specific membrane lipids of Prochloron, in order to establish whether some distinctions can be made between the algae infecting different hosts. A number of criteria, which provide evidence for strain variation, have been established for the symbiotic dinoflagellate, G. microadriaticum (C,D,E,F,G), including the content of eicosapentaenoic acid in thylakoid galactolipids and the relative levels of octadecatetraenoic acid in MGG and DGG (F). However, in the case of Prochloron, the present results do not allow a similar conclusion. Although the lipid composition of Prochloron is characterized by a high content of SL, and a high MGG:DGG ratio, and each of the four major lipids has a characteristic fatty acid composition (Table I and ref. M), variations in the fatty acid compositions of individual lipids in samples from different areas, and from different times in the same area (Table II) do not at this stage permit fatty acid composition to be used as a taxonomic criterion. It must be recalled however that in eucaryotes, MGG, DGG and SL are located only in chloroplast membranes, whereas in cyanobacteria and presumably Prochloron, these lipids are present in both thylakoid and cyto-

plasmic membranes. Both the cyanobacteria (Q,R) and Prochloron (Table I and ref. M) lack lipids such as phosphatidylcholine and phosphatidylethanolamine both of which are characteristic of eucaryotic membranes. They also synthesize MGG by a pathway involving epimerization of monoglucosyldiacylglycerol (M,N,O) whereas eucaryotes utilize a pathway involving UDP-galactose.

While the present results do not justify the use of fatty acid content in the taxonomy of Prochloron, the variations found in the lipids of cells from the same host harvested from different areas, or at different times in the same area, suggest that a study of the effects of temperature and light intensity on lipid composition would be rewarding. The lipid and fatty acid composition of the cyanobacteria Anacystis hidulans which does not synthesize polyunsaturated fatty acids, are both affected by temperature (Q) and the ability to culture Prochloron in the laboratory (B) now makes such a study feasible.

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ABBREVIATIONS

DGG, digalactosyldiacylglycerol

MGG, monogalactosyldiacylglycerol

PG, phosphatidylglycerol

SL, sulphoquinovosyldiacylglycerol

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TABLE I MAJOR FATTY ACIDS OF LIPIDS OF *PROCHLORON* ISOLATED FROM *DIPLOSOMA SIMILIS*[a]

Fatty acid	Total lipid	MGG	DGG	SL	PG
14:0	7.6	~~2.8~~ 2.1	2.0	14.5 7	18.5 3
14:1	19.7	~~40.2~~ 42.3	40.8	7.2	13.1
16:0	33.2	~~10.9~~ 11.4	11.8	60.6	38.8
16:1	31.9	~~41.6~~ 43.7	45.3	17.5	30.7
18:0	+	-	+	-	+
18:1	1.2	+	+	-	+

[a] One-Tree Island, November 1982.

TABLE II. MAJOR FATTY ACIDS OF *PROCHLORON* GLYCOLIPIDS

Host	*Lissoclinum*[a] *patella*		*Lissoclinum*[b] *patella*		*Lissoclinum*[c] *patella*		*Diplosoma*[d] *similis*		*Diplosoma*[e] *similis*		*Lissoclinum*[f] *vaelzkowii*		*Trididemnum*[g] *cyclops*		*Didemnum*[h] *molle*	
	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG
14:0	2.4	4.2	2.1	0.5	2.3	3.3	3.5	5.9	4.2	2.5	2.9	6.2	2.3	3.6	1.1	
14:1	24.4	20.0	24.3	31.2	25.1	27.2	39.4	32.7	30.3	16.8	23.0	21.7	27.8	27.7	12.3	
16:0	13.7	13.0	10.5	4.9	29.4	23.9	18.4	18.0	18.8	9.3	21.9	20.9	24.6	18.8	31.1	
16:1	50.5	60.1	58.2	60.8	43.3	45.5	38.8	43.9	38.7	68.4	47.6	46.9	45.3	48.4	54.7	
14:1 + 16:1	74.9	80.1	82.5	92.0	68.4	72.7	78.2	76.6	69.0	85.2	70.6	68.6	73.1	76.1	67.0	

[a] Palau, 1979; [b] Palau, 1981; [c] Palau, 1982; [d] Palau, 1982; [e] One-Tree Island, July 1982; [f] Palau, 1982; [g] Palau, 1982; [h] Lizard Island, 1979, data from Bishop *et al.* (1980).

Prochloron--A Status Report

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Summary

Prochloron is a genus of prokaryotic algae with photosynthetic pigments like those of chlorophytes. Prochlorophytes are almost invariably found associated as symbionts with marine protochordates (didemnid ascidians), and so far none has been successfully grown in sustained culture away from its host. Based on materials collected from nature, information of various sorts (biochemical, physiological, cytological and fine-structural) has been obtained, indicating many resemblances (and probably close phylogenetic affinities) between prochlorophytes and cyanophytes. Nevertheless they are distinguished by certain unique combinations of characters. Some of the data support the symbiogenesis theory for the origin of green-plant chloroplasts. Other possibilities are briefly discussed.

Prokaryotic algae with two chlorophylls, a and b and lacking accessory phycobilin pigments, have been found associated with protochordate invertebrates on tropical sea shores (Lewin and Cheng, 1975; Newcomb and Pugh, 1975). First described as zoochlorellae (Smith, 1935) and later as Synechocystis (Lewin, 1975), they have now been assigned to the genus Prochloron (Lewin, 1977) in a newly established algal division, the Prochlorophyta (Lewin, 1976). An earlier review of this subject, which I prepared in 1978, was published in 1981a; in this one I review work published in the years up to 1982. Since, by the characteristics described above, prochlorophytes differ from cyanophytes (the only prokaryotic algae known hitherto), they have recently elicited considerable interest, especially in regard to their phylogeny (e.g., Björn and Björn, 1982; Cavalier-Smith, 1982; Chadefaud, 1978; Kremer, 1980; Chapman and Trench, 1982; Seewaldt and Stackebrandt, 1982). Two specific topics have engaged attention. (1) Did prochlorophytes arise independently of cyanophytes, or did one of these classes arise from the other? (2) If eukaryotic chlorophytes originated by symbiogenesis (which is still debatable), might their plastids have arisen from symbiotic, endophytic prochlorophyte-like ancestors? In theory, various kinds of evidence on these points could now be obtained by electron microscopy, comparative biochemistry, molecular biology and even biochemical engineering--if we had at our disposition viable and preferably axenic cultures. Unfortunately, up until now no such cultures of Prochloron have been available, though one recent report (Patterson and Withers, 1982) indicates that this situation may soon be remedied.

Symbiotic associations of Prochloron with didemnids occur on shores along the north-west coast of Mexico, in the Caribbean, on some coasts of the Galápagos, Hawaii, etc., but they tend to be relatively inconspicuous, and

only small quantities of algal cells can be harvested from them (see references to earlier work cited by Lewin, 1981a; Lewin, Cheng and Lafargue, 1980). However, rich and varied sources of these algae, which normally live in symbiotic association with certain colonial ascidians, mostly didemnids, have now been discovered in certain areas of the Indo-Pacific (Lewin, 1979; Kott, 1980). The algae from different hosts seem very similar in most respects, though certain differences have been noted in their cell diameter and fine structure (Pugh, 1976). Lissoclinum patella, in particular, is an excellent source, since dense suspensions of the algae can be readily obtained, almost free from contaminating cells of other kinds, by simple mechanical pressure. We have prepared freeze-dried preparations of Prochloron from such symbioses and have made samples available to many scientific colleagues in various laboratories around the world. Much of the following information was obtained from such material.

To begin with, the oligonucleotide catalogues of 16S rRNA from Prochloron cells from four different host species are almost identical (Stackebrandt et al., 1982), suggesting that they are, if not conspecific, at least very closely related. A growing body of evidence indicates that there are close biochemical similarities, and presumably phylogenetic relationships, between Prochloron and a variety of blue-green algae. Its cell walls contain muramic acid, some 1.3 fg μm^{-2} of cell surface (Moriarty, 1979), the peptidoglycan type being identical to that found in cyanophytes and many Gram-negative bacteria (Stackebrandt and Kandler, 1982). Prochloron contains a variety of carotenoids, predominantly β-carotene and zeaxanthin, generally like that of blue-green algae except for the absence of glycosidic carotenoids (present in most cyanophytes), and differing in important respects from that of eukaryotic chlorophytes (Withers et al., 1978; Withers, Vidaver and Lewin, 1978). The

polar lipid composition is typical of that of cyanophytes in that the major lipids are galactolipids, sulfoquinovosyldiacyl-glycerol and phosphatidylglycerol, while phosphatidylcholine and phosphatidylethanolamine are absent (Perry et al., 1978; Murata and Sato, 1983). Monogalactolipid is apparently synthesized via monoglucolipid as in cyanophytes, not by the pathway characteristic of eukaryotic plants (Murata and Sato, 1983; Kenrick et al., Sydney; personal communication). Polyunsaturated fatty acids are not present in significant amounts (Perry et al., 1978; Murata and Sato, 1983). Small amounts of sterols, with a high proportion (22%) of 5 α-stanols, have been found in Prochloron, as in cyanophytes. The relatively high proportion of cholesterol (0.03% of cell dry weight) suggested to Johns et al. (1981) that they may not have been produced by the alga itself but by its animal host.

Ecological considerations, and data on photosynthetic activities of Prochloron cells in hospite and in vitro, have been presented and discussed by Tokioka (1942), Thinh and Griffiths (1977), Thinh (1978b), Fisher and Trench (1980), Pardy and Lewin (1981), Thinh, Griffiths and Ngan (1981), Kremer, Pardy and Lewin (1982), Pardy, Lewin and Lee (1982) and Lewin, Cheng and Alberte (in preparation). It appears that the alga could derive at least some of its requirements for CO_2 and reduced nitrogenous compounds including perhaps tryptophan (Patterson and Withers, 1982), from the animal host, and that the latter could benefit by the photosynthetic production of organic solutes and oxygen by the Prochloron cells in their extracellular spaces (Pardy and Lewin, 1981; Griffiths and Thinh, 1983).

The particularity of the interdependence of alga and animal may vary greatly from one host species to another.

In cells of Prochloron, as in many blue-green algae and autotrophic bacteria, there are polyhedral bodies with a crystalloid substructure (Schulz-Baldes and Lewin, 1976; Whatley, 1977) probably composed of ribulose

bis-phosphate (RuBP) carboxylase molecules. RuBP-carboxylase has been extracted from Prochloron, and it appears immunologically identical to that of the cyanophyte Microcystis (G. A. Codd, Dundee; personal communication). The nature of the RUBP-carboxylase in Prochloron is of particular interest for the following reason. In green plants generally, the enzyme is made up of 16 subunits, 8 large (encoded in the chloroplast DNA) and 8 small (synthesized on nRNA originating in the nuclei). But Prochloron has neither plastids nor nuclei; so how does it make its RuBP-carboxylase? (Some cyanophytes make an enzyme of the 8L+8S type--but some do not.) Berhow and McFadden (1983) have confirmed that RuBP carboxylase is indeed present in Prochloron, along with phosphoribulokinase, and that it has large and small subunits much like those in higher green plants. The Prochloron RuBP carboxylase dissociates (probably to 4L + 4S halves) during polyacrylamide-gel electrophoresis, being in this respect unique among all RuBP-carboxylase enzymes so far examined. Activities of superoxide dismutase (L. Henry, King's College, London), nitrite reductase (H. Bothe, Cologne) and succinate thiokinase (P. D. J. Weitzman, Bath) have been demonstrated in lyophilized Prochloron cells (personal communications), as have glucose-6-phosphate and 6-phosphogluconate dehydrogenases (Fall, Lewin and Fall, in preparation). However, the isolation of many other enzymes from Prochloron species preparations seems to be hampered by the phenomenon of intracellular coagulation, which occurs apparently as the cells die (Fall, Lewin and Fall, in preparation).

Nucleotide sequence data from analyses of 5S and 16S ribosomal RNA point to close phylogenetic affinities between Prochloron and cyanophytes, including filamentous types such as Nostoc (MacKay et al., 1982; Seewaldt and Stackebrandt, 1982), though a re-evaluation of the 16S RNA data led Van Valen (1982; see also Van Valen and Maiorana, 1980) to dispute this phylogenetic

conclusion.

On these bases we could conclude that Prochloron is a deviant cyanophyte in which unusual features of its photosynthetic pigment complement had been acquired, perhaps, relatively recently. Basing his considerations on the limited preliminary data then available, Antia (1977) therefore argued against the nominal creation of an algal division, the prochlorophytes, separate from the cyanophytes.

However, in addition to the pigment composition of Prochloron, anomalous for a prokaryote, there are other features to be considered which distinguish it from blue-green algae. In Prochloron the polyhedral bodies lie peripherally among the thylakoids (Schulz-Baldes and Lewin, 1976), whereas in cyanophytes they are generally in the centroplasm. The locations of the DNA bodies, as revealed by staining with fluorescent dyes such as "DAPI," differ in the same way: there are many peripheral lumps in Prochloron (Coleman and Lewin, 1983), but only a single condensed nucleoid in the centroplasm of cyanophytes. The thylakoids of Prochloron are, of course, devoid of phycobilisomes (Thorne, Newcomb and Osmond, 1977), which are a characteristic feature of cyanophytes. They are paired or stacked (Whatley, 1977; Thinh, 1978a), whereas in the cells of virtually all cyanophytes examined electron-microscopically the thylakoids are separated singly. The size distributions of photosynthetic particles on the inner Prochloron thylakoid faces, as visualized by freeze-fracturing and electron microscopy, are much closer to those of chloroplasts (of eukaryotic green algae or higher plants) than to those of cyanophytes (Giddings, Withers and Staehelin, 1980; Cox and Dwarte, 1981). Correlated perhaps with these structural features is a physiological feature, a burst of fluorescence exhibited when dark-adapted cells of

Prochloron or other green algae are re-illuminated; this response is not shown by cyanophytes (W. Vidaver, personal communication).

Although the "spectrum" or variety of photosynthetic products in Prochloron is somewhat closer to that of blue-green algae than to that of chlorophytes, a notable feature is the apparent absence of sucrose, which is produced by most other plants that contain chlorophyll b. Published results of short-term photosynthesis experiments with $^{14}CO_2$ differ somewhat, perhaps partly because the algal cells studied came from different host species and partly because of the different experimental techniques employed. Akazawa, Newcomb and Osmond (1978) found that, within the first half-minute or so, 3-phosphoglyceric acid was the major photosynthetic product of Prochloron in Diplosoma virens; after that, more acid phosphates and dicarboxylic acids were formed; and after an hour some 85% of the total fixed carbon was in the form of a water-soluble oligosaccharide. Fisher and Trench (1980), who studied Prochloron cells isolated from D. virens, reported that after 1 h of incubation in the light the primary end-products were glucose, maltose, fructose, sugar phosphates and polyglucans; the main extracellular product was glycollic acid. By contrast, Kremer, Pardy and Lewin (1982), who worked with Prochloron cells isolated from Lissoclinum patella incubated in light for periods up to 1 h, found no free sugars; more than 50% of the fraction soluble in aqueous alcohol consisted of amino acids, and about 20% comprised acids of the tricarboxylic acid cycle and glycollic acid.

Both branched and relatively short-chain, unbranched 1,4 alpha-glucans (starches) occur in Prochloron cells (Akazawa, Newcomb and Osmond, 1978; Fredrick, 1980; Kremer, Pardy and Lewin, 1982) as in many green algae. (Unbranched polyglycans have not been reported from cyanophytes.) Two

phosphorylase and synthase enzymes, and three branching isozymes of the glucosyl-transferase system, have also been found in Prochloron (Fredrick, 1981).

Gas vacuoles, which occur in many but by no means all cyanophytes, have not been found in Prochloron; nor has cyanophycin (R. D., Simon, Rochester, Rochester; personal communication) (a polymer of aspartic acid and arginine, found in many cyanophytes); nor has poly-beta-hydroxybutyric acid (G. Gottschalk, Göttingen; personal communication) (which occurs in some cyanophytes as well as in many non-photosynthetic prokaryotes). But in considerations of phylogeny the absences of such features may not be as telling as positive characteristics. The production of 3-(N-methylamino) glutaric acid in Prochloron (Summons, 1981) is apparently unique; its metabolic (or osmotic) role remains to be established.

On the basis of present data, the decision as to the taxonomic position of Prochloron has to be made subjectively, according to the gravity with which we weigh the various similarities and differences reviewed above. But even if we are led to conclude that Prochloron is a "relatively" close relation of the cyanophytes, we have still to account somehow for its ability to synthesize chlorophyll b (now a well-established fact: Lewin and Withers, 1975; Thorne, Newcomb and Osmond, 1977; Thinh and Griffiths, 1977) and the thylakoid proteins to which it is bound (Withers et al., 1978). Is this ability monophyletic, ancestral to both divisions of prokaryotic algae (as well as to eukaryotic chlorophytes)? Possibly the prochlorophytes retained it while the cyanophytes (now much more widespread and "successful" on ecological and physiological grounds) somehow lost it and developed other accessory pigments, phycobilins, instead. In the pre-Cambrian era there may have been

free-living prochlorophytes as well as cyanophytes. The fossil record cannot tell us which came first: prokaryotic algal remains in pre-Cambrian rocks could as well be fossilized prochlorophytes as cyanophytes, as experimentally demonstrated by the laboratory silicification of Prochloron cells (Francis, Barghoorn and Margulis, 1978). Or did Prochloron evolve from a cyanophyte stock and somehow--independently of the other green algae but by some parallel biochemical evolution--"re-invent" the synthesis of chlorophyll b and associated proteins and, earlier or later, dispense with accessory phycobilin pigments? (Mutants of blue-green algae with reduced abilities for biliprotein synthesis have been reported, e.g., by Stevens and Myers, 1976.) Or could it have picked up the system for chlorophyll b as a plasmid from some neighbouring green plant?

The second major question posed by Prochloron - did green-plant chloroplasts arise, by endosymbiosis, from a prochlorophyte ancestor of this sort - was briefly discussed three years ago (Lewin, published in 1981b). A little more relevant information has come to light since then. The data of Herdman (1981), critical to this point, indicate that Prochloron has a genome size (3.59×10^9 daltons) similar to that of many other autonomous prokaryotes, some 30-40 times as big as that of a chloroplast genome in higher plants (Herdman and Stanier, 1977). If chloroplast ancestors were prochlorophytes, they seemingly lost some 95% of their genetic information coding, including much of that required for the functioning of the photosynthetic apparatus, after the postulated act of symbiogenesis. This, of course, is conceivable. The fact that the apparent "plastids" of the flagellate Cyanophora have a DNA genome size of only 1.17×10^8 daltons (Herdman and Stanier, 1977), yet nevertheless have (or retain) at least traces of a peptidoglycan envelope, indicates that such a reduction may have occurred

more than once in evolution. There seems good evidence, from 16S RNA base-sequence data, that red algal plastids did in fact originate from cyanophytes (Bonen and Doolittle, 1975; Bonen et al., 1979), losing over the years some 90-95% of their genetic material. On the other hand, Cavalier-Smith (1982) argues, though less plausibly, that heterogeneity of the pigment contents of plastids in various algal classes could have arisen after a single primordial act of symbiogenesis.

Outlining problems of this kind helps us to formulate fresh approaches and to suggest new lines of research whereby they may ultimately be resolved. And meanwhile we should retain "the ability to see things in a long-term perspective...and the willingness to concede that there just may be another point of view" (Queen Elizabeth's jubilee speech, 1977).

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Prochloron-Ascidian Symbioses: Photosynthetic Potential and Productivity

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Abstract - We determined the chlorophyll content of ~~several species of~~ didemnid ascidians ~~possessing~~ with symbiotic algae (Prochloron sp.) from oligotrophic tropical marine waters around Palau, Western Caroline Islands. Several species contain as much chlorophyll per unit dry weight as many herbaceous crop plants and more than do other symbiotic associations such as lichens, green Hydra, etc. Their chlorophyll a/b ratios (3-9) were generally much higher than those of angiosperms (2-4). Where they abound, Prochloron-ascidian symbioses could make a major contribution to the productivity, especially in localized areas of tropical marine waters characterized by low nutrient levels and high irradiance.

Introduction

Until a few years ago, didemnid ascidians with "zoochlorellae" were barely mentioned in lists of symbiotic associations of invertebrates with algae (e.g., Droop, 1963; Taylor, 1973; Trench, 1979). More recently, interest in these protochordates has grown, largely because of the unexpected discovery that their algal symbionts (Prochloron sp.) are prokaryotes capable of synthesizing chlorophyll b as well as chlorophyll a, a feature hitherto thought to be confined to eukaryotic chlorophytes such as green algae and higher plants (see review by Lewin, 1981). However, even among marine biologists familiar with many of the diverse symbiotic associations found in tropical coastal waters and reefs, few are yet aware of the potential ecological importance of symbiotic didemnids in ~~many~~ such regions (Lewin, 1979; Kott, 1980).

In fringing reef areas and shallow lagoons surrounding the islands of Palau, Western Caroline Islands, several species of symbiotic didemnids are abundant. Along some of the lagoon shores, many rhizophores and lower

branches of the mangrove Sonneratia bear a mosaic coating of a soft green didemnid, Diplosoma virens (Tokioka, 1942). On nearshore coastal flats, leaves of the seagrasses Halophila and Enhalus can be found partly enveloped by grey-green colonies of Lissoclinum voeltzkowi, while in nearby areas these seagrasses can be equally heavily laden with spherical colonies of yet another symbiotic species, Didemnum molle. Along parts of the Kamori Channel, at depths of about 1 m, virtually all of the dead coral surfaces are blanketed by another green didemnid, Trididemnum cyclops. This same species also carpets large patches of the bottom of at least one of the marine lakes (Hamner, 1982).

In the localized areas where they are found, symbiotic didemnids could contribute significantly to primary productivity, since they are abundant and contain large numbers of photosynthetic cells (Lewin, 1981) with high rates of photosynthesis (Fisher and Trench, 1980; Thinh et al., 1981; Griffiths and Thinh, 1983; Alberte et al., in preparation). There is evidence that these algal symbionts, which line cloacal cavities and other spaces of their host colonies (see Kott, 1980; Lewin, 1981), release fixed carbon (Thinh and Griffiths, 1977; Fisher and Trench, 1980; Griffiths and Thinh, 1983) which can be taken up by their hosts at levels between 7% (Pardy and Lewin, 1981) and 51% (Griffiths and Thinh, 1983). These values are comparable to those, sometimes exceeding 20-30%, determined for hermatypic corals (Taylor, 1973; Muscatine, 1980). In coral reef systems, the net productivity (g $C/m^{-2}/d^{-1}$) may be 5 to 50 times as high as in the overlying waters (Marsh, 1976; Lewis, 1977; Trench, 1979), and may reach levels comparable to those reported for agricultural systems, kelp beds and seagrass communities (Dawes, 1981). We present here evidence, based on the chlorophyll contents in eight species of symbiotic didemnid ascidians, indicating an important role of Prochloron-ascidian associations in the primary productivity of certain tropical marine benthic communities.

Materials and Methods

Didemnid ascidians were collected in February, 1982, from a small coastal reef flat in the Kamori Channel, Palau, Western Caroline Islands. Colonies immersed in seawater were transported from the field to running seawater tanks at the laboratory of the Micronesian Mariculture Demonstration Center, Koror. Prochloron sp. cells were removed from the various host species by squeezing the colonies. The ratios of fresh (wet) to ash-free dry weight were determined on the same samples. Ash weight were determined after calcining at 330°C for 1 hr. Chlorophyll contents and chlorophyll a/b ratios were determined in extracts in 90% (v/v) acetone by using equations of Jeffrey and Humphrey (1975). Some comparative data were obtained from collections made in Singapore in 1977, or from the published literature.

Results and Discussion

Among the 8 species of didemnids examined, the dry weight/wet weight ratios are lower (Table 1) than those of most invertebrates (ref. ?) but are roughly equivalent to those of many angiosperms (Evans, 1972). Most of the values are less than 17%, the lowest values being those of Didemnum molle (8%) and Diplosoma similis (>3%) which are respectively full of mucilaginous material and a less viscous algal suspension. The one high value (42%), that of Lissoclinum bistratum, is attributable to the high content of calcareous material (84% of the dry weight) in the colonies of this species.

The chlorophyll a/b ratios (Table 1), 3.1 to 8.8, are in the same range as those reported for various Prochloron preparations examined earlier (summarized by Lewin, 1981), being higher than those typical of terrestrial plants (2.2 to 4.0; Kirk and Tilney-Bassett, 1978) and of marine and fresh -

water green algae (1.5 to 2.8; Wood, 1979; Alberte, unpublished). The amounts of chlorophyll a (per gm ash-free dry weight or per unit biomass) of the symbiotic association range from 0.8 to 5.3 mg/gm (Table 1). These values are comparable to those found in the symbiotic Paramecium bursaria + zoochlorella complex (5 mg/gm; D. Weis, Cleveland, Ohio, pers. commun., July 1982) and in some whole angiosperms (comprising nonphotosynthetic as well as photosynthetic tissues) such as duckweed (Lemna minima, 5.5 mg/gm, our data) and barley (Hordeum vulgare leaves (5.0-7.0 mg/gm, our data); they are higher than that of the highly productive seagrass, Zostera marina (Mazzella et al., 1981; Dennison and Alberte, 1982). The average value, 2.8 mg for the chlorophyll a content/gm, is higher than those reported for lichens (e.g. 0.4-1.2 mg/gm in Peltigera canina, Wilhelmsen, 1959), the symbiotic green sponge Spongilla lacustris (0.55-1.2 mg/gm; Frost and Williamson, 1980), and the dinoflagellate-containing sea-anemone Anthopleura elegantissima (~1.0 mg/gm, estimated from data of Fitt et al., 1982). The relatively high levels of chlorophyll in ~~these~~ symbiotic didemnids indicate a considerable potential for photosynthetic activity, which could make a significant contribution to their nutrition and thereby add to the overall primary productivity of ~~such~~ certain tropical benthic communities.

A high content of chlorophyll is not always correlated with a high rate of photosynthesis (Kirk and Tilney-Bassett, 1978; Richardson et al., 1983), though in many plants it reflects, fairly accurately, their photosynthetic rates (Patterson, 1980). Light-saturated rates of photosynthesis in Diplosoma virens have been reported to be ~~6-fold~~ considerably greater than the dark respiration rates (P:R = 8, Tokioka, 1942; Fisher and Trench, 1980; Thinh et al., 1981); ~~while~~ in the other species listed in Table 1 (except for Didemnum molle and Lissoclinum bistratum, which were not examined) the P:R ratios are 10 or greater (Alberte et al., in preparation). Since in Palau these ascidians experience daily

periods of light-saturated photosynthesis of about 12 hr (Alberte et al., in preeparation), their daily (24 hr) P:R ratios are at least 5. Similar daily P:R ratios are found in such terrestrial angiosperms as wheat (Triticum) and maize (Zea) (C. Gudin, pers. commun.; de Witt et al., 1970) and in the seagrass Zostera marina (Dennison and Alberte, 1983). In most of these symbiotic didemnids, net photosynthesis per unit chlorophyll a is comparable to, or exceeds, that of many marine macrophytic algae (Kirk and Tilney-Bassett, 1978; Dawes, 1981). On the same basis, net photosynthetic rates of the symbiont alone are comparable to those of typical cyanobacteria (e.g., Anacystis nidulans, Vierling and Alberte, 1980; a marine Synechococcus sp., Wood et al., 1983; Alberte et al., in preparation).

In Palauan reef shelf waters, the majority of the symbiotic didemnid species, especially Lissoclinum patella, Diplosoma virens and Didemnum molle, are found at depths of less than 2-3 m where there is little attenuation of light intensity by the water column. (E.g., at solar noon, under a cloudless sky, the light intensity [400-700 nm] at 2 m is 98% of that at 2 cm; Alberte, unpublished data). In isolated Prochloron cells and in Lissoclinum patella and Diplosoma virens colonies, photosynthesis saturates at light intensities of 500 to 1000 $\mu E\ m^{-2}\ s^{-1}$ (Alberte et al., in preparation). These symbiotic associations are evidently adapted to high-light environments, maximizing their photosynthesis, and hence their primary production, under the light environments prevailing in shallow reef waters.

Another feature of some of these symbiotic ascidians which may help them to maintain high rates of photosynthesis is their motility with positive phototaxis. Colonies of Diplosoma virens can move along substrate surfaces at rates up to a few mm per day (Birkeland et al., 1981; Thinh et al., 1981).

This could help to keep them in well illuminated situations and reduce ~~and/or~~ or even eliminate potential shading by other organisms or from burial under sediment. ~~It would be highly adaptive for a species with photosynthetic symbionts to maximize photosynthesis.~~

Relatively high growth rates around 2.3% per diem have been reported for Diplosoma virens (Thinh et al., 1981); similar rates have been documented for such photoautotrophs as macrophytic algae (deBoer et al., 1978). Some of the nutrients supporting the growth of these animals presumably derive from photosynthetic activity of their algal symbionts. However, the presence of fecal pellets in their guts indicates that ~~colonies of~~ D. virens ingests microplankton (e.g. bacteria and algae) providing an additional source of nutrition for the maintenance and growth of the host. ~~Certainly,~~ future work ~~in this area~~ is needed to clarify the nutritional economy of symbiotic didemnids.

Although the presence of symbionts in didemnid ascidians has been recognized for some time, their uniqueness has been only recently appreciated ~~(see Lewin, 1981)~~. The nutritional role of these prochlorophyte symbionts in the growth of their hosts remains unclear, but there is now considerable evidence indicating that they can contribute significantly to the primary production of some tropical littoral communities. In the present report we demonstrate that at least eight species of Prochloron-didemnid symbioses have photosynthetic potentials (based on their chlorophyll content) equalling or exceeding those of other marine or terrestrial species ~~and symbioses~~.

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Diurnal Rhythm in the Cell-division Frequency of Prochloron (Prochlorophyta) in Nature

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Abstract

The proportion of dividing cells of Prochloron living symbiotically in colonies of a didemnid, Diplosoma virens, rises from about 4% during the night (20.00-04.00 hrs) to about 13% in the morning (08.00-12.00 hrs.), and then falls again in the afternoon. Similar, though less pronounced, changes have been observed among Prochloron cells in two other symbiotic didemnids, Lissoclinum patella and L. voeltzkowi.

Introduction

Prochloron (Prochlorophyta) is a unicellular, prokaryotic alga with photosynthetic pigments similar to those of eukaryotic chlorophytes. Since it has been suggested that ancestral green-plant chloroplasts may have been prochlorophytes (reviewed by Lewin, 1981), this alga is of more than usual interest in considerations of the origins of eukaryotic plants. It has not been grown in sustained culture, though Patterson & Withers (1982) reported limited success in its cultivation in their laboratory, where cells divided on the average once every 5 days. Almost all other studies, biochemical and physiological as well as cytological, have involved Prochloron cells collected from Nature and mechanically separated from the didemnid ascidian colonies with which they are normally associated as extracellular symbionts. Of necessity, such studies have been based on materials collected in tropical coral-reef areas. One of the richest of such areas is the Palau Archipelago, in the western Pacific Ocean, where at least six symbiotic didemnids occur in relative abundance within a few metres of the sea surface.

Prochloron cells divide exclusively by binary fission (Lewin, 1975). Most of the samples of Prochloron didemni, collected by Lewin (1975) from the surfaces of didemnid colonies (Didemnum sp.) in the Gulf of California, showed only about 1% of the cells undergoing division, but in three samples (fixed respectively at 08.00, 12.00 and 20.00 hrs), the proportions were considerably higher, around 10-20%. We report here observations on the

frequencies of cell-division stages in suspensions of Prochloron cells, expressed at regular intervals throughout a natural day-night cycle from several colonies of four species of host didemnid.

Colonies of three species of symbiotic didemnid ascidians (Diplosoma virens, Lissoclinum patella and L. voeltzkowi), still attached to their natural substrates, were collected from the Kamori reef site near the Nikko-Palau Hotel, Koror, Palau, Western Caroline Islands, in March 1983. They were kept in gently running sea water at 29°, in 10,000-litre holding tanks open to the sky. The days were mostly sunny. At 2-hour intervals, for a 32-hour period spanning more than one day and one night (14-15 March), whole colonies or colony fragments of each species were removed and pressed in a vial to extrude algal cells, which were preserved with ethanol (90%). Droplets of cell suspension from each sample were later examined at a magnification of x100, and in each subsample the percentage of dividing cells was estimated on the basis of 400 cells counted. All stages from the first appearance of a centripetal cross-wall (early division stage) to incipient separation of paired, spherical daughter cells were counted as "division stages" (embodying two cell elements). The counts as percentages were plotted against the time of day. (A fourth species, Trididemnum cyclops, collected and sampled in the same way, yielded too few algal cells for meaningful consideration.)

Results

The data obtained for Prochloron cells in Dip. virens are shown in Fig. 1. In the morning and until early afternoon (06.00-14.00) some 13% of the algal cells exhibited stages of division; in some samples almost 16% of the cells were undergoing division. Later in the day and at night the proportions fell to around 4-6%. Algal cell divisions in the other two didemnids showed slight but similar tendencies (Figs. 2 and 3). In algae from the epiphyllous L. voeltzkowi the percentage of dividing cells ranged from about 10% (02.00-12.00) to 5% (14.00-22.00). In algae from the giant benthic didemnid L. patella the ratios, usually around 4-6%, showed a slight decrease around 20.00-24.00 hrs.

Unfavourable circumstances did not permit us to continue this series of samples for more than 32 hours beyond 16.00 hrs on 15 Mar 83. However, we were able to obtain some additional data from samples of Dip. virens and L. patella taken hourly, between 06.00 hrs and 18.00 hrs, on 16 and 17 Mar 83. On both days there were more cells undergoing division during the morning hours (06.00 - 12.00 hrs) than later in the day. There was a more pronounced difference between the high and low proportions of dividing algal cells in Dip. virens (19% and 2% respectively) than in L. patella (8% and 0% respectively).

Discussion

Although the three symbiotic ascidians under consideration here are found in the same intertidal lagoon, they occupy different habitats. Dip. virens, which has the smallest colonies of the three species, seldom exceeding 2 cm in diameter or 0.3 cm in thickness, occurs highest on the shore, on rocks or mangrove roots exposed daily to full tropical sunlight (Lewin, Cheng & Alberte, 1983). L. voeltzkowi forms colonies several cm long and 1-3 cm wide on leaves of a seagrass (Enhalus sp.) in about 1 m of water, where it is never totally exposed even at low spring tides. As in Dip. virens the colonies are thin, less than 0.5 cm in thickness. L. patella, on the other hand, is a giant didemnid that grows either attached to loose sandy gravel among coral rubble or on calcareous algae (Halimeda spp.). The colonies are usually more than 1 cm thick and may exceed 10-15 cm in diameter. In the reef-flat area where we carried out most of our observations, some of the colonies of this species were totally exposed at low spring tides. Since colonies of L. patella generally occur 10-20 m further offshore than those of Dip. virens, their exposure to full tropical sunlight is generally much less than that of the latter species. Such ecological differences may account for the differences that we observed in the cell division frequencies of Prochloron cells in these different didemnid hosts. Dip. virens, which shows the highest cell division frequencies and most pronounced diurnal rhythm, is also the most motile, being able to shift its position relative to light (Kirkeland, Cheng and Lewin, 1981). It probably has the highest

rate of vegetative reproduction. Thus the high proportion of dividing algae in this didemnid may be a reflection of the relatively high growth rate of the animal host (Thinh et al., 1981). Although in this host species we observed more cells in early division stages at 04.00 hr than at any other time during the day, we are not yet in a position to assess its rate of reproduction in Nature since we do not know how long it takes for a single cell of Prochloron to divide into two.

However, since there is evidence for a diurnal rhythm, with a maximum around 16%, it seems reasonable to conclude that the rate of cell multiplication cannot be less than one doubling in six (100/16) days. It is possible that Dip. virens grows equally rapidly, so that the animal cells grow at the same rate as their algal symbionts. Field observations indicate that the other two species (L. voeltzkowi and L. patella) grow much more slowly. We must therefore conclude either that the excess algal cells are consumed in the colonies of their hosts (for which there is some evidence from electron microscopy: Cox, 1982) or that they are expelled through the cloacal apertures into the surrounding water.

We have been unable to find references to comparable studies of cell-division frequencies in other prokaryotes under natural conditions but have learned of some relevant, unpublished data from Dr. John J. Waterbury, Woods Hole, Massachusetts. He observed that a marine Synechococcus, collected at frequent intervals from surface waters in Woods Hole harbor, shows evidence for a diurnal rhythm of cell division: the frequency of dividing

cells rises during daylight hours, reaching a peak of about 25% at dusk, and then declines sharply to a more or less constant value of 5% during the night.

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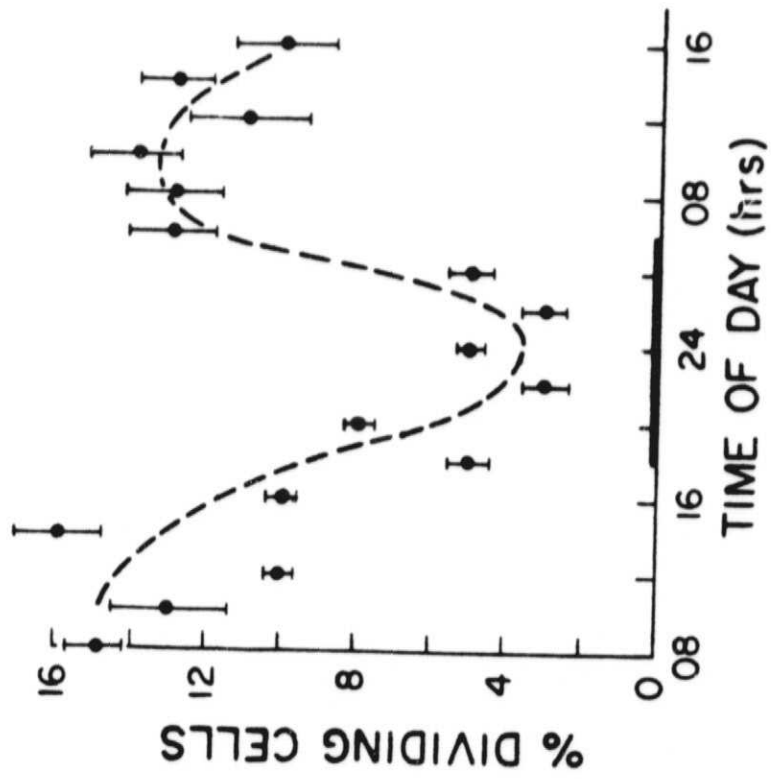
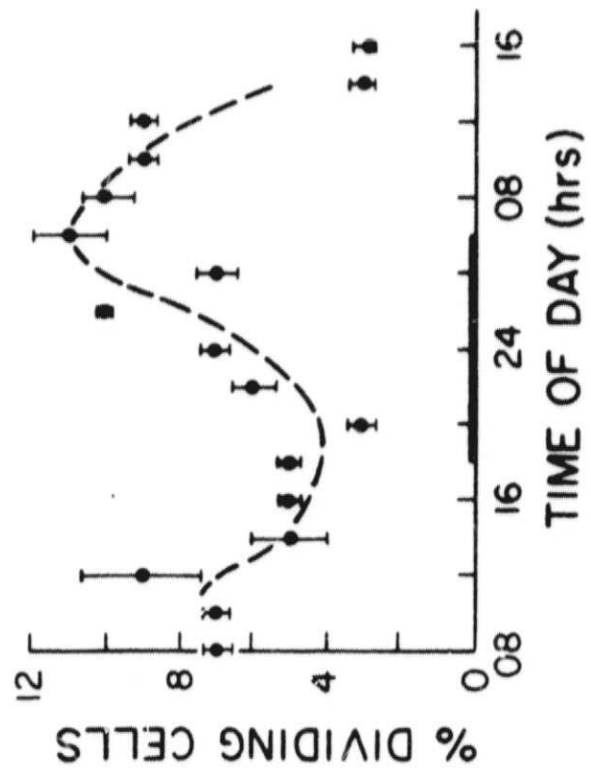
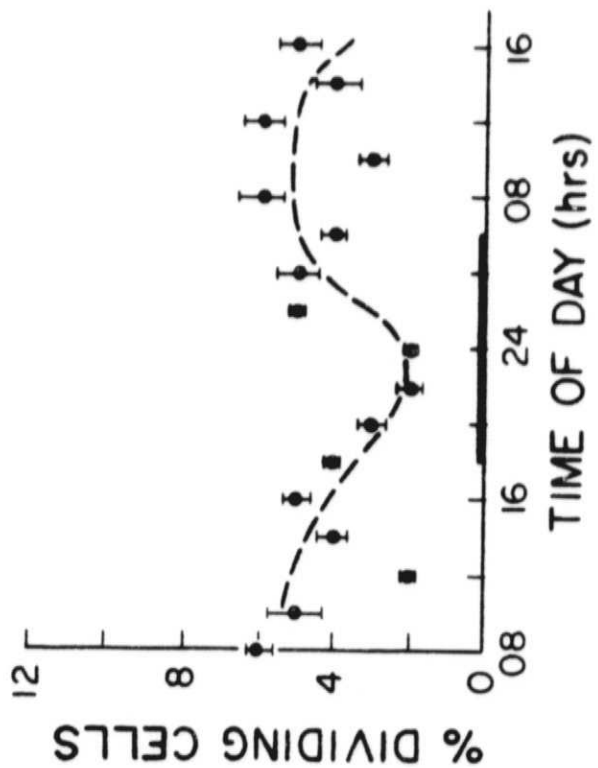
Legend

Fig. 1-3 Proportions of dividing cells of Prochloron in colonies of (1) Diplosoma virens, (2) Lissoclinum voeltzkowi and (3) L. patella, based on counts made at 2-hr intervals in a 32-hr period. Each point represents a value based on 400 cells counted (respectively, 100, 200 and 100 by each of the authors): the ranges are indicated by bars.







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Chlorophyll and carotenoid pigments of *Prochloron* (Prochlorophyta)

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Abstract

High-performance liquid chromatography (HPLC) with a gradient-elution technique was utilized to separate and quantify chlorophylls a and b as well as major carotenoid pigments present in freeze-dried preparations of Prochloron-didemnid associations and in Prochloron cells separated from host colonies. Results confirm earlier spectrophotometric evidence for both chlorophylls a and b in this prokaryote. Chlorophyll a:b ratios range from 4.14 to 19.71; generally good agreement was found between ratios determined in isolated cell preparations and in symbiotic colonies (in hospite). These values are 1.5 to 5-fold higher than ratios determined in a variety of eukaryotic green plants. The carotenoids in Prochloron are quantitatively and qualitatively similar to those found in various freshwater and marine blue-green algae (cyanophytes) from high-light environments. However, Prochloron differs from cyanophytes by the absence of myxoxanthophyll and related glycosidic carotenoids. Its pigment characteristics are considered sufficiently different from those of cyanophytes to justify its assignment to a separate algal division..

Key words: carotenoids, chlorophyll a and b, cyanophyte, HPLC, Prochlorophyta.

Introduction

Among photosynthetic algae, Prochloron, a genus of coccoid, non-motile, single-celled algae, 7-25 μm in diameter, generally living as symbionts in or on tropical didemnid ascidians, is morphologically and biochemically unique (Lewin 1976). In cell structure it resembles cyanophyte algae, since no membrane-bound organelles or nuclei can be seen under the microscope, and its layered photosynthetic lamellae are not associated in chloroplasts. Nevertheless, Prochloron is distinguishable from cyanophytes by the absence of

phycobilisomes and the presence of chlorophyll b (in addition to chlorophyll a) (Lewin & Withers 1975). In a study based on spectrophotometric absorption scans and thin-layer chromatography (TLC), Withers et al. (1978) established the presence of chlorophyll b in Prochloron samples from at least six different host species. They observed a similarity in the complement of carotenoid pigments between Prochloron and cyanophytes, and confirmed the absence of phycobilisomes. We further confirm these findings and present data

We also present comparisons between Prochloron and other prokaryotic and eukaryotic algae for ecological and taxonomic considerations.

Pigments of Prochloron cells, both associated in symbiosis and separated from their didemnid hosts, were extracted from a variety of samples; see Table 1. For comparative purposes we included samples of freeze-dried and fresh cyanophytes and chlorophytes. Axenic clones of Nostoc entophytum and Anacystis montana were grown in the laboratories of the Culture Collection of Algae and Protozoa, Cambridge, U.K. The following algae were cultured at the laboratory in North Carolina under 400 μEm^{-2} sec^{-1} PAR illumination (cool white). Anabaena oscillarioides (Cyanophyta) was grown in Chu-10 (minus combined nitrogen) (Chu 1942). Chlorella vulgaris and Scenedesmus quadricauda (Chlorophyta) were axenically grown in ASM-1 medium (Parker 1982). Spirogyra spp., Chlorococcum humicola (Chlorophyta) and Navicula spp. (Chrysophyta), all obtained from Chowan River, N.C., were grown non-axenically on ASM-1 medium diluted 1:3. Microcystis aeruginosa (Cyanophyta) was collected from a surface bloom in the

* Photosynthetically active radiation (400 - 700 nm).

Neuse River, N.C., where it constituted over 95% of the phytoplankton biomass. Codium decortatum and Ulva lactuca (Chlorophyta) were freshly sampled from Bogue Sound, N.C. The spermatophytes analyzed were obtained from the H.W.P.'s garden.

Among the plant materials discussed, carotenoid and chlorophyll pigments were all examined by HPLC, and in specified cases, also by TLC and scanning spectrophotometry. The pigments were extracted from centrifuged algal cells or freshly ground higher plants by sonication for 4 min in 90% HPLC-grade acetone (Fisher) buffered with $MgCO_3$ to pH 8.0, and then allowed to stand at 4°C in darkness for 30 min. For Scenedesmus and Ulva a second sonication was necessary in order to quantitatively remove the pigments, but for all freeze-dried samples only a single extraction was needed. Acetone volumes used for extraction were varied according to the quantities of plant material being extracted. Dry weights of all plant materials were also determined. Extracts were centrifuged twice at 2600 RPM and the supernatants were filtered through diameter 0.2-µm porosity Nuclepore filters in preparation for both HPLC and TLC.

HPLC Methodology: For each HPLC analysis 20 µl of filtrate was injected into an Altex model 110-A liquid chromatograph equipped with dual pumps, a 25-cm long Ultrasphere ODS-18 reverse-phase column and a Hitachi 100-10 spectrophotometer (detector) with an 8-µl micro-flow cell. A linear solvent gradient program was used. The following mobile-phase gradient steps were employed: 0-7 min = 100% of a 90:10 methanol:acetonitrile mixture (solvent A), 7-11 min = a linear increase to 60% acetone and 40% of solvent A, 11-20 min = 60% acetone and 40% solvent A, 20-28 min = a linear decrease in 60% acetone until solvent A was again the sole solvent. The flow rate was

adjusted to 1.5 ml min^{-1}. Absorption peaks were graphically recorded and integrated, and total peak areas were determined with a Hewlett-Packard model 3390A recording integrator-plotter. Integration analyses allowed for quantification both of specific pigments and of relative contributions of the individual pigments to total pigments detected.

Carotenoids and chlorophylls were identified and quantified by comparing their retention times and absorbance characteristics to known reference standards, which included: i) purified chlorophyll a (from Anacystis nidulans) from Sigma Chemical Company; ii) purified β carotene, also from Sigma; iii) zeaxanthin, one sample from Phormidium persinicum (donated by Dr. D. J. Chapman, U.C.L.A.), a second from freeze-dried corn (Zea mais), and a third synthesized by Hoffman La Roche, Switzerland; iv) myxoxanthophyll, two samples respectively from Anabaena oscillarioides and Microcystis aeruginosa; v) purified echinenone from Hoffman La Roche. Respective R_f values were also obtained by TLC separations (Paerl et al. 1983) and compared to corresponding values determined by other workers (Stransky and Hager 1970; Jeffrey 1974, 1981).

Chlorophylls a and b revealed their respective absorption maxima at 663 nm and 642 nm. Maximum absorption peaks for carotenoids were close to 475 nm; accordingly this wavelength was chosen for specifically detecting carotenoids. Together, chlorophyll a and carotenoids were effectively detected at 440 nm. We used values for molar extinction coefficients for chlorophyll a and b published by Jeffrey and Humphrey (1975) and for pertinent carotenoids values published by Davies (1976).

TLC Methodology: Two-dimensional TLC was used both for pigment purification and for identification of selected samples on Avicel micro-

crystalline cellulose-coated glass plates 25 x 20 x 20 cm (Analtech, Inc.). All TLC and PHLC reagents were Fisher HPLC or A. R. grade. The first-dimension separation was in n-propanol:ligroine (2.5:97.5 v/v), the second in ligroine:chloroform:acetone (70:30:0.5 v/v/v). The only deviation from Jeffrey's technique (1981) was in the use of ligroine instead of light petroleum, which was unavailable. Pigments from filtrates were initially concentrated by extraction and partition between equal volumes of petroleum ether and 10% aqueous NaCl at -20°C for 20 min. The ether phase containing the pigments was collected and concentrated to 30 µl by volatilizing the ether under a stream of helium or argon. Any water remaining after this concentration step was further separated by centrifugation and discarded.

Concentrated pigment solutions were applied 2 cm from a corner of each plate and the solvent front was allowed to move 15 cm. Plates were dried between first- and second-dimension separations. Clearly separated spots were localized and removed by carefully scraping them off the plates and preserving them in 90% acetone at -20°C. (Reference values were as follows: chlorophyll a = 0.843/0.239, chlorophyll b = 0.512/0.101). Pigments purified in this manner were processed further by HPLC for identification or purification.

Scanning Spectrophotometry: Absorption characteristics of filtered extracts were determined with a Bausch and Lomb model 2000 U.V.-Vis. double-beam scanning spectrophotometer. We used quaratz microcurvettes 1 cm in width. Scans from 800 to 350 nm were plotted on an x-y recorder.

Results

Evaluations of analytical procedures. Chlorophyll and carotenoid pigments remained well preserved in recently freeze-dried didemnid colonies

and in isolated Prochloron samples. No degradation product of chlorophyll a or b, respectively pheophytin a (abs max = 667 nm) or pheophytin b (abs max = 655 nm), was detected by spectrophotometry of extracts from HPLC separations. Quantitative values for chlorophyll a and b and for major carotenoids decreased less than 8% during 6 months of storage in the dark at -20°C. From all samples except Scenedesmus and Ulva, complete pigment extraction was achieved by a single sonication. In these two algae, included for comparative purposes, approximately 70% of the pigment content was extractable after the first sonication and a further 20% was extracted after the second. Extraction efficiencies for chlorophylls and carotenoids were similar, ±4.5%. The total elution time for all chlorophylls and carotenoids in the gradient described above was 19 min (Fig. 1). Baseline drift during a single run proved to be less than 3%. Repeat HPLC injections during a 2-h period after pigment extraction and centrifugation had a standard error of ±4.9%, indicating that extraction and storage procedures yielded pigment samples stable for at least 2 h.

Chlorophyll a and b results. Table 1 lists chlorophyll a:b ratios and specific chlorophyll a contents of the materials examined. The chl a:b ratios in whole colonies were very close to those in separated cells of Prochloron, indicating that no significant amounts of these pigments were contributed by other epiphytic or endozoic algae associated with the didemnids, and that, for chlorophyll determinations by HPLC, prior isolation of Prochloron is necessary only for determining pigments per unit of algal biomass.

The specific chlorophyll a content of Prochloron appears close to the mean value for most of the green plant materials examined, but the chl a:b ratios are generally higher (Table 1). While in eukaryotic plants these

ratios ranged from 1.76 to 4.45, those of Prochloron ranged from 4.14 to 19.71. Prochloron derived from diverse didemnid hosts revealed dramatic, but consistent, differences in chl a:b ratios (Table 1).

The types of carotenoid pigments found in Prochloron were in good agreement with previously published observations of Withers et al. (1978). In all samples examined R_f values the following major carotenoids were identified: zeaxanthin (R_f = 0.380/0.340), echinenone (R_f =0.860/0.415), and β carotene (R_f = 0.960, 0.890). A minor carotenoid, possibly cryptoxanthin (R_f = 0.72, 0.41), was also consistently detected by both TLC and HPLC, but no reference standards for its confirmation (cf. Hertzberg and Liaaen Jensen 1966; Stransky and Hager 1970). Myxoxanthophyll, which is commonly found in a wide range of cyanophytes (Goodwin 1980), was conspicuously absent from Prochloron (Fig. 1,2), as previously noted by Withers et al. (1978). In other respects the carotenoid pigment types in Prochloron proved more similar to those of cyanophytes than to those of eukaryotic green plants (Fig. 2). Carotenoids typically found in such eukaryotes, including lutein, neoxanthin, violaxanthin and antheraxanthin (Hager and Stransky 1970; Jeffrey 1981), have not been detected in Prochloron. Likewise, the relative proportions of carotenoids, estimated from integrated peak data (Table 2), indicate that in its carotenoid composition Prochloron more closely resembles cyanophytes than chlorophytes (Fig. 1,2,3), and like cyanophytes, has a ratio of β carotene to chl a higher than that of chlorophytes (Table 3, Figs. 1,2) (cf. Stransky and Hager 1970, Hager and Stransky 1970 and Goodwin 1980). Echinenone, reported as an extra-plastidic carotenoid produced under unfavorable environmental conditions in certain chlorophytes (Goodwin 1980), occurs in Prochloron and cyanophytes (Fig. 1,2) but not in any of the samples of eukaryotes examined here. Lutein, commonly present in eukaryotic chlorophytes (Fig. 3), was not

found in Prochloron or in any of the cyanophytes examined (Fig. 1,2). Prochloron contains rather large quantities of zeaxanthin, as do most prokaryotic and eukaryotic plants (Figs. 2,3).

The presence of both chlorophylls a and b as well as carotenoid pigments is reflected in spectrophotometric absorption scans of 90%-acetone extracts. Characteristic features in the spectra appear in the 350-475 nm region, where Prochloron exhibits a relatively wide absorbance band (Fig. 4). Per unit of chlorophyll, the absorption of near-UV light was higher in acetone extracts of whole didemnid colonies than in extract of the algae alone, indicating the presence of some UV-absorbing material originating from the animal (host) cells.

Discussion

HPLC is well suited to rapid qualitative and quantitative analyses of photosynthetic pigments, combining high resolution, good sensitivity and excellent repeatability. Comparative analyses of Prochloron, cyanophytes, chlorophytes and higher-plant materials revealed both unique and common pigment characteristics of Prochloron. Although its chlorophyll a content is not strikingly different from that of other prokaryotic or eukaryotic algae, the relative content of chlorophyll b is generally lower by a factor of 2 to 5. We cannot yet say whether the observed variations in chl a/chl b ratios reflect genetic differences or contrasting environmental conditions in the different didemnid hosts.

Prochloron contains a range of carotenoid pigments; with the exception of the absence of myxoxanthophyll, the spectrum is like that of most cyanophytes thus far examined (Stransky and Hager 1970, Goodwin 1980). Carotenoids may

serve for photosynthetic energy transfer (as an accessory pigment: Warburg and Negelein 1922, Emerson and Lewis 1943, Haxo and Blinks 1950, Goedheer 1959, Cho and Govindjee 1970, Cox and Bendall 1974) and/or for photoprotection. In Prochloron, which may inhabit didemnids in shallow oligotrophic tropical waters exposed to high levels of irradiation, accessory carotenoids might serve in either role.

Spectral absorbance curves of acetone extracts of Prochloron resembled those of cyanophytes and terrestrial plants (Fig. 4), with strong absorption maxima in the 350-450 nm region, as reported earlier by Withers et al. (1978). The presence of abundant carotenoids and the strong low-wavelength absorption may reflect Prochloron's well-illuminated habitats, where it might be able to exploit the light at relatively high PAR saturation levels ($P_{opt.} \cong 700\ \mu E\ m^{-2}\ sec^{-1}$ PAR; R. Alberte, personal communication). It has been shown that energy from low-wavelength radiation captured by β-carotene may be efficiently transferred to chlorophyll in the photosystem I of cyanophytes (Goedheer 1964), whereas xanthophylls may serve more as photoprotective pigments (Goodwin 1980). The relatively high proportion of β-carotene in this alga may be considered of ecological relevance. Prochloron thus shows several well-defined biochemical and ecological similarities to cyanophytes, complementing the more obvious similarities in their structural features.

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Table 1. Chlorophyll a:chlorophyll b ratios and chlorophyll a content of Prochloron determined in hospite in all but 3 samples and various eukaryotic plants.

Class and species	Source	Chl a:b	Chl a (mg g^{-1} dry wt)
Prochlorophyta			
Prochloron	in Didemnum molle (1982)	5.23	4.94
	in Didemnum molle (1983)	4.98	5.01
	from Diplosoma similis	4.14	6.32
	in Diplosoma virens (1982)	14.0	5.41
	in Diplosoma virens (1979)	13.20	5.03
	in Diplosoma virens (1983)	13.65	4.78
	in Lissoclinum bistratum	7.2	3.28
	Lissoclinum patella (1983)	10.41	4.21
	from Lissoclinum patella	10.62	6.15
	in Lissoclinum voelzkowii (1982)	9.29	5.05
	in Lissoclinum voelzkowii (1983)	9.36	5.12
	from lissoclinum voelzkowii (1982)	10.41	7.05
	in Trididemnum clinides	(19	19.71
	in Trididemnum cyclops	5.83	3.49
Chlorophyta	Chlorococcum humicola	2.25	7.41
	Codium decortatum	2.09	7.52
	Scenedesmus quadricauda	1.96	6.05
	Spirogyra sp.	1.76	5.90
	Ulva lactuca	2.61	4.48
Spermatophyta	Brassica oleracea	3.22	4.25

	Spinacia oleracea	2.29	8.26
	Cichorium endivia	2.41	8.19
	Impatiens sp.	3.85	5.23
Chrysophyta	Navicula sp.	3.11	4.95

Table 2. Relative proportions of major carotenoids in various Prochloron strains, as determined by HPLC.

	Carotenoids (% of total)			
Prochloron from:	Zeaxanthin	Echinenone	Cryptoxanthin	β-carotene
Didemnum molle	26	12	6	52
Diplosoma similis	29	9	3	50
Diplosoma virens	20	10	4	58
Lissoclinum patella	24	9	2	63
Lissoclinum voelzkowii	31	6	3	59

Table 3. Chlorophyll a and carotenoid contents of various algae. (Published data were recomputed for this comparison.)

Organisms (by genus)	Chl a ($mg\ g^{-1}$)	Total carotenoids ($mg\ g^{-1}$)	Carotenoid: chl a	β-carotene: total carotenoids	Referen
Cyanophyta					
Anacystis	9.46	7.67	0.81	0.24	1
Synechococcus	3.96	3.59	0.91	0.22	1
Microcystis	3.91	3.14	0.80	0.42	2
Merismopedia	6.11	3.64	0.60	0.40	1
Phormidium	2.99	2.95	0.99	0.28	1
Anabaena	3.95	2.82	0.71	0.40	
Aphanizomenon	3.25	2.19	0.67	0.45	
Oscillatoria	6.88	2.32	0.34	0.59	1
Calothrix	3.06	2.63	0.86	0.19	1
Chlorophyta					
Dunaliella	8.60	3.39	0.40	0.26	3
Eudorina	4.55	2.85	0.63	0.20	3
Ankistrodesmus	22.02	4.10	0.19	0.02	4
Scenedesmus	12.16	5.12	0.42	0.13	
Chlorella	6.95	2.96	0.43	0.09	
Zygnema	6.33	3.56	0.56	0.18	3
Chara	2.05	0.84	0.41	← 0.31	
Prochlorophyta					
Prochloron					

in Did. **molle**	5.01	2.91	0.58	0.51
in Dip. **similis**	4.14	2.43	0.59	0.50
in Dip. **virens**	4.78	2.86	0.60	0.58
in L. **patella**	4.21	2.77	0.66	0.63
in L. **voelzkowii**	5.12	2.30	0.45	0.59

[1] Stransky and Hager 1970

[2] Paerl *et al.* 1983

[3] Hager and Stransky 1970

[4] Goodwin 1980

D R A F T

INTERNATIONAL PROCHLOROPHYTA EXPEDITION VII

MIDC, KOROR, PALAU, 5 FEB - 3MAR

1982

RESEARCH REPORTS

Dr. Randall S. Alberte, University of Chicago.
Dr. Ray Fall and Lana Fall, University of Colorado.
Dr. Ralph A.Lewin and Dr. Lanna Cheng, University of California, San Diego.
Dr. Grover C.Stephens, University of California, Irvine.

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Prochloron Expedition

R. S. Alberte

Purpose

Prepare *Prochloron* photosynthetic membranes for the isolation of the two major chlorophyll-proteins, the P700-chlorophyll *a*-protein and the light-harvesting chlorophyll *a*/*b*-protein, using SDS-polyacrylamide gel electrophoresis. The prepared proteins (purified) will then be examined for their cross-reactivity to polyclonal antibodies prepared from higher plant proteins. In addition, material will be prepared for 1) electron microscopy, 2) isolation of the DNA for determination of its general complexity (COT analysis) and similarity to barley chloroplast DNA and *Anabaena* DNA by using restriction-endonuclease analysis, 3) Kleinschmidt spreads of the DNA will be examined in the EM to identify and measure the extent and size of the circular DNA.

Experimental Design

Photosynthetic membranes were prepared from *Prochloron* cells in of *Lissoclinium patella* by squeezing the animals and collecting cells Tris-HCl or Bicine (pH 8.4)-buffered seawater. Cells were washed in the same buffer and resuspended in 0.1 M Tris-HCl (pH 8.4) containing 0.3 M NaCl. This suspension was French pressed at <500 psi (1 passage) to obtain membrane fragments. Since we were unable to pellet these fragments, sufficient amounts of 10% SDS were added to bring the final concentration of SDS in the preparation to 1% (w/v). This material was stored for 15-30 min and then centrifuged to remove cellular debris.

The green supernatant was layered on a modified Tennali SDS-gel system and subjected to electrophoresis for 30 min at 150 v (constant current). Three pigment zones were resolved. The slowest migrating band was the P700-chl a-protein, the zone of intermediate mobility was the chl a/b-protein and the fastest migrating band was free pigment. The zones were removed from the gel, dialyzed against distilled water and then lyophilized for blotting on vitrocellulose. The vitrocellulose blot will be leached with Staph A-coupled antibody to which ^{125}I has been bound. A positive antigen-antibody reaction will be visualized on less than 1 µg protein by audioradiography.

Additional Studies

1. Effect of light and dark on photosynthetic (PS) performance of Prochloron cells in situ.

Exp - Colonies of L. patella were placed in sunlight and in total darkness. Each day for 6 days, Prochloron cells were extruded and their PS activity measured on a Clark-type oxygen electrode (Rank Bros.).

Results - It was found that L. patella colonies kept in darkness for up to 4 days showed no significant effect on the PS activity of the symbiont. On day 5 and 6 the PS rate dropped to 1/5-1/7 of light controls.

2. Photosynthesis vs. Irradiance Characteristics of Prochloron from L. patella.

Exp - Prochloron cells were isolated from L. patella (as above) and examined for their oxygen evolution rates in relation to light

intensity. Net rates were obtained from gross photosynthesis minus dark respiration.

Results - The P_{max} of Prochloron is obtained only under high illumination levels. No evidence of photoinhibition was obtained. The saturation-light intensity was similar to light levels in the field in their natural habitats. Specific details await further calculations and calibration of the lamp to $\mu Ein^{-2}sec^{-1}$ (PAR).

3. The extent of DOC release from Prochloron

Exp - Prochloron cells were isolated and washed twice in Millipore (0.45 μ)-filtered and buffered seawater to renove extraneous material and to reduce bacterial contamination. Cells were placed in the above medium in vials and incubated in the presence of ^{14}C-labelled $NaHCO_3$ for 30 min, 60 min and 120 min. Dark controls were run for each time point. At the end of the incubation periods, cells were pelleted and the supernatants (S-N) discarded. This was repeated with cold seawater (chase) and the S-N I and II corbined. Cells and S-N were killed with boiling ethanol and acidified (to remove any unreacted CO_2 or HCO_3^-) with 6N HCl. Samples were packed for counting later in Chicago.

Results (To be obtained in Chicago.)

4. Photosynthesis and respiration in larvae of L. patella.

Exp. - Six to 8 swimming larvae of L. patella were placed in a charber with the oxygen electrode to determine whether they were capable of net photosyntheis.

Results - In these experiments it was shown that the larvae were at compensation; that is, the respiration rates of each larva plus the 50-80 million algal cells were equal to the PS rate of the algae.

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Report of Prochloron Research

R. & L. Fall

I. General Goals

Our major goal was to assay enzymes in situ in Prochloron, and to prepare active enzyme preparations for future use. In addition, we wanted to measure photosynthesis as an indicator of whole cell viability, and use this to monitor extended survival of Prochloron cells after their removal from the host animal.

II. Results

A. Cell coagulation: We observed cell coagulation (gross, irreversible cellular changes) under many conditions, including the following:

1. Squeezing cells from host into an inadequately buffered media. (Sea water buffered with 40 mM Tris (TBSW) or Bicine at pH 8.4, using a 1:1 ratio of buffer and squeezate gave apparently satisfactory living cells.)

2. Freezing the cells.

3. Treating them with 1% toluene.

4. Allowing cells to sit at high density, such as in a pellet from centrifugation.

5 Suspending cells in hypotonic or hypertonic media.

B. Enzymes assayed in situ: The following enzymes were assayed in whole cell preparations.

Enzyme	Result
Catalase	+
Guiacol peroxidase	+
G-6-P dehydrogenase	-
Isocitrate dehydrogenase	-
Malic enzyme	-
α-galactosidase	-
β-galactosidase	-

C. Enzyme extracts: All the above enzymes (part B), and superoxide dismutase (SOD) were also assayed in broken cell preparations. Only SOD was conclusive detected before the spectrophotometer failed! Soluble proteins were extracted by French pressing the cells and by hypotonic shock. The extracted protein was detected by Lowry protein assay or precipitation with ammonium sulfate.

D. Cell survival: We tried various schemes to stabilize cells so that they could carry out photosynthesis. Additions of a variety of antioxidants or thiols to cells in TRSW did not preserve cells. Monitoring the kinetics of loss of photosynthetic capacity over 24 hours showed that photosynthesis was best preserved in solutions above pH 8, with shaking to prevent settling of the cells. Bicine+bicarbonate buffered sea water seemed promising. pH 6.4 buffering, no buffering and high cell density led to more rapid declines in photosynthetic capacity.

E. Cell thiols: Fresh cell extracts made in aqueous buffer, ethanol or methanol contained no detectable thiols as measured by the DTNB test. (This is hard to understand.)

F. Cell aggregation: Prochloron cells aggregated when squeezed into TBSW or isotonic NaCl. Cells in isotonic NaCl + 10-20 mM EDTA did not. Such cells, returned to sea water, aggregated. The factor or factors responsible were not completely identified, but is seems that aggregation is probably linked to calcium levels in the media.

Report of Prochloron Research - IPF-VII (Palau, Feb. 1982)

Ralph A. Lewin and Lanna Cheng

1. At suitable low-tide periods about 5-6 new sites were surveyed as possible convenient sources of symbiotic didemnids. The Ngerdorl Channel site previously surveyed during IPF-VI remains by far the best, in terms of species, quantities and accessibility. It was not necessary to use a boat, since there was ready access by road to the Nikko-Palau Hotel dock. Representative colonies were photographed in situ.

2. Prochloron from the six major species of symbiotic didemnids was compared, in terms of yield and ease of expression, pH of the expressed serum, cell size and vacuolation, etc. Chlorophyll a/b ratios and DNA (hybridization studies) will be carried out later on lyophilized material. Comparative data are being tabulated.

3. Tadpoles from Lissoclinum patella colonies were observed emerging from cloacal apertures; about 400 were collected. All but 4 carried a girdle of symbiotic Prochloron cells (about 40,000 per larva). Observations were made on motility, phototaxis, and settling, in light and darkness. Samples were fixed for later morphological and cytological study.

4. Observations made on cell viability (the oxygen electrode was used by L. Fall to measure photosynthesis) indicated that a marked increase in protoplasm viscosity of the cell contents was associated with cell death. Living cells, in 5 microlitres of buffered sea water under a coverslip, when pressed with a 2 kg

weight for 10 sec., squashed completely, whereas the contents of dead cells, apparently congealed to a rubbery consistency, retained their spherical form under pressure. Since cells expressed in buffered sea-water (kept at a pH value above 7) remained squashable, this was routinely used for cell preparations (below).

5. Attempts were made to culture Prochloron in sea-water media, enriched with low concentrations of ammonium chloride and sodium glycerophosphate and supplemented with various concentrations of sodium bicarbonate or Tris buffer (pH 8.0). Eighty tubes with various combinations of these supplements, inoculated with various numbers of cells ($1-10^4$/tube) were incubated in diffuse daylight (ca. 2500 lux) at 30°. Another set of 80 tubes, set up with sterilized nutrient sea water, was supplemented with various concentrations of catalase, cysteine, glutathione and a sterile-filtered extract of host tissue (Lissoclinum patella), and inoculated with single Prochloron cells isolated by micromanipulation on agar. (About 70% proved bacteria-free.) So far no sign of growth has appeared in any tube (after 3 weeks).

6. For eventual studies of various enzymes, Prochloron cells from L. patella were disrupted by hypotonic media (Tris, EDTA and NaCl, each 0.01 M) or by ammonium formate (5%, approx. isotonic with sea water). One passage through a French pressure cell (squirting Prochloron suspensions from 3-4 MPa to atmospheric pressure) completely disrupted more than 99% of the cells. These lysates were frozen or lyophilized for later analyses of nucleic acids, enzymes and sulphydryl compounds.

7. In addition to the above, whole animals (didemnid + algal symbionts) and expressed Prochloron cells were treated and preserved in various ways for later studies. A total of about 130 scintillation vials were prepared; most contained 50-500 µg of cells. Vials included untreated frozen didemnids or algae, frozen or frozen and lyophilized; acetone powders of Prochloron; cells boiled briefly (to inactivate nucleases) before lyophilization; etc. (A tabular summary of these preparations is appended.) They will be distributed to various colleagues, who have solicited such materials for special studies in laboratories around the World. Results of their collaborative analyses should be forthcoming within the next year.

Summary of Prochloron/Didemnid samples from IPE-VII

		Frozen	Lyophilized	Otherwise Preserved
Lissoclinum patella	whole	2	3	
	expressed	(ca. 10 kg)		
	Prochloron cells		27	12
	lysed	4	6	2
	French-pressed	5	6	
	extracted		4	1
Lissoclinum voelzkowii	whole	1	2	
	Prochloron cells		6	
Lissoclinum punctatum	whole	-		
Trididemnum cyclops	whole	2	3	1
	Prochloron cells		5	
Trididemnum clinides	whole	12		
Diplosoma virens	whole		3	
	Prochloron cells		1	
Diplosoma similis			3	1
	Prochloron cells		6	
Didemnum molle	whole		4	
	Prochloron cells		3	1
Didemnum spp. (not symbiotic)	whole		2	
TOTAL		27	84	18

C-2

N84 20123

I.P.E. VII

(Some of this might be included in the section on Materials and Methods:)

The cells of Prochloron (which has not yet been successfully grown in laboratory culture) were obtained and prepared in the following way. Colonies of the symbiotic host, the giant didemnid ascidian Lissoclinum patella, were collected by L. Cheng and R. A. Lewin at low-tide level on reef-flat sand between Kamori Island and Koror, Palau, Western Caroline Islands, in Feb.-Mar. 1982. The animal colonies were taken, immersed in sea water, to an 8,000-litre holding tank in the Micronesian Mariculture Demonstration Centre and kept with constantly running sea water at 30°, in which they remained healthy for several days. Individual colonies were picked clean of contaminants (chiefly gravel and segments of Halimeda), rinsed in sea water buffered with 40 mM or 100 mM Tris buffer at pH 8.4, and squeezed by hand to express the algal cells from the cloacal atria. The algae were received in about an equal volume of the same buffered sea water; this neutralized the acids liberated by the bruised ascidians and thereby maintained the pH high enough to keep the algal cells green. (Without such buffering, the pH dropped below 6, as noted also for other ascidians [Stoecker, 1980: Mar. Ecol. progr. Ser. 3, 257-265], and the algae tended to die within a few minutes and turn brown due to the production of phaeophytin [Thorne, Newcomb and Osborn, 1977: P.N.A.S. 74, 575-578].) The Prochloron cells were washed twice with buffered sea water and concentrated by centrifugation at about 50 g for 90 seconds. Microscopic examination revealed that contamination by animal host cells or bacteria was negligible (much less than 1%).

(INVESTIGATOR: Please check this on your sample after rehydration.) The supernatant solution was carefully decanted, and the cell paste (1-5 ml), in glass or polypropylene vials, was frozen to -20° overnight. The contents were then dried in a Virtis L-100 lyophilizer under low pressure (130 mtorr) for 15-20 hours. (Occasional power failures may have somewhat interfered with the freeze-drying process in some samples.) The vials were then quickly capped, sealed with conforming tape, and stored at -20° except during transportation by air between Palau and La Jolla. Henceforth they have been kept in darkness at -20°.

When you publish the results of your research on this material, please include among the acknowledgements something on the following lines:

> The Prochloron material was collected and supplied by Drs. L. Cheng and R. A. Lewin, with occasional help from other members of the 7th International Prochlorophyte Expedition, Palau, Western Caroline Islands (Feb.-Mar., 1982), using facilities of the Micronesian Mariculture Research Centre at Koror. The I.P.E. VII received financial support from the National Aviation and Space Administration (Planetary Biology Section), under grant No. NAGW-181 to Ralph A. Lewin, Scripps Institution of Oceanography A-002, University of California, La Jolla, CA 92093 U.S.A.

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Prochloron Expedition

C. Stephens

N84 20124

I. Rationale

Prochloron requires a source of N. It can obtain this from its didemnid symbiont as NH_4^+ or amino acids or both. It may obtain it from sea water as do free-living algae (FAA), but this is not likely to be the major source (biomass considerations).

II. Methods and Observations

1. Influx of ^{14}C-glycine into:

   a. symbiont pair,

   b. Prochloron, and

   c. aposymbiotic didemnids

2. Net influx of asp, ser, ala, gly into a, b, c (as above).

3. Net production or influx of ammonia (as in #1).

4. Availability of NH_4^+ and FAA to the symbiont pair from natural sources.

   a. free in water column

   b. at sediment-water interface

c. in interstitial water of sediments

5. Rate of incorporation of ^{14}C-glycine into protein and other compounds in Prochloron.

Techniques for #1 and #5 are scintillation counting, solvent fractionation, and TLC. Technique for #2, 3, 4 is HPLC analysis.

III. Samples collected.

1. Influx and net influx for all local didemnids.

2. Influx, net influx and incorporation for Prochloron.

3. Environment samples from:

MIDC site,

Nikko Palau Hotel site.

IV. Results

To be obtained at U.C., Irvine.