

INCORPORATION OF H³-THYMIDINE INTO CHLOROPLAST DNA OF MARINE ALGAE

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ABSTRACT

The chloroplasts of three genera of marine algae, *Dictyota*, *Padina*, and *Bryopsis*, were labeled with tritiated-thymidine for various time periods during culture in "Erd-Schreiber's" solution. Autoradiographs were prepared from both smeared and sectioned material. They revealed that almost all of the radioactivity was in the cytoplasm and associated with the chloroplasts, as detected in the overlying silver halide crystals. Deoxyribonuclease, ribonuclease, and hot trichloroacetic acid treatments indicated that the loss of radioactivity corresponded to the removal of DNA and not RNA. Quantitative studies of silver grain distribution suggested that the radioactivity of the labeled DNA originated from the edge of the pyrenoids on either side in the longitudinal direction of *Bryopsis* chloroplasts. Nuclei did not incorporate H³-thymidine even though cells were dividing rapidly in the three genera examined. It is postulated that the enzyme, thymidine kinase, is absent as a coding sequence of nuclear DNA in algae, but is present in chloroplast DNA. When the chloroplasts of *Dictyota* and *Padina* in various stages of division were scored for labeling, there appeared to be a DNA synthesis period, analogous to S period in cell division. This chloroplast-labeling period occurred just previous to fission. Many of the criteria seem to have been satisfied to establish the self-reproducing and semi-autonomous nature of chloroplasts, especially when combined with the chemical, genetic, and morphological evidence.

INTRODUCTION

Chloroplasts of algae were observed to divide and to possess many unique properties by nineteenth century cytologists and physiologists. These observations led to the development of an explicit hypothesis, now termed the Meyer-Schimper hypothesis. A wide variety of plastids throughout the plant kingdom were examined independently by Meyer in 1883 (10) and Schimper in 1883 and 1885 (17, 18). In general, the hypothesis states that chloroplasts are derived from each other by division and possess a variety of autonomous chemical and developmental properties.

Not long after the rediscovery of Mendel's paper, Correns (3) in 1909, one of the rediscoverers, recognized the difference between nuclear and cytoplasmic inheritance while ex-

amining the "status albomaculatus," which results in the green and white sectoring of plants. The mode of this inheritance depended on the presence or absence of plastids in the maternal cytoplasm. Further studies have provided many substantial cases for cytoplasmic heredity as clearly demonstrated by Rhoades (13), Michaelis (11), Sager and Ramanis (16), and others cited by Granick (8, 9).

Current dogma states that the chemical basis of heredity is DNA (or RNA in many viruses) and that this nucleic acid must be present in a structure to accomplish an autonomous or semi-independent reproduction. Following this tenet, one would expect to find DNA in chloroplasts, if they are indeed self-replicating. Nucleic acids have

been detected in small amounts by many workers in cellular fractionation of chloroplasts (8, 9). Baltus and Brachet (1) and Gibor and Izawa (5) found DNA in the chloroplast of enucleate *Acetabularia*. Only in the last few years have chemical methods improved to the point at which nuclear-DNA contamination could be excluded on the grounds of differences in base composition. Recently Chun, Vaughan, and Rich (2) isolated DNA from enriched chloroplast preparations from the leaves of two higher plants, *Beta* and *Spinacia*, and from two algae, *Chlamydomonas* and *Chlorella*. This distinctive DNA, termed beta and gamma DNA, was shown to possess considerably higher percentages of guanine plus cytosine than nuclear DNA. A specific chloroplast DNA with similar base composition was reported by Sager and Ishida (15) for the chloroplasts of *Chlamydomonas*. It is termed satellite DNA by the latter investigators and amounts to about 6 per cent of the total DNA of the cell. Ris and Plaut (14) have demonstrated Feulgen-positive regions on either side of the pyrenoid in *Chlamydomonas*. They found that the characteristic green fluorescence from the complex of DNA and acridine orange was obtained in this same region of the chloroplast in which they also observed DNA-like fibrils adjacent the pyrenoid in electron micrographs.

The incorporation of tritiated-thymidine into the chloroplast of *Spirogyra* was first observed by Stocking and Gifford (21) as indicating incorporation into DNA. The experiments presented here concern enzymatic and acid hydrolysis, to test whether the tritium is in DNA. Attempts are made to localize the source of radioactivity in the chloroplasts and to find the time of incorporation into dividing chloroplasts.

As Schimper (18) pointed out, the large chloroplasts of *Bryopsis* and *Padina* divide much like a cell divides. The labeling data to be presented suggest that nuclear-like DNA synthesis may occur in chloroplasts before their fission, analogous to chromosome duplication before cell division.

MATERIALS AND METHODS

The preliminary experiments were done with *Bryopsis plumosa* (Chlorophyceae, Siphonales) collected in Buzzard's Bay, Massachusetts. Further studies were done with *Bryopsis pennata* collected at Tucker's Town Bay, Bermuda. The experiments with brown algae were done with *Padina* species and *Dictyota dichotoma* (Dictyotales) collected at various locations on St. George's Island and Tucker's Town Bay,

Bermuda. The identification of the marine algae was done with the aid of a recent classification by Taylor (22).

The algae were cultured in "Erd-Schreiber's" solution (19) made with filtered seawater. After the algal cultures were established, frequent subculturing was employed to reduce any bacterial and diatom contamination. Individual fronds were examined under a stereomicroscope for growth rate and lack of surface contamination. The appearance of new cells could be determined directly. The amount of thymidine-methyl- H^3 (Schwarz BioResearch, Inc., Orangeburg, New York; specific activity 3 to 6 C/mm) in the "Erd-Schreiber's" solution when the algae were labeled from 1 to 10 $\mu\text{C}/\text{ml}$.

Most of the algae used for sectioned material was fixed in 10 per cent formalin in seawater. Some fixation was done in alcohol-acetic acid (3:1) but this fixative was unsatisfactory for the preservation of chloroplasts and cytoplasm. The removal of RNA was accomplished with ribonuclease A, RAS (Worthington Biochemical Corp., Freehold, New Jersey). The reaction was carried out in saline-citrate buffer at pH 7.0 for a period of 2 hours at 37°C. Removal of DNA was done with deoxyribonuclease I (Worthington Biochemical Corp.) using 10 mg per 100 ml of solution, containing 0.003 M magnesium sulfate at pH 6.0, reacting for 2 hours at 37°C. The removal of both RNA and DNA was accomplished with 5 per cent trichloroacetic acid (TCA) in a boiling water bath for 15 minutes. After exposure and processing of autoradiographs, all of the algal smears and sections were processed by the azure B method, at a pH of 3.4 or 4.0, for distinguishing both nucleic acids. Squash preparations of chloroplasts were made by fixing slides overnight in formalin vapor, a simple method since free, green chloroplasts could be observed directly before fixation.

Autoradiographs were prepared by the dipping method using two nuclear emulsions: the first emulsion was Ilford Nuclear Research Emulsion-type G.5, prepared as a 1:1 dilution by weight with distilled water and the mixture heated to 45°C in a water bath. The second emulsion type employed was Kodak Nuclear Track Emulsion, NTB, used undiluted and melted at 40°C. Each emulsion was stirred and poured into a flat tube. Two slides, back to back, were dipped into one of the mixtures, inverted, dried, and then stored at about 5°C. After exposure, the slides were developed in a Kodak D19 at 20°C and cleared in Kodak fixer approximately twice the time to clear.

RESULTS

At first, these experiments were initiated to label meiotic chromosomes in order to examine the mechanism of chromatid exchange with crossing-

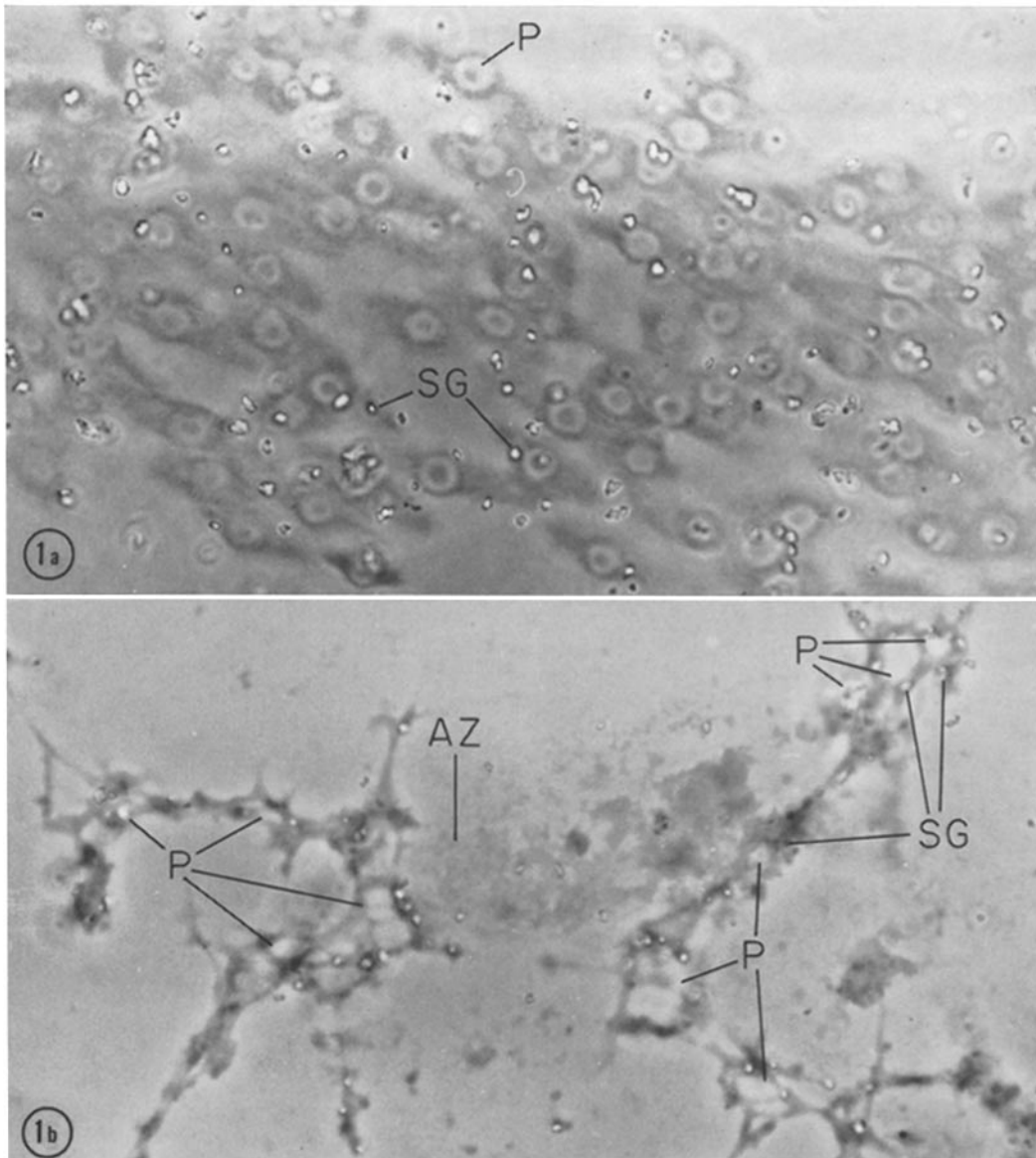


FIGURE 1 Smears of chloroplasts labeled with H^3 -thymidine. *a*, *Bryopsis* chloroplasts with pyrenoids (*P*) and silver grains (*SG*). $\times 1,700$. *b*, *Dictyota*, showing silver grains (*SG*) over chloroplast material and near pyrenoids (*P*), while the basophilic regions (*AZ*), stained with Azure B, are unlabeled. $\times 825$.

over. However, as it will be shown, no incorporation into nuclear-DNA was observed with H^3 -thymidine even though cells were in rapid division in these and later experiments. During these preliminary experiments with *Bryopsis plumosa* at Woods Hole, it was observed that the cytoplasm became labeled.

Further study on the nature of the cytoplasmic

labeling was done at Bermuda the following year. Examples of cytoplasmic labeling, appearing in smear preparations, are shown in Fig. 1 *A* from chloroplasts of *Bryopsis pennata* in Figs. 1 *B* and 3 from chloroplasts of *Dictyota dichotoma*. The radioactivity from tritium beta particles, other than an occasional silver grain attributed to background, was in close proximity to the pyrenoids.

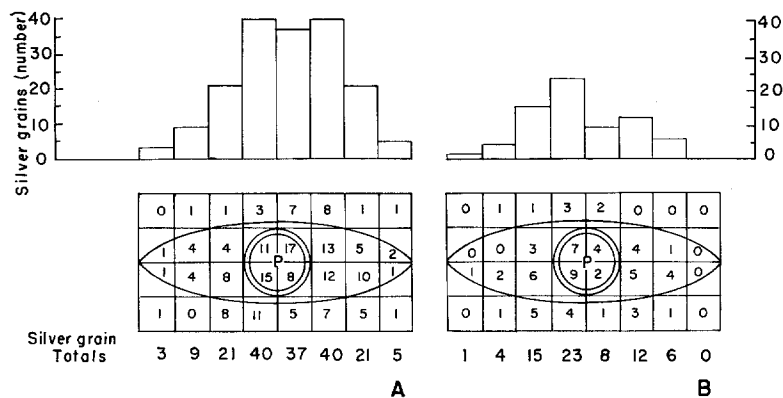


FIGURE 2 The histograms (A and B) show the distribution of radioactivity (silver grains) in smears of chloroplasts of *Bryopsis* labeled with H^3 -thymidine. The data were obtained by projecting Kodachrome lantern slides, and interposing a grid. The distribution of radioactivity appears to closely associate with the pyrenoid, perhaps on either longitudinal edge.

The cytoplasmic label seemed to be associated only with the chloroplasts. An analysis of the distribution of the silver grains over *Bryopsis* chloroplasts is provided in Fig. 1 B. These data were obtained by projecting Kodachrome slides on a screen and interposing a grid oriented with the long axis of the chloroplast, as indicated in the Fig. 2. The two histograms (Fig. 2) show silver grains to be closely associated with the pyrenoid, the peaks occurring at each side. The double peak is probably real because chloroplasts with 2 or more silver grains showed a fit to a 1:1 left to right distribution from the middle of the pyrenoid, with an absence of asymmetric distributions, such as 5:0 or 4:0 in the sample of 18 chloroplasts.

The growth of *Dictyota* is more favorable for experimental purposes than that of *Bryopsis* since numerous small fronds will regenerate from a cut piece of mature frond of *Dictyota*. These rapidly dividing cells are a necessity for labeling studies. The question arises whether there is a synthetic period (S) in the dividing chloroplast similar to that in the dividing cell. In order to test this possibility, an arbitrary numerical sequence of stages of chloroplast division from 0 to 6 was set up and is given in Fig. 3 and Table I. Stage 0 shows no sign of chloroplast enlargement or division, while stages 1 through 5 are progressive stages from enlargement to fission. The chloroplast data in Fig. 3 and Table I were obtained from rapidly dividing fronds labeled for 24 hours with H^3 -thymidine and fixed immediately. The chloroplasts in stage sequences 3, 4, and 5 are definitely

labeled in Fig. 3, and the quantitative data in Table I support this conclusion. Probably no incorporation of H^3 -thymidine occurs in chloroplasts at stages 0 and 1, while chloroplasts in stages 2, 3, 4, and 5 are labeled. Stage 3 is likely to be the middle of the incorporation period (S). The most heavily labeled chloroplasts were in stage 4, indicating the completion of a full period of DNA synthesis.

A similar analysis was done with another brown alga, *Padina*, whose chloroplasts show a slightly different mode of division. Autoradiographs were prepared from sectioned material. After scanning the processed slides, it was obvious that only chloroplasts in the process of fission incorporated H^3 -thymidine. Mature, non-dividing chloroplasts were unlabeled and at background radiation levels. Regions of the thallus containing dividing chloroplasts were selected, and then all of these chloroplasts were scored for overlying silver halide crystals to obtain the data in Table II. The chloroplasts of *Padina* are asymmetrical at stage 2. Almost all of the radioactivity above background is at the pyrenoid side of these asymmetric chloroplasts. The chloroplast DNA must be located in the pyrenoid region, probably at the periphery as in *Bryopsis* and *Chlamydomonas* (14). From Table II, the synthetic period in *Padina* appears to start in stage 1 and continue through stage 2.

Cytochemistry

It goes without saying that H^3 -thymidine could be incorporated into compounds other than DNA.

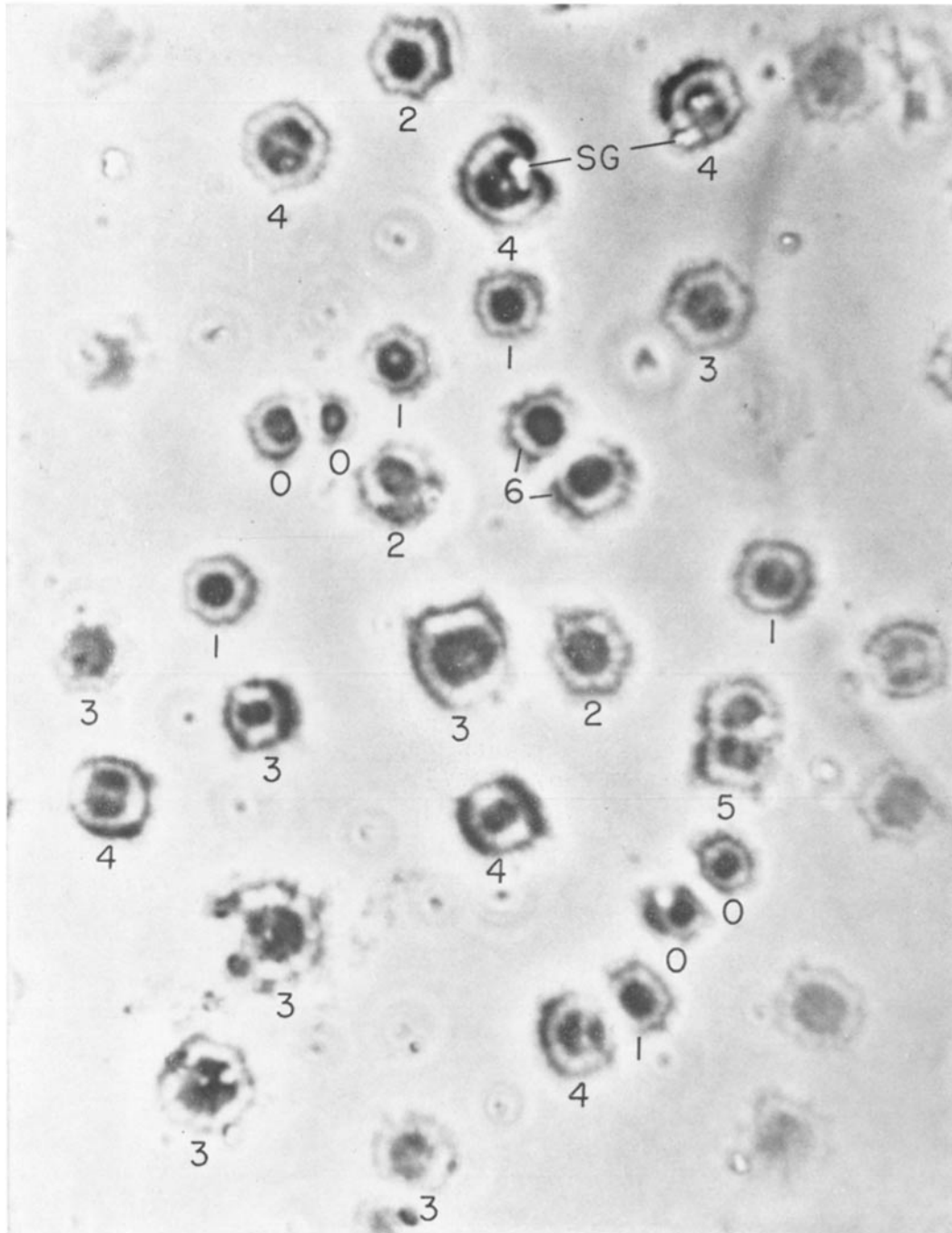


FIGURE 3 Smear of *Dictyota* showing H^3 -thymidine-labeled chloroplasts in division. The silver grains (SG) generally appear as white spots. The sequential stages of division are indicated by the numbers 0 through 6, with 0 being the products of division, 1 through 5 the progressive stages, and 6 the completing of division. Note the furrow forming across the chloroplast in stage 4, which is more evident at stage 5 and is completed by stage 6. In general, stages 3, 4, and 5 are most radioactive while stage 0 is unlabeled. $\times 3,500$.

TABLE I
Distribution of Silver Grains over Dividing Dictyota Chloroplasts after 24 Hours in H³-Thymidine as Analyzed from Smears













No. of silver grains per chloroplast	Numerical classes indicating different stages of division					
	0	1	2	3	4	5
						
0	24	29	15	5	0	0
1	4	12	11	3	0	1
2	1	3	16	7	0	2
3		2	7	6	2	0
4			3	6	2	2
5			3	1	4	0
6					4	1
7					1	
Total chloroplasts	29	46	55	28	13	6
Average number of silver grains per chloroplast	0.21	0.52	1.38	2.28	4.46	3.17

TABLE II
*Distribution of H³-Thymidine Radioactivity from Dividing Padina Chloroplasts Examined in Sections**

No. of silver grains	Numerical classes indicating different stages of division					
	0	1	2	3	4	5
						
0	47	13	1	0	1	1
1	6	18	10	2		
2		5	4	3		
3		1	2	1		
4			1			
5			1			
Total chloroplasts	53	37	19	7	1	1
Average number of silver grains per chloroplast	0.11	0.97	1.74‡	2.14	0	0

* Labeled for 24 hours in 3 μ c H³-thymidine/ml of solution, fixed in formalin-seawater, and sectioned. Only silver grains over the chloroplasts and within a micron radius from the pyrenoids were recorded.
 ‡ Silver grains are almost exclusively over the pyrenoid side of these chloroplasts at this asymmetric stage.

To rule out this possibility, a series of enzymatic and acid hydrolysis studies were done. Adjacent sections made from the same labeled *Dictyota* fronds were randomized among all four types of treatments—DNase, RNase, hot 5 per cent TCA, and buffer controls. The *Dictyota* tissue had been labeled for 12 hours and fixed immediately.

The first test was to see whether H^3 -thymidine could be converted into labeled RNA. Darkfield illumination was employed in order to distinguish silver grains from ovoid granules, the latter perhaps being storage bodies, probably of polysaccharide composition. It is apparent from Table III that the enzyme, RNase, removes little if any of the label. It is certain that RNA was removed, since the usual dark blue staining of the RNA-azure B complex from nucleoli and the cytoplasmic basophilia was absent, while DNA nuclear staining remained. Additional determinations on the effectiveness of both RNase and DNase were done by using smears of *Vicia faba* roots or sections of *Lilium* ovaries in the same reaction mixtures.

The radioactivity from H^3 -thymidine-labeling of chloroplasts was removed with DNase and hot 5 per cent trichloroacetic acid, indicating without much doubt that the label was incorporated into chloroplast DNA (Table III).

TABLE III
Cytochemical Treatments of Dictyota Cells Indicating Loss of H^3 -Thymidine Label from Chloroplasts with DNase and Hot Trichloroacetic Acid Hydrolysis

Treatment	Slide	Average number of silver grains per unit area*	Cell growth stage
Buffer controls	1	11.6	Enlarging
	2	12.7	
	3	24.4	
RNase	1	9.2	Enlarging
	2	27.1	
DNase	1	1.78	
	2	1.04	
Hot 5% TCA	1	1.04	Enlarging
	2	1.0	

* Unit area of cytoplasm approximately $230 \mu^2$. All slides were exposed for the same time period of about 8 months.

In all of the experiments with three genera of algae, nuclear-DNA labeling was never detected, even though high specific activity thymidine at high concentrations was used and some autoradiographs exposed as long as a year.

Some of the developmental aspects should be considered regarding the chloroplast during cell division and differentiation. The three cell-layer thallus of *Dictyota* grows from single apical cells. The peak of chloroplast labeling occurs in the middle cell layer just after the daughter cells have been cut off from the apical cell and when these proximal cells begin rapid enlargement (Table III). Within the cytoplasm itself, the most active sites of thymidine incorporation occur in the zones of highest chloroplast concentration, particularly surrounding the nucleus. The upper and lower layers of epidermal-like cells were never labeled as might be expected since no chloroplast development was observed.

DISCUSSION

Chloroplasts of algae seem to go through synthesis, enlargement, and division just like a cell. Although a detailed analysis is required for substantiation, it would appear that H^3 -thymidine is incorporated into DNA during an interphase-like synthesis period, referred to as S, followed by division and assortment of the label to daughter chloroplasts.

The probable location of DNA in the marine chloroplasts studied compares favorably with the ultrastructural and staining sites for DNA fibrils immediately adjacent the pyrenoid in *Chlamydomonas* as shown by Ris and Plaut (14). Gibor and Granick (6) have gone further to suggest that both chloroplasts and mitochondria have chromosomes, a conclusion brought forth from a variety of evidence. The data presented here tend to support the hypothesis that chloroplasts have chromosomes.

At first sight, the failure to incorporate tritiated thymidine (thymidine-methyl- H^3) into the nuclei of *Spirogyra* (21), *Bryopsis*, *Padina*, and *Dictyota* would appear unusual. However, this was the case in the fungi, *Elythroderma* (7) and *Uromyces* (20), and the failure to take up thymidine-methyl- H^3 in *Neurospora* was shown to be due to the absence of an enzyme which phosphorylates thymidine to thymidylic acid (4). The main pathway to thymidylic acid into *Neurospora* DNA includes the methylation of uridylic acid. Thymidine, however, is demethylated and channeled into nucleo-

tides for both RNA and DNA synthesis, and thereby loses the tritium as a methyl group label. Tritiated uridine and labeled amino acids were taken up by *Neurospora* with no difficulty. According to Okazaki and Kornberg (12), the enzyme, thymidine kinase, is never an intermediate in the biosynthesis of DNA *de novo*, but is a "salvage" mechanism for thymidine by direct phosphorylation. One might expect to show the lack of this enzyme in the aforementioned genera of algae, at least absent from their nuclear gene control but regulated by a postulated "chloroplast chromosome." Most bacteria can incorporate thymidine (12), and one might imagine this

relationship to be connected to the previous evolution of the chloroplast from bacteria as suggested by workers at the turn of the century, a hypothesis recently revived by Ris and Plaut (14). This speculation that photosynthetic bacteria gave rise to chloroplasts by a process of endosymbiosis has many interesting connotations in plant and animal evolution.

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