CHLOROPLASTS AS FUNCTIONAL ORGANELLES IN ANIMAL TISSUES

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ABSTRACT

The marine gastropod molluscs *Tridachia crispata*, *Tridachiella diomedea*, and *Placobranchus ianthobapsus* (Sacoglossa, Opisthobranchia) possess free functional chloroplasts within the cells of the digestive diverticula, as determined by observations on ultrastructure, pigment analyses, and experiments on photosynthetic capacity. In the light, the chloroplasts incorporate $H^{14}CO_3^-$ *in situ*. Reduced radiocarbon is translocated to various chloroplast-free tissues in the animals. The slugs feed on siphonaceous algae from which the chloroplasts are derived. Pigments from the slugs and from known siphonaceous algae, when separated chromatographically and compared, showed similar components. Absorption spectra of extracts of slugs and algae were very similar. The larvae of the slugs are pigment-free up to the post-veliger stage, suggesting that chloroplasts are acquired *de novo*. with each new generation.

INTRODUCTION

The occurrence of plant chloroplasts in animal cells was first demonstrated by Kawaguti and Yamasu (1965) in the marine gastropod *Elysia atroviridis*. The evidence that the structures observed were chloroplasts and not endosymbiotic algae was based on electron microscope examination, and on comparison of the plastid structure in the animals with that in the siphonaceous alga *Codium fragile* upon which the animals feed. No evidence was presented to show that the chloroplasts were functional.

Taylor (1968) studied the ultrastructure of chloroplasts in the digestive gland cells of *Elysia viridis*, and showed the ultrastructural similarity between the chloroplasts in the slug and the chloroplasts in *Codium tomentosum*, the alga upon which it feeds.

This paper describes experiments which demonstrate that the marine gastropods *Tridachia crispata*, *Tridachiella diomedea*, and *Placobranchus ianthobapsus* possess chloroplasts in the cells of their digestive diverticula, and that these chloroplasts are functional in the sense that they photosynthetically fix $^{14}CO_2$ and release the products of photosynthesis to the animal tissues. The chloroplasts may be maintained in a functional state for relatively extended periods of time. Evidence that the structures in the animals are chloroplasts is drawn from their ultrastructure and from a comparative study of pigments in the animals and pigments in known siphonaceous algae.

MATERIALS AND METHODS

Collection of Specimens

Tridachia crispata was collected on the shallow reef flat off Port Royal, Jamaica, West Indies. *Tridachiella diomedea* was collected at San José Island, Gulf of California, and *Placobranchus ianthobapsus* was collected from Kaneohe Bay, Oahu, Hawaii. Animals were

brought into the laboratory and maintained in well aerated seawater aquaria. Under no circumstances were animals kept for more than 4 days before experimentation.

Histology and Histochemistry

Many conventional fixatives were used in this study (see Gray, 1964), but Bouin's solution, made up without urea and chromic acid, proved most successful.

Two different techniques were used to dehydrate and clear tissues prior to embedding: (*a*) dehydration in methyl cellosolve (ethylene glycol monomethyl ether) by three changes, 1 hr each, after removal of the fixative, followed by methyl benzoate (two changes, 1 hr each) as a clearing agent and then by benzene (two changes, 1 hr each); and (*b*) dehydration in serial changes of ethyl alcohol and clearing in xylene. Tissues were always embedded *in vacuo* in paraffin (mp 56–58°C). Serial sections 5–7 μ thick were prepared.

Hematoxylin and eosin, Mallory trichrome, Mallory phosphotungstic acid, and hematoxylin were among the general stains used. Toluidine blue and alcian blue were used to detect mucopolysaccharide-like materials. The periodic acid–Schiff (P.A.S.) technique was used to detect the presence of alpha-glycols (Pearse, 1959). The osmium tetroxide method (Gray, 1964) was used to demonstrate the presence of lipids.

Electron Microscopy

Small pieces of tissue were fixed in sodium cacodylate-buffered glutaraldehyde at pH 7.4, at 4°C for 3 hr and washed in cacodylate buffer at pH 7.4 (Bensch et al., 1962; Sabatini et al., 1963, 1964). Tissues were postfixed in phosphate-buffered osmium tetroxide for 2 hr (Lauritis, personal communication), then dehydrated and embedded in Araldite (Luft, 1961).

Sections were cut on a Porter-Blum MT-2 ultramicrotome, and were viewed and photographed with the Hitachi 11B electron microscope.

Photosynthesis

Freshly collected specimens were incubated in millipore-filtered seawater (pH 7.9) in beakers, with $NaH^{14}CO_3$ added to give an initial activity of 10 μc $\times$ ml^{-1}. Constant illumination was delivered by a bank of 40-watt Grolux fluorescent tubes (General Electric Company, Cleveland, Ohio). The temperature was maintained at 25.5°C. Periods of incubation varied from 1 to 5 hr, and control specimens incubated in the dark were included in all experiments.

Oxygen production was determined on *Tridachia* by the Winkler technique, with slight variations of the method described by Strickland and Parsons (1960).

Radioautography

Specimens incubated as above for periods of from 15 min to 9 hr were removed from the ^{14}C-labeled seawater, washed in clean seawater, and fixed. Specimens were dehydrated by one of the methods previously described. Sections of specimens 7 μ thick were prepared for radioautography with Kodak AR-10 stripping film and Kodak NTB2 liquid emulsion, exposed for 4–5 days, and developed as advised by the manufacturer. Control specimens, incubated with isotope in darkness, were processed for radioautography in the same manner.

The reagents used in the preparation of sections for radioautography will remove soluble radioactive substances from the tissues. To determinine the extent of this loss, measurements were made of the ^{14}C extracted at each stage of fixation, dehydration, and clearing (Table I).

TABLE I

Loss of Organic ^{14}C Due to Leaching by Histological Solvents During Preparation of Radioautographs of $H^{14}CO_3^-$ Incubated Tridachia crispata

Reagent		^{14}C lost
		%
Bouin's solution		25
Ethyl cellosolve	1.	7
	2.	4
	3.	2
Methyl benzoate	1.	1
	2.	0
Benzene	1.	0
	2.	0
Total Loss		39

Note: Specimens were fixed, dehydrated, and cleared in known volumes (10 ml) of histological reagents. An estimate of the level of photosynthetic ^{14}C fixation was made by determining the difference between the activity added to the incubation medium and that remaining after a known period of photosynthesis. Per cent loss due to leaching was calculated from $\frac{\text{cpm}}{\text{Ps}}$ (reagent) $\times$ 100. 100 λ samples of each reagent were counted in duplicate. The percentage loss is a minimum estimate due to the possibility of exchange of ^{14}C with the atmosphere and with animal respiratory $^{14}CO_2$.

Paper Chromatography of Tissue Extracts

Following 1–5-hr incubations, whole animals were extracted in 20 ml of hot 80% ethanol until no further radioactivity could be detected in the changes of solvent. The ethanol-insoluble residue was then hydrolyzed in 1 N HCl at 100°C for 3 hr, and alcoholic and HCl extracts were combined and evaporated to dryness. So as to exclude salt, soluble organic ^{14}C was taken up from the residue in dry absolute ethanol, and this extract was stored at −17°C until chromatographed.

Alcoholic extracts were analyzed for labeled organic material by paper partition chromatography in two dimensions on Whatman No. 4 paper (46 × 57 cm), with use of phenol-water (100:30, w/v) for the first dimension, and *n*-butanol-propionic acid-water (142:71:100, v/v/v) for the second dimension. Radioactive compounds were located on chromatograms by radioautography with Kodak single-coated, blue-sensitive medical X-ray film (Bassham and Calvin, 1957).

Pigment Analysis and Characterization

Pigments from *Tridachia crispata* and *Placobranchus ianthobapsus* (the sea slugs) and from *Caulerpa racemosa* var. *uvifera* and *Codium fragile* (Chlorophycophyta: Siphonales) were extracted in cold (3–4°C) absolute methanol under nitrogen gas. The pigments in the methanolic extracts were transferred to diethyl ether in a separatory funnel and 10% aqueous NaCl was added to effect phase separation. The diethyl ether phase was further washed with aqueous salt for removal of any remaining methanol. Petroleum ether (bp 60–80°C) was added to the diethyl ether, and the mixture was washed with distilled water; the water was removed with anhydrous Na_2SO_4. The extracts were then concentrated by evaporation under nitrogen gas. All extraction procedures were conducted in a darkened room, and the flasks containing the pigments were wrapped in aluminum foil to shield the extracts from direct light.

Diethyl ether–petroleum ether samples (100–200) were spotted on thin-layer silica gel-G plates (250 μ thick), dried under nitrogen gas, and developed in the dark by ascending chromatography with 15% hexane in diethyl ether.

Comparisons were made between whole pigment extracts from sea slugs and algae, as well as between individual pigment bands eluted from the thin-layer chromatograms. The pigments from the chromatograms were taken up in diethyl ether, and their absorption spectra were determined on the Cambridge Unicam S.P. 600 and S.P. 700 spectrophotometers, and the Beckman DU spectrophotometer.

RESULTS

Morphology of the Chloroplast Association

The anatomy of the digestive system of sacoglossan opisthobranchs has been described (Fretter, 1940; Yonge and Nicholas, 1940; Marcus and Marcus, 1960, 1962). Briefly, the buccal mass is attached to the stomach by a short esophagus (in *Tridachia* and *Tridachiella* the esophagus possesses an esophageal pouch of unknown function). From the stomach, four major ducts arise and proliferate throughout the body. These ducts constitute the digestive glands or digestive diverticula (Fig. 1 *a*). Transverse sections taken at random through the body always reveal the tubules of the digestive glands in section (Figs. 1 *b* and *c*).

Sacoglossan opisthobranchs characteristically feed on algae by puncturing single cells and sucking out the fluid contents (Fretter, 1940). In so doing, they acquire the algal chloroplasts which become located within the cells of the tubules of the digestive glands (Fig. 2). The plastids average 3 μ in diameter, and appear to be bounded by a double membrane (see Taylor, 1968). They are

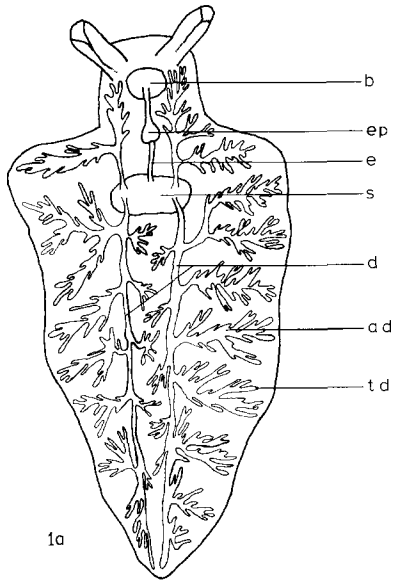

FIGURE 1 *a* *Tridachia crispata*, schematic diagram of the digestive system. *ad*, "absorbing" region of the tubules of the digestive diverticula; *b*, buccal mass; *d*, major duct of digestive diverticula; *e*, esophagus; *ep*, esophageal pouch; *s*, stomach; *td*, terminal bulbs of the tubules of the digestive diverticula. Enlarged about 3 times.

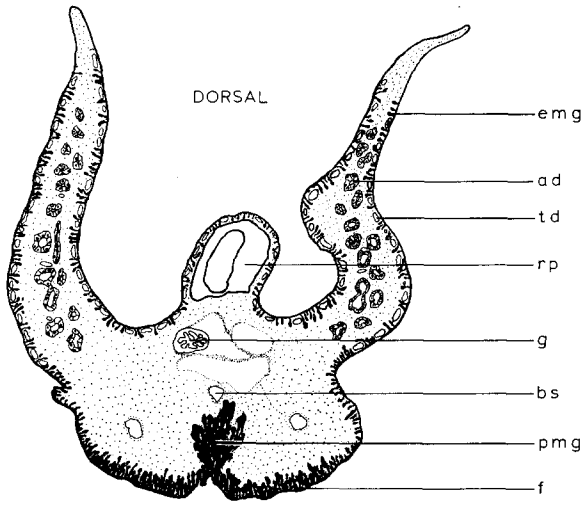

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FIGURE 1 *b* Schematic diagram of a transverse section through the anterior portion of *T. crispata* showing the disposition of tissues referred to in the text. *bs*, blood sinus; *emg*, epidermal mucus gland; *f*, foot; *g*, gut; *pmg*, pedal mucus gland; *rp*, renopericardium. Enlarged about 5 times.

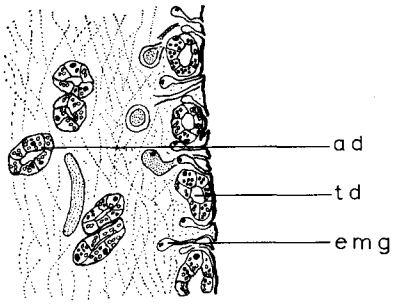

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FIGURE 1 *c* High magnification diagram of the edge of a parapodium. Enlarged about 400 times.

closely associated with what is interpreted as animal cell membranes (Fig. 3), thought to be endoplasmic reticulum. Kawaguti and Yamasu (1965) also observed the association of the chloroplasts with animal cell membranes in the digestive gland cells of *Elysia atroviridis*.

The plastids contain groups of from two to nine closely appressed thylakoids. Osmiophilic granules (probably lipid in nature) were often observed in the plastids, as were other structures which have been provisionally characterized as starch granules.

The size of the plastids, the apparent absence of nuclei within them, and the presence of thylakoid structure bounded by double membranes are consistent with the interpretation that the structures are free chloroplasts.

Origin of the Chloroplasts

The morphology of the feeding apparatus of sacoglossan opisthobranchs indicates that they are adapted "for puncturing plant cells and sucking out their contents" (Yonge and Nicholas, 1940). In the case of *Elysia atroviridis*, the chloroplasts are thought to be derived from *Codium fragile*, a siphonaceous marine alga (Kawaguti and Yamasu, 1965), while in *E. viridis* they are derived from *C. tomentosum*, another siphonaceous alga (Taylor, 1967, 1968).

In an attempt to verify these observations, pigments from *Tridachia* and *Placobranchus* were compared chromatographically and spectrophotometrically with those of the siphonaceous algae *Caulerpa racemosa* var. *uvifera* and *Codium fragile*. Total pigment extracts from the slugs and from the green algae in 80% ethanol showed identical absorption maxima at 416, 435, 465, 650, and 558 mμ. Fig. 4 shows that the pigments separated from the slugs *Tridachia* and *Placobranchus* have the same chromatographic mobility as those separated from the algae *Caulerpa* and *Codium*. It is significant that the chromatographic patterns of pigments separated from the two siphonaceous algae and from the two opisthobranchs are almost identical. Furthermore, xanthophyll pigments characteristic of siphonaceous algae, namely siphonoxanthin and siphonein (Strain, 1965), have been tentatively identified (by R_f values and spectrophotometrically) in each of the animals tested.

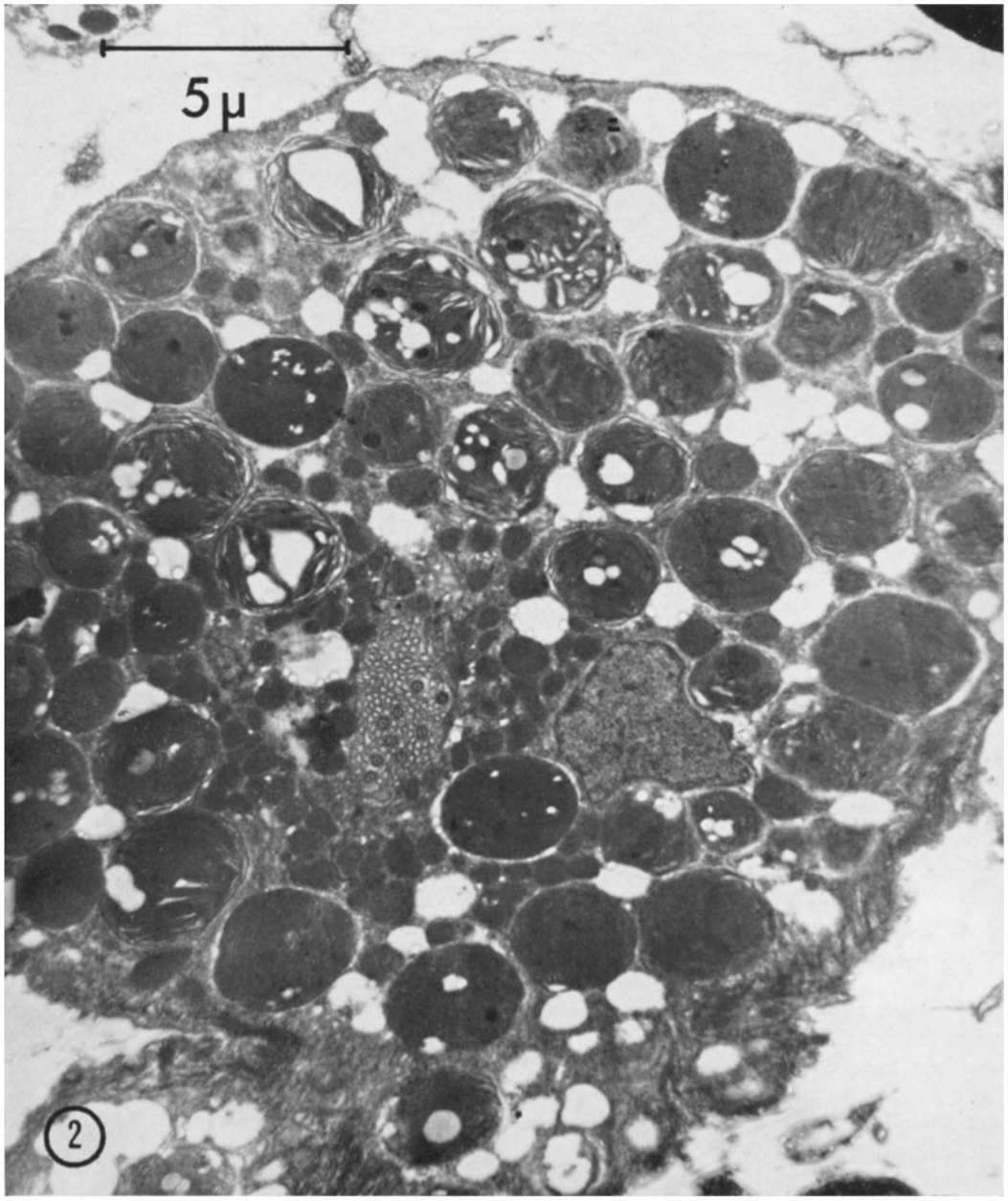

FIGURE 2 Electron micrograph of a slightly oblique section across the terminal bulb of a tubule of the digestive diverticula of *T. crispata*. Uranyl acetate, lead citrate stained. Approximately × 1,500.

Chloroplast-free *Tridachia* have never been observed in the field, and at this time only two specimens of *Placobranchus* have been collected which lacked chloroplasts altogether. Attempts to free the animals of the plastids by dark treatment for periods of up to 6 wk in the laboratory have proven unsuccessful. The eggs of *Tridachia* and *Placobranchus*, observed with the light microscope, appear to be free of chloroplasts. After hatching, the chloroplast-free larvae survive up to the post-veliger stage, and attempts to prolong their survival in the laboratory have not been successful. The attempts

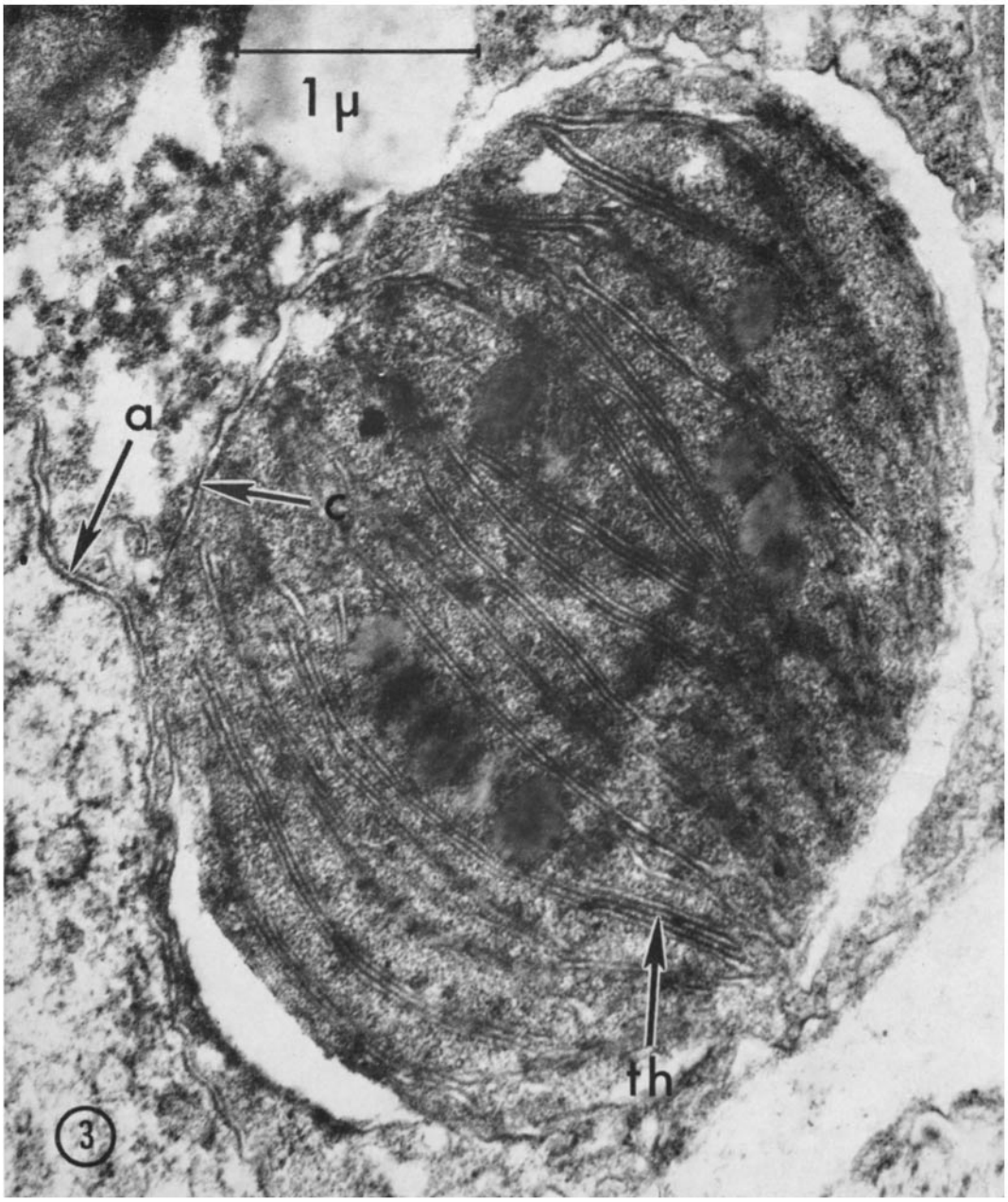

FIGURE 3 Electron micrograph of a single chloroplast in a digestive gland cell of *T. crispata*. *a*, animal cell endoplasmic reticulum; *c*, double chloroplast membrane; *th*, chloroplast thylakoids. Uranyl acetate, lead citrate stained. Approximately × 85,000.

of Kawaguti and Yamasu (1965) to grow the larvae of *Elysia atroviridis* also proved unsuccessful.

These data are consistent with the interpretation that the sacoglossan opisthobranchs *Tridachia crispata* and *Placobranchus ianthobapsus* derive their chloroplasts from siphonaceous algae, and that the chloroplasts appear not to be inherited, but are acquired anew with each generation.

Photosynthetic Capacity of the Chloroplasts in Situ

Preliminary experiments in which the Winkler technique (Strickland and Parsons, 1960) was used established that there was net oxygen production in the test vessel when specimens of *Tridachia* and *Placobranchus* were kept in the light. The slugs also showed a dull red fluorescence under long wavelength (365 mμ) ultraviolet light. These observations are consistent with the presence of chloroplasts in the slugs' tissues. For further demonstration of this condition, whole animals were incubated in seawater with $H^{14}CO_3^-$ in the light for periods of up to 5 hr (with dark controls in all cases), and then extracted in hot 80% ethanol. The ethanol extract was subsequently analyzed by paper partition radiochromatography.

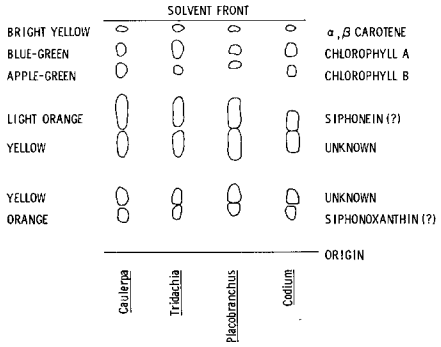

FIGURE 4 Ascending thin-layer chromatogram of photosynthetic pigments from the algae *Caulerpa* and *Codium* (Siphonales) and the slugs *Tridachia* and *Placobranchus* (Sacoglossa). The chromatograms were developed in 15% hexane in diethyl ether.

Radioautographs of the chromatograms showed that the ^{14}C became incorporated into a wide range of metabolic intermediates when the animals were incubated in the light. Although the radioactive compounds separated have not been identified, the pattern of ^{14}C fixation was very similar in many respects to that obtained with photosynthetic plants (Bassham and Calvin, 1957; Muscatine, 1965). Since the level of ^{14}C fixation by the dark controls was only 3–4% of that fixed in the light, these results support the hypothesis that a func-

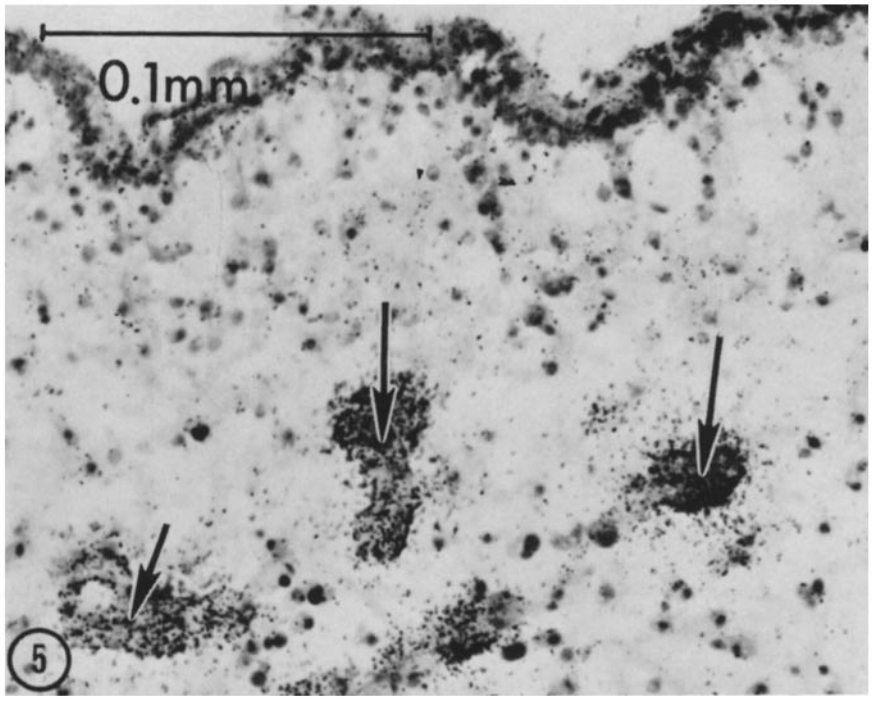

FIGURE 5 Radioautograph of section of parapodium of *T. diomedea* (hematoxylin and eosin stained), showing the uptake of radioactivity by the chloroplasts in the end-bulbs of the tubules of the digestive diverticula after 15 min of photosynthesis in $^{14}CO_2$. Arrows point to the end-bulbs. Approximately × 350.

tional photosynthetic system is present in the animals.

In order to determine in a preliminary manner the functional life-span of chloroplasts in *T. diomedea*, animals were maintained away from a supply of chloroplasts for periods of up to 6 wk, and their photosynthetic capacity was determined by incubation in $H^{14}CO_3^-$ in the light (with dark controls), and subsequent tissue radioautography.

After 6 wk away from a potential source of plastid replenishment, the chloroplasts in *T. diomedea* photosynthetically incorporated ^{14}C, and the label could be followed progressively into chloroplast-free tissues. Dark controls did not incorporate detectable quantities of the isotope.

These observations suggest very strongly that the chloroplasts in *Tridachiella* may have a functional life-span of up to 6 wk in the cells of the molluscan host.

Movement of Photosynthate from Chloroplasts to Host

The possibility that products of chloroplast photosynthesis become available to the animal tissues was examined by radioautography of sections of various portions of chloroplast-free organs of animals which had been previously exposed to $H^{14}CO_3^-$ in the light, with controls incubated in the dark. Specimens of *T. crispata* were sampled hourly for 5 hr: specimens of *T. diomedea* were sampled after 15, 30, 45, 60, 120, 240, and 540 min of incubation.

It is of the greatest importance to stress that the technique involved in preparing sections for radioautography involves the removal of materials soluble in the reagents used, i.e. nearly all the compounds of small molecular weight including those which are involved in intermediary metabolism. Table I illustrates the extent of the loss of ^{14}C from tissues during this process.

When specimens of *T. diomedea* were incubated in the light in $H^{14}CO_3^-$, the first tissues to become labeled were the terminal bulbs of the tubules of the digestive diverticula which contain the chloroplasts. Radioactivity could be detected in this tissue and not elsewhere in the animal tissues, following incubation periods of 15 min, (Fig. 5).

After 60 min of photosynthesis, in both *T.*

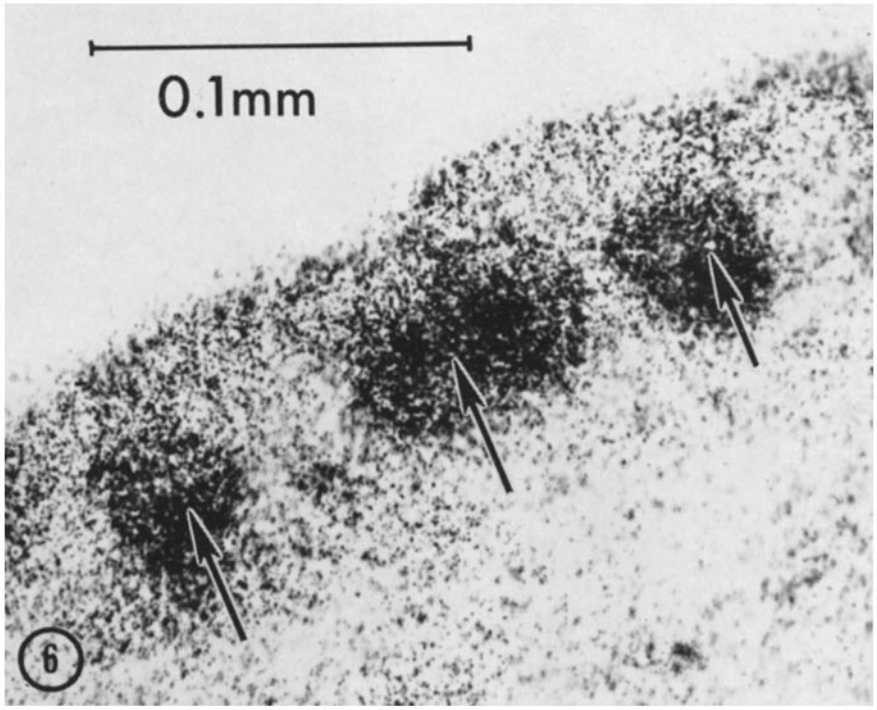

FIGURE 6 Radioautograph of a portion of the parapodium of *T. crispata* (unstained), showing the uptake of radioactivity by the chloroplasts within the end-bulb of the tubules of the digestive diverticula after 300 min of photosynthesis. Arrows point to the end-bulbs. Approximately × 350.

crispata and *T. diomedea*, label was detected in the tubules of the digestive diverticula and in the renopericardium. After 120 min, the incorporation of label into the cephalic neural tissue and the mucus-secreting pedal gland was detectable. After 300–540 min of incubation, the tissues found to incorporate the label were the tubules of the digestive diverticula (Fig. 6), the renopericardium (Fig. 7), the cephalic neural tissue (Fig. 8), particularly the osmiophilic sheaths surrounding the brain ganglia, and the intestine (Fig. 9). The highest level of radioactivity, as judged by the intensity of siliver grain reduction of the emulsion, was associated with the pedal gland (Fig. 10). This gland is PAS positive, shows strong γ-metachromasia with toluidine blue, and stains positively with Alcian blue. Homologous glands in other elysiids synthesize and secrete mucus (Fretter, 1940).

Dark control specimens, incubated and processed the same way as the light-incubated specimens, showed no evidence for high levels of $^{14}CO_2$ fixation, as demonstrated by the radioautograph in Fig. 11.

The foregoing data are consistent with the interpretation that the products of photosynthetic ^{14}C fixation appear to become translocated from their sites of fixation in the chloroplasts to a variety of chloroplast-free animal tissues.

DISCUSSION

The results of this study show that chloroplasts from siphonaceous algae can establish a functional association within animal cells. It is of great significance that the chloroplasts can maintain their structural and functional integrity outside the parent plant cells, suggesting that they can continue to function for a considerable time in a foreign environment if the proper milieu and other

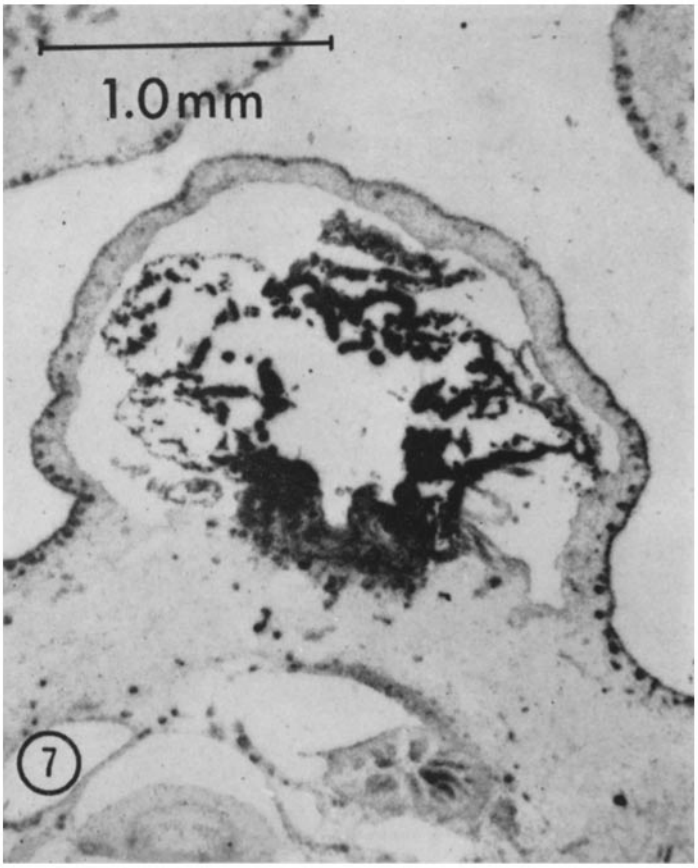

FIGURE 7 Radioautograph of a transverse section through the renopericardium of *T. crispata* (unstained) after 300 min of photosynthesis. Approximately × 30.

conditions essential to plastid metabolism are available.

The only other studies on the morphology of symbiotic chloroplasts are those of Kawaguti and Yamasu (1965) and Taylor (1968). Kawaguti and Yamasu (op. cit.) drew attention to the fact that the chloroplasts are closely associated with the endoplasmic reticulum of the host cells. This has been demonstrated in algae by Gibbs (1962), and by Wooding and Northcote (1965) in higher plants.

The observations of both Kawaguti and Yamasu (1965) and Taylor (1968) show that the plastids are limited by a double membrane, but it is difficult to determine whether the plastids are enclosed in a vacuole limited by animal membranes or whether they lie free in the host cell cytoplasm in intimate association with host cell endoplasmic reticulum. Double membranes associated with the chloroplasts have been detected (Fig. 3), but whether these are host cell endoplasmic reticulum or membranes limiting a vacuole is unclear. High-resolution electron microscopy studies are now in progress to resolve this problem.

The mode of maintenance and the fate of the derived chloroplasts in the cells of the host remain obscure. The lifespan of the chloroplasts in the cells of the digestive diverticula of the slugs is not known. Taylor (1968) found, in the elysiids he studied, that the turnover time was as short as 24 hr. However, our data show that in *Tridachiella* the chloroplasts remain functional for at least 6 wk after the animal has been removed from a potential source of plastid replenishment. Again, J. K. Testerman (unpublished), on the basis of oxygen production, found that the chloroplasts in *Placobranchus* also continue to function after animals have been "starved" for up to 6 wk. Aspects of the

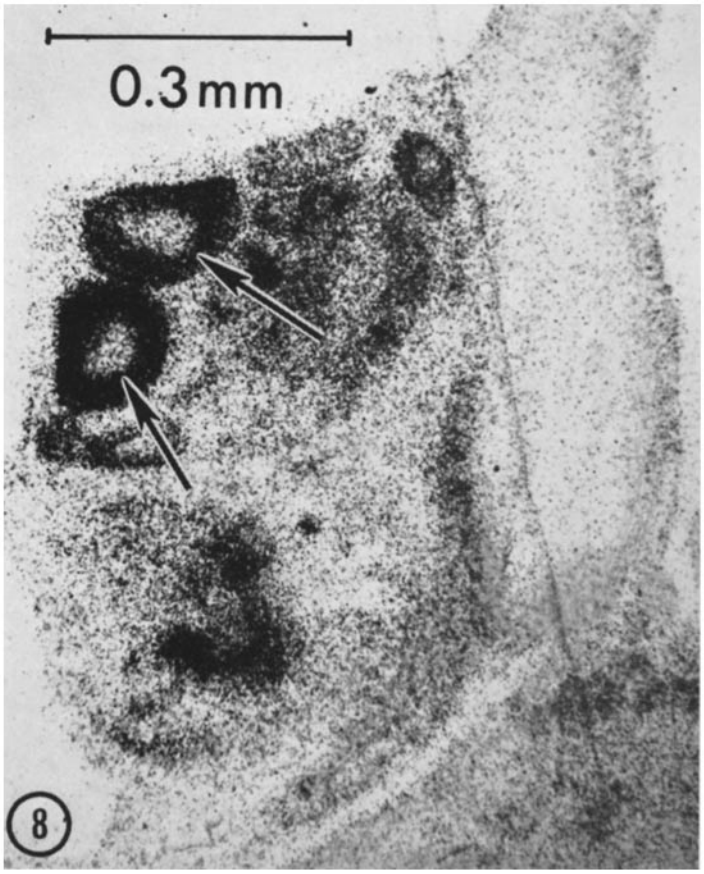

FIGURE 8 Radioautograph of a section through the brain mass of *T. crispata* (unstained). Arrows point to the osmiophilic sheaths surrounding the nerve ganglia. Approximately × 150.

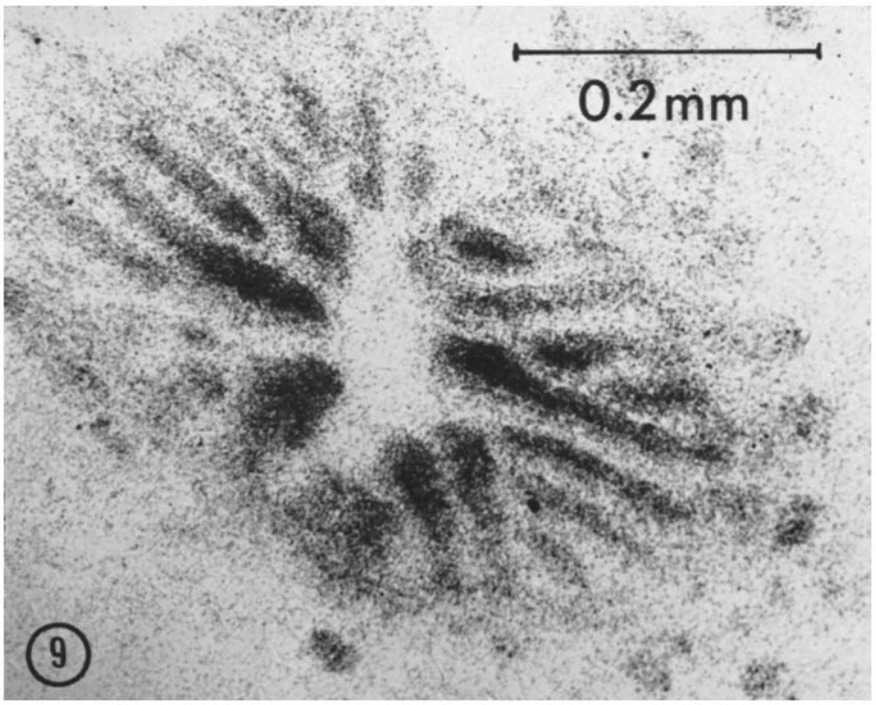

FIGURE 9 Radioautograph (unstained) of a section through the gut of *T. crispata* after 300 min of photosynthesis. Most of the label appears over the folded gut epithelium. Approximately × 150.

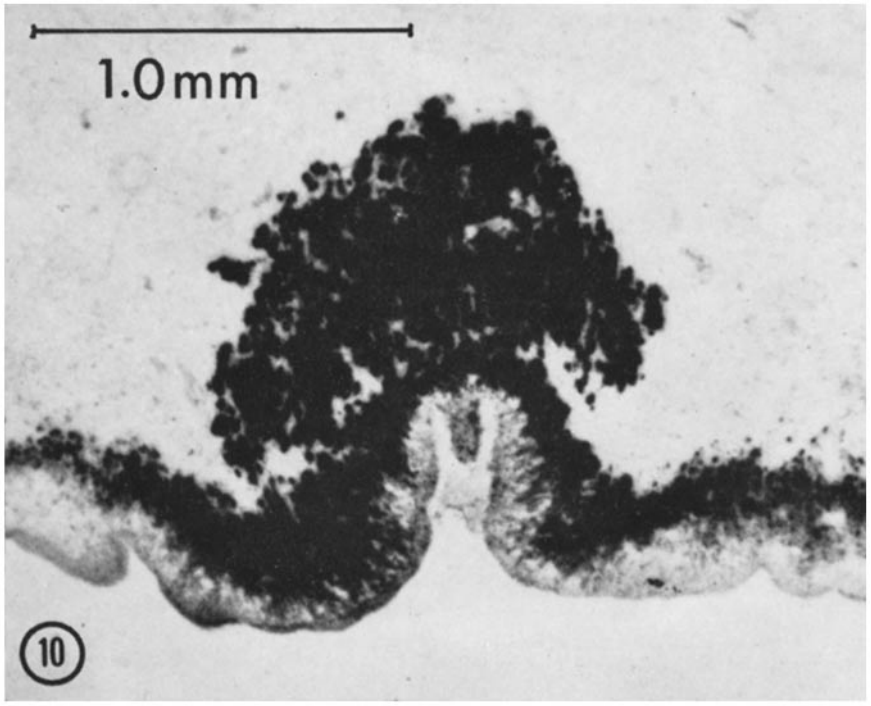

FIGURE 10 Radioautograph of a section through the mucus-secreting pedal gland of *T. crispata* (unstained) after 300 min of photosynthesis. Approximately × 30.

comparative survival time of chloroplasts in different slugs are currently under study by R. W. Greene.

There is no evidence that the chloroplasts are being digested. Taylor (1968) speculates that in *Elysia* the reason for this may be "the unspecialized nature of the animal's digestive enzymes, which seem to be intended more for the breakdown of cell sap than for action on more complex structures such as the chloroplast membranes." The moribund appearance of some chloroplasts in electron micrographs prompted Kawaguti and Yamasu (1965) to suggest that the chloroplasts in *Elysia atroviridis* were being digested. Without concomitant biochemical evidence, such a conclusion, based on structural appearances alone, is not yet warranted.

The separation by thin-layer chromatography of chlorophylls *a* and *b* from the chloroplasts in the slugs is evidence that the plastids originated from a green alga. That the chloroplasts also contained the xanthophylls siphonoxanthin and siphonein is highly suggestive that the chloroplasts are of siphonaceous algal origin. The close resemblance of the chromatographic patterns of the animals' chloroplast pigments and the algal chloroplast pigments, bearing in mind the animals' mode of feeding, indicates that the plastids in the slugs were derived from the siphonaceous algae upon which they feed. Taylor (1968) also separated and identified siphonoxanthin and siphonein from *E. viridis*. The implication that *Tridachia* feed specifically on *Caulerpa racemosa* or that *Placobranchus* feeds on *Codium fragile* is not intended. The precise plants upon which these organisms feed are not known.

The movement of products of photosynthesis from an autotrophic symbiont to the tissues of its host is now a well established phenomenon (Muscatine and Hand, 1958; Goreau and Goreau, 1960; Muscatine and Lenhoff, 1963; Smith and Drew, 1964; Goreau, Goreau, and Yonge, 1965), but its occurrence in chloroplast associations has only recently been reported (Trench, 1969).

There seems to be an interesting parallel in the fate of photoreduced $^{14}CO_2$ in *Tridachia* and *Tridachiella*, and in the giant clam *Tridacna*, in which the symbionts are zooxanthellae (i.e. encysted unicellular Dinophyceae). In all cases, the main target

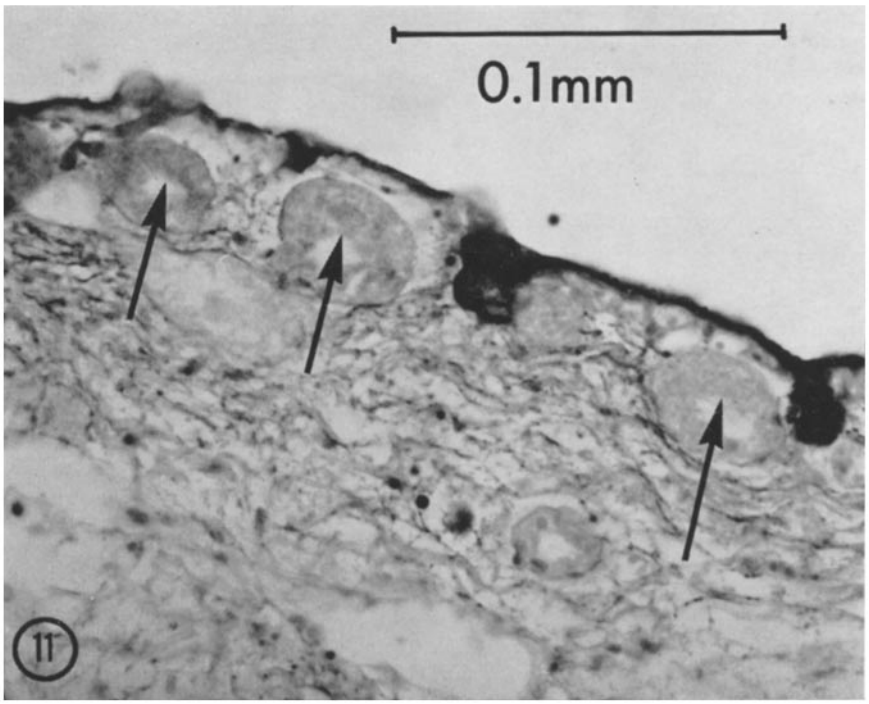

FIGURE 11 Radioautograph of the edge of a parapodium of *T. crispata* (toluidine blue stained), incubated in $^{14}CO_2$ in the dark for 300 min. Arrows point to the end-bulbs of the tubules of the digestive diverticula. Note the absence of evidence for high levels of heterotrophic $^{14}CO_2$ fixation. Approximately × 350.

organ for the photosynthetic products of the autotrophic symbionts appears to be mucus-producing tissues, which presumably have a high metabolic turnover. In *Tridacna*, Goreau, Goreau, and Yonge (1965) have shown that the soluble photosynthetic products of zooxanthellae rapidly appear in the "secretory organs such as the mucus glands, the byssal glands, digestive diverticula, heart and intestine, the epithelium responsible for the secretion of the crystalline style, and the crystalline style itself." The crystalline style in other bivalves is known to be composed of large quantities of mucopolysaccharide (Doyle, 1966). Studies are now underway to determine the labeled moiety in the mucus and in the crystalline style.

A "built-in" source of reduced carbon for those metabolically active tissues may have selective advantages to the host in the long run, and may favor the perpetuation of the association. However, it appears that infection with chloroplasts occurs *de novo* with each generation since the larvae seem to be devoid of the organelles. The possibility that the plastids are passed on as proplastids cannot, however, be ruled out at this time.

The phenomenon described here is consistent with the hypothesis that chloroplasts may have arisen through an originally endosymbiotic association between the free-living predecessors of these organelles and their hosts (see Mereschkowsky, 1905, and Famintzin, 1907, in Ris, 1961). More recently, with the discovery of DNA in chloroplasts (for an historical review, see Kirk and Tilney-Bassett, 1967), the concept has been regenerated that endosymbiotic associations range from organisms which carry on an independent existence to "true cell organelles" (Lederberg, 1952; Karakashian and Siegel, 1965; Ris, 1961; Sagan, 1967).

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REFERENCES

BASSHAM, J. A., and M. CALVIN. 1957. The Path of Carbon in Photosynthesis. Prentice-Hall, Inc., Englewood Cliffs, N.J.

BENSCH, K. G., D. D. SABATINI, and R. J. BARRNETT. 1962. New fixatives for cytochemistry and electron microscopy. Proceedings, 51st Annual Meeting International Academy of Pathology, Montreal.

DOYLE, J. 1966. Studies on the chemical nature of the crystalline style. *In* Some Contemporary Studies in Marine Science. H. Barnes, editor. George Allen and Unwin Ltd., London. 253.

FRETTER, V. 1940. On the structure of the gut of the ascoglossan nudibranchs. *Proc. Zool. Soc. Lond. Ser. B* **110**:185.

GIBBS, S. P. 1962. The ultrastructure of the chloroplasts of algae. *J. Ultrastruct. Res.* **7**:418.

GOREAU, T. F., and N. I. GOREAU. 1960. Distribution of labeled carbon in reef-building corals with and without zooxanthellae. *Science.* **131**:668.

GOREAU, T. F., N. I. GOREAU, and C. M. YONGE. 1965. Evidence for a soluble algal factor produced by the zooxanthellae of *Tridacna elongata* (Bivalvia, Tridacniidae). *Int. Conf. Trop. Oceanography* (abstract).

GRAY, P. 1964. Handbook of Basic Microtechnique. McGraw-Hill Book Co., New York. 3rd edition.

KARAKASHIAN, S. J., and R. SIEGEL. 1965. A genetic approach to endocellular symbiosis. *Exp. Parasitol.* **17**:103.

KAWAGUTI, S., and T. YAMASU. 1965. Electron microscopy on the symbiosis between an elysioid gastropod and chloroplasts of a green alga. *Biol. J. Okayama Univ.* **11**:57.

KIRK, J. T. O., and R. A. E. TILNEY-BASSETT. 1967. The Plastids. W. H. Freeman and Co., San Francisco.

LEDERBERG, J. 1952. Cell genetics and hereditary symbiosis. *Physiol. Rev.* **32**:403.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**: 409.

MARCUS, E., and E. MARCUS. 1960. Opisthobranchs from American Atlantic warm waters. *Bull. Mar. Sci. Gulf Carib.* **10**:129.

MARCUS, E., and E. MARCUS. 1962. Opisthobranchs from Florida and the Virgin Islands. *Bull. Mar. Sci. Gulf Carib.* **12**:450.

MUSCATINE, L. 1965. Symbiosis of Hydra and algae. III. Extracellular products of the algae. *Comp. Biochem. Physiol.* **16**:77.

MUSCATINE, L., and C. HAND. 1958. Direct evidence for the transfer of materials from symbiotic algae to the tissues of a coelenterate. *Proc. Nat. Acad. Sci. U.S.A.* **44**:1259.

MUSCATINE, L., and H. LENHOFF. 1963. Symbiosis: On the role of algae symbiotic with Hydra. *Science.* **142**:956.

PEARSE, A. G. E. 1959. Histochemistry. Churchill Ltd., London. 2nd edition.

RIS, H. 1961. Ultrastructure and molecular organization of genetic systems. *Canad. J. Genet. Cytol.* **3**:95.

SABATINI, D. D., K. G. BENSCH, and R. J. BARRNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19.

SABATINI, D. D., K. G. BENSCH, and R. S. BARRNETT. 1964. Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope. *J. Histochem. Cytochem.* **12**:57.

SAGAN, L. 1967. On the origin of mitosing cells. *J. Theoret. Biol.* **14**:225.

SMITH, D., and E. A. DREW. 1964. Studies in the physiology of lichens. V. Translocation from the algal layer to the medulla in *Peltigera polydactyla*. *New Phytol.* **64**:195.

STRAIN, H. H. 1965. Chloroplast pigments and the classification of some siphonalean green algae of Australia. *Biol. Bull.* **129**:366.

STRICKLAND, J. D. H., and T. R. PARSONS. 1960. A manual of sea water analysis. *Fish. Res. Board Can. Bull. No. 125.* 185 pp.

TAYLOR, D. L. 1967. The occurrence and significance of endosymbiotic chloroplasts in the digestive glands of herbivorous opisthobranchs. *J. Phycol.* **3**:234.

TAYLOR, D. L. 1968. Chloroplasts as symbiotic organelles in the digestive gland of *Elysia viridis* (Gastropoda: Opisthobranchia) *J. Mar. Biol. Ass. U. K.* **48**:1.

TRENCH, R. K. 1969. Symbiosis: functional chloroplasts in the tissues of *Tridachia crispata* (Sacoglossa: Opisthobranchia). *Nature.* In press.

WOODING, F. B. P., and D. H. NORTHCOTE. 1965. Association of the endoplasmic reticulum and the plastids in *Acer* and *Pinus*. *Amer. J. Bot.* **52**:526.

YONGE, C. M., and H. M. NICHOLAS. 1940. Structure and function of the gut and symbiosis with zooxanthellae in *Tridachia crispata* (Oerst.) Bgh. *Pap. Tortugas Lab.* **32**:287.