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Symbiosis in sacoglossan opisthobranchs: functional capacity of symbiotic chloroplasts

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Abstract

It has been previously reported that many species of the order Sacoglossa (Mollusca: Opisthobranchia) contain algal chloroplasts within the cells of their digestive gland and maintain them in a symbiotic condition. In the present study, two species, *Elysia hedgpethi* MARCUS and *Placobranchus ianthobapsus* GOULD, were compared as to their abilities to retain functional chloroplasts in their tissues. Animals were starved for varying lengths of time, and the functional capacity of the plastids was ascertained at intervals. The chlorophyll content of whole animals, and the ability to incorporate $^{14}CO_2$ were used as the assay for functional capacity. *E. hedgpethi* decreased in chlorophyll content during starvation until the tenth day, when no chlorophyll was detectable spectrophotometrically. Incorporation of $^{14}CO_2$ paralleled the decline in chlorophyll, and was at control levels by the tenth day. *P. ianthobapsus* showed no decline in chlorophyll content over 27 days starvation, although the ability to incorporate $^{14}CO_2$ showed a decrease.

Introductior

Since 1965, the presence of algal chloroplasts living symbiotically within the digestive gland cells of a variety of sacoglossan opisthobranchs has been well documented (KAWAGUTI and YAMASU, 1965; TAYLOR, 1967, 1968; GREENE, 1968, in press; TRENCH, 1969; TRENCH et al., 1969). The chloroplasts in all associations studied appear to be derived from the animals' algal food, and the plastids remain within the digestive cells for varying periods, depending on the species. Estimates of the length of time the chloroplasts may remain functional within the cells of a given species range from 24 h or less (TAYLOR, 1968), to 6 weeks (TRENCH et al., 1969) depending on the species investigated.

This study compares the abilities of chloroplasts in two species of sacoglossan slugs to remain functional following ingestion.

Materials and methods

Experimental animals

Both of the animals used in this study belong to the order Sacoglossa (Mollusca: Opisthobranchia). *Elysia hedgpethi* MARCUS was collected intertidally at Flat Rock, Palos Verdes, Los Angeles County, California, USA. This species lives and feeds upon either *Codium fragile* HARIOT or *Bryopsis corticulans* SETCHELL, both siphonaceous green algae.

Placobranchus ianthobapsus GOULD was obtained in Kaneohe Bay, Oahu, Hawaii, where it occurs on reef-flats composed of fine sand. The animals were found at about 1 m depth and were not observed feeding on algae at any time (GREENE, in press).

Maintenance of animals

Specimens of *Elysia hedgpethi* were maintained in the recirculating sea-water system at the Zoology Department of the University of California at Los Angeles (UCLA). Water temperature was kept at 13 °C. Prior to experimentation, *E. hedgpethi* was allowed to feed continuously on *Bryopsis corticulans*.

Placobranchus ianthobapsus was maintained in plastic tubs (27 × 32 × 13 cm) containing seawater at room temperature (about 22 °C) which was changed every 2 days. Specimens of *P. ianthobapsus* were not fed during captivity.

Experimental procedures

Determination of functional-residence time of chloroplasts

At the start of the experiment, animals were divided into 2 groups: a starved experimental group, and a starved dark-control group. By incubating the animals from each group with $^{14}CO_2$ as described below, it was possible to assess the chloroplasts' ability to fix carbon. Starved animals incubated with isotope in the dark served as controls for heterotrophic carbon fixation.

Incubation with ^{14}C

Following various periods of starvation, whole animals were incubated in 25 ml Erlenmeyer flasks with Millipore-filtered (porosity 0.45 μ) seawater containing $NaH^{14}CO_3$ (Calbiochem, sp. act. 35 mc/mM)

at an initial concentration of 10 μc/ml. Light intensity during incubations was adjusted to 500 foot-candles measured at the bottom of the incubation flasks (Weston Illumination Meter, Model 756). Light was supplied by 4 photoflood lamps (G.E. 150 W., 115 V.) controlled by a rheostat. Dark control animals were incubated in 25 ml flasks light-proofed with Scotch-brand vinyl electrical tape. All flasks were immersed in a trough of running water to maintain constant temperature during the experiments. Specimens of *Elysia hedgpethi* were incubated at 14 °C, while *Placobranchus ianthobapsus* was incubated at 22 °C. Animals were rinsed twice with fresh seawater following incubation, to remove excess isotope prior to further analysis.

aliquot was assayed for radioactivity. The remaining insoluble material was resuspended in ethanol, an aliquot was withdrawn and assayed.

Analysis of chlorophyll sample

The chlorophyll was quantified spectrophotometrically according to the following equation (after STRAIN and SVEC, 1966):

Total chlorophyll (μg/ml) = 7.12 (A_s660) + 16.8 ($A_s642.5$). Absorbance readings were made on a Beckman-Gilford spectrophotometer in diethyl ether. A 0.1 ml aliquot of the ether-soluble phase was plated on a planchet, acidified by addition of 1 drop of 0.1 N HCl, and assayed as below.

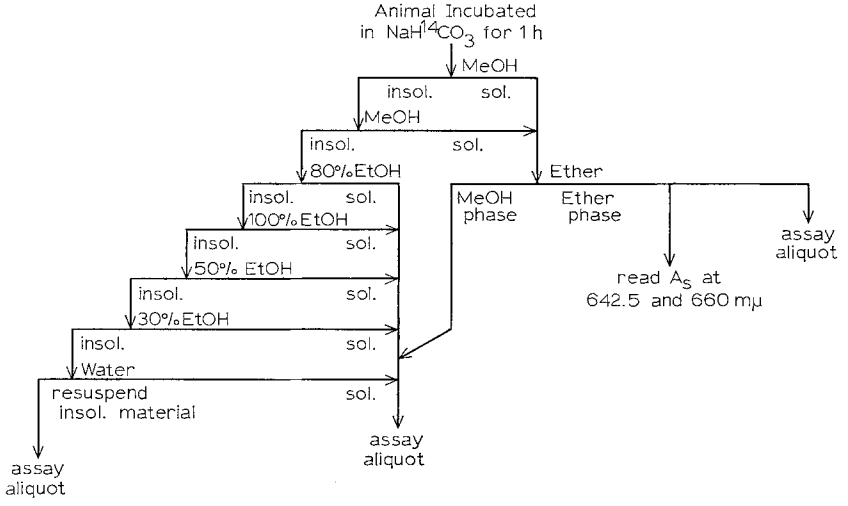

Fig. 1. Flow diagram of assay for functional capacity of symbiotic chloroplasts

^{14}C-fixation assay

After incubation in isotope for 1 h, animals were placed in cold absolute methanol (3° to 4 °C) and were homogenized with a glass rod. To extract the chlorophyll pigments from the methanolic supernatant, 3 to 4 ml diethyl ether were added, the extract was gently swirled, and phase separation was effected by addition of about 5 ml distilled water. The methanol extraction was carried out a second time, and the ether phase was carefully drawn off and saved for chlorophyll analysis. The residual animal tissues were then placed in 2 ml hot 80% ethanol, extracted again, and the insoluble material was sedimented in an International Clinical Centrifuge (Model CL). The alcoholic supernatant was drawn off, and the process was repeated using 100%, 50% and 30% ethanol and hot water. All alcoholic-aqueous extracts were pooled with the original methanol extract (see flow diagram, Fig. 1), the volume was recorded, and a 0.1 ml

Assay of radioactivity

Radioactivity was measured with a transistorized scaler (Nuclear Supplies, Model SA-250) and thin end-window G.M. tube (LND Inc., No. 733). All determinations were corrected for background and self-absorption. Liquid aliquots of 0.1 ml were plated on ringed nickel-plated planchets. They were acidified with 0.1 N HCl to drive off unbound $^{14}CO_2$, and were dried under an infra-red lamp.

Results

In order to assess the abilities of the chloroplasts to remain functional within the host tissues, it was necessary to prevent chloroplast replenishment. Animals were, therefore, starved and then incubated with $NaH^{14}CO_3$ in the light, with controls in the dark. Their ability to incorporate carbon-14 was determined, and their chlorophyll content was calculated.

Chlorophyll determinations

Fig. 2a shows the results of the chlorophyll determinations on *Elysia hedgpethi* starved for up to 10 days. The values represent averages of determinations on 4 individuals. Animals selected for this experiment were chosen for similarity in size and, during the study, all specimens decreased in size and chlorophyll content. Freshly fed animals maintained high levels of chlorophyll for the first 24 h and then began to pale visibly. As the animals were starved for longer periods, the amount of chlorophyll decreased until, by the tenth day, there was no detectable absorbance at

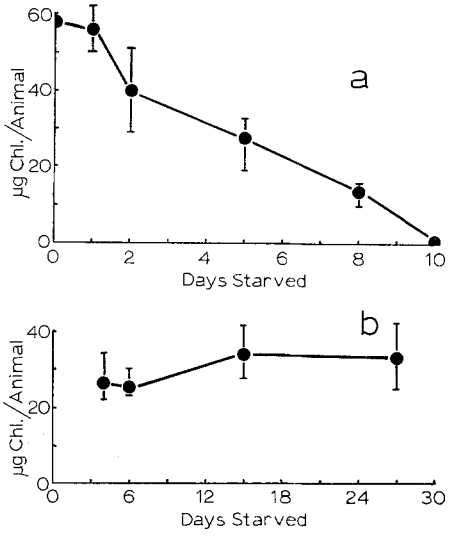

Fig. 2. Total chlorophyll content of *Elysia hedgpethi* (a) and *Placobranchus ianthobapsus* (b) during starvation. Points represent mean and range of 4 replicate determinations

either 642.5 or 660 mμ in the animal extracts. By this time the animals had paled from dark green to bright yellow-orange coloration.

Placobranchus ianthobapsus exhibited a relatively high chlorophyll content for a much longer time. Fig. 2b shows the average chlorophyll contents for specimens of *P. ianthobapsus* "starved" for 27 days (see discussion). The experiment was terminated at this point. The chlorophyll content of the animals was apparently unaffected by total starvation for this period of time. Animals starved in the dark showed no decrease in chlorophyll content for the duration of the experiment. This observation is in agreement with the work of J. K. TESTERMAN (unpublished data) who found that *P. ianthobapsus* starved in the dark began to lose pigment only after a period of 8 weeks.

Incorporation of ^{14}C

Fig. 3 shows that the level of ^{14}C-fixation by *Elysia hedgpethi* closely paralleled the decrease in chlorophyll content during starvation. Fig. 3a shows that the animal-chloroplast's ability to fix carbon-14 began to decline rapidly after 2 days starvation (circles). Finally, after being starved for 10 days and losing all their chlorophyll (Fig. 2a), animals incubated with isotope in the light could incorporate no more ^{14}C than control animals incubated in darkness (triangles).

Specimens of *Placobranchus ianthobapsus* incubated in the light (Fig. 3b, circles) were still able to incorporate more ^{14}C than dark-controls (triangles) after 27 days. However, during the period of starvation, it was evident that the photosynthetic ability of the chloroplasts was diminished.

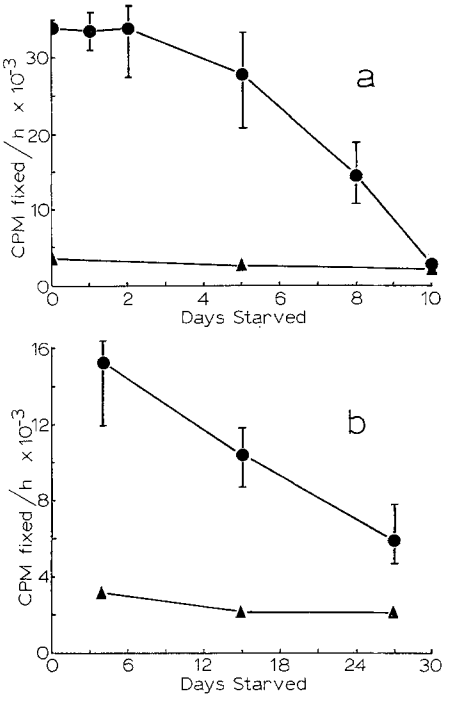

Fig. 3. Incorporation of ^{14}C by starved *Elysia hedgpethi* (a) and *Placobranchus ianthobapsus* (b). Values represent mean and range of 4 replicate determinations on animals of roughly equal size. Circles are values for animals incubated in the light, while triangles represent values obtained in the dark

Chlorophyll content versus ^{14}C-incorporation

Fig. 4 shows the data for *Elysia hedgpethi* and *Placobranchus ianthobapsus* expressed as incorporation of ^{14}C/µg chlorophyll/h. Fixation of carbon in starved *E. hedgpethi* increased per unit chlorophyll up to 2 days, then remained constant up to 8 days, while in *P. ianthobapsus*, incorporation of carbon decreased. Thus, it appears that *E. hedgpethi* is losing its chloro-

plasts, since both carbon fixation and chlorophyll content decrease with starvation. *P. ianthobapsus*, however, seems to contain chloroplasts which maintain their chlorophyll, but lose their functional integrity.

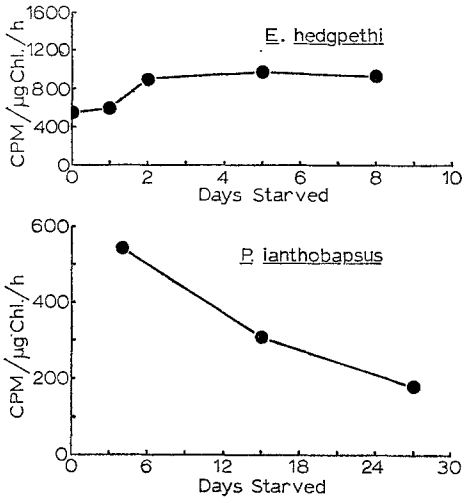

Fig. 4. Relation between chlorophyll content and ^{14}C-incorporation by starved *Elysia hedgpethi* and *Placobranchus ianthobapsus*

Discussion

The data presented show that the 2 species of sacoglossans considered vary in their abilities to retain functional chloroplasts in their digestive gland cells. *Elysia hedgpethi* lost its capacity to fix ^{14}C photosynthetically when starved for longer than 8 days. *Placobranchus ianthobapsus*, on the other hand, was still able to fix ^{14}C after 27 days of starvation.

In both species, the "starvation" time must be considered to be the minimum time the organisms were isolated from a source of fresh chloroplasts. Specimens of *Elysia hedgpethi*, both in the field and in the laboratory, may have a continuous supply of fresh algae upon which to feed, but it is impossible at the time of collection to tell when feeding last occurred. *E. hedgpethi* does, however, appear to feed continuously in captivity.

Placobranchus ianthobapsus presents a different problem. Specimens of this animal do not appear to feed on algal material as adults. It has been reported previously that *P. ianthobapsus* from Hawaii, has not been observed crawling upon or feeding on any species of algae in the field (GREENE, in press). KAWAGUTI et al. (1965) also have reported upon *P. ianthobapsus* from the reef-flats in Hawaii, and make no mention of their association with any species of algae. In an earlier report, KAWAGUTI (1941) discussed a close relative of this species, *P. ocellatus* from Palao Island, and mentioned the sacoglossan's frequent proximity to *Halimeda*, a green alga (Chlorophyta: Siphonales). GREENE (in press) has identified the plant pigments from *P. ianthobapsus* as characteristic of a siphonaceous green alga. Thus, since this species in Hawaii is not associated with siphonaceous algae, the problem becomes enigmatic, and it is impossible to determine when the animals last acquired fresh chloroplasts.

The results of the present study are consistent with previous reports on chloroplast function in sacoglossans. J. K. TESTERMAN (unpublished) demonstrated that the symbionts in the digestive gland cells of *Placobranchus ianthobapsus* from Hawaii were functional for "at least several weeks". In his work, oxygen production was used as an index of functional capacity. Preliminary experiments on the same species indicated that *P. ianthobapsus* could incorporate carbon-14 in the light after being starved for 2 months (personal observation). Thus, the decline in chloroplast function in *P. ianthobapsus* observed in the course of this study may be due, in part, to the maintenance conditions at UCLA as opposed to those in Hawaii.

TRENCH et al. (1969) reported that *Tridachiella diomedea* could still fix carbon-14 photosynthetically after 6 weeks away from a source of chloroplast replenishment.

TAYLOR (1968) reported that *Elysia viridis* from Great Britain, could incorporate ^{14}C (as $H^{14}CO_3^-$) in the light for 24 h after ingestion of fresh chloroplasts. Animals starved for periods longer than 24 h showed no more incorporation of isotope than those incubated in the dark, when compared using radioautographic methods. In the present study, *Elysia hedgpethi* showed a reduction in photosynthetic capacity after 48 h, but fixation continued for 7 more days (Fig. 3a).

KAWAGUTI and YAMASU (1965) have reported that the ultimate fate of chloroplasts in *Elysia atroviridis* is digestion by the animal. TAYLOR (1968) says that chloroplasts do not appear to be digested by *E. viridis*. He also points out that starved animals do not appear to excrete chloroplasts in the fecal pellets.

The data presented for *Elysia hedgpethi* in Fig. 4 suggest that the maintenance of ^{14}C-fixation per unit of chlorophyll from 2 to 8 days is caused by the fact that the chlorophyll content (Fig. 2a), and the animals' ability to incorporate carbon (Fig. 3a) both decrease during starvation. Consistent with this conclusion is the observation that starved *E. hedgpethi* produce green fecal pellets. It is not known whether defecated plastids are still functional.

The data for *Placobranchus ianthobapsus* in Fig. 4 must result from plastids maintaining their chlorophyll, but losing their functional capacity, since Fig. 2b shows that the pigment content remains the same while fixation of carbon decreases during starvation (Fig. 3b). There is no evidence of egestion of green plastids by *P. ianthobapsus* in fecal pellets. The

possibility does exist that animals, once having fed on an alga and obtained their complement of chloroplasts, need not replenish them. Work is in progress to determine whether or not the plastids are capable of dividing while within the cells of *P. ianthobapsus*.

Summary

1. *Elysia hedgpethi* MARCUS and *Placobranchus ianthobapsus* GOULD contain algal chloroplasts in their digestive gland cells. The chloroplasts remain functional for various lengths of time following ingestion, depending upon the species of animal.

2. During starvation, the chlorophyll content of *E. hedgpethi* decreases to zero after 10 days, while chlorophyll in *P. ianthobapsus* remains relatively constant for at least 27 days.

3. Both species were able to incorporate carbon-14 in the light. Chloroplasts in *E. hedgpethi* could fix carbon for 8 days, after which they were no longer functional. Chloroplasts in the tissues of *P. ianthobapsus* showed a decline in the ability to fix carbon-14 during starvation, but after 27 days were still capable of carbon fixation.

4. Carbon-fixation and chlorophyll content showed a parallel decline in *E. hedgpethi*, suggesting that chloroplasts are being egested in the feces, a conclusion which is verified by observation in this species. Chloroplasts in *P. ianthobapsus* retain their chlorophyll while functional capacity decreases, suggesting that non-functional chloroplasts are being retained in the tissues.

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