Phagosome-Lysosome Fusion Inhibited by Algal Symbionts of *Hydra viridis*

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ABSTRACT   Certain species of *Chlorella* live within the digestive cells of the fresh water cnidarian *Hydra viridis*. When introduced into the hydra gut, these symbiotic algae are phagocytized by digestive cells but avoid host digestion and persist at relatively constant numbers within host cells. In contrast, heat-killed symbionts are rapidly degraded after phagocytosis. Live symbionts appear to persist because host lysosomes fail to fuse with phagosomes containing live symbionts. Neither acid phosphatase nor ferritin was delivered via lysosomes into phagosomes containing live symbionts, whereas these lysosomal markers were found in 50% of the vacuoles containing heat-killed symbionts 1 h after phagocytosis.

Treatment of symbiotic algae before phagocytosis with polycationic polypeptides abolishes algal persistence and perturbs the ability of these algae to control the release of photosynthate in vitro. Similarly, inhibition of photosynthesis and hence of the release of photosynthetic products as a result of prolonged darkness and 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) treatment also abolishes persistence.

Symbiotic algae are not only protected from host digestive attack but are also selectively transported within host cells, moving from the apical site of phagocytosis to a basal position of permanent residence. This process too is disrupted by polycationic polypeptides, DCMU and darkness. Both algal persistence and transport may, therefore, be a function of the release of products from living, photosynthesizing symbionts. Vinblastine treatment of host animals blocked the movement of algae within host cells but did not perturb algal persistence: algal persistence and the transport of algae may be initiated by the same signal, but they are not interdependent processes.

A variety of unicellular organisms, including bacteria, protozoa, fungi, and algae, survive and persist as intracellular symbionts in host cells which otherwise display normal intracellular digestion. The means by which these symbionts survive host digestive attack are not understood in all cases but appear to involve either the growth of endosymbionts at a rate greater than that of their digestion by the host (cf. reference 12) or avoidance of host digestion by any of the following mechanisms: (*a*) escape from the phagosomal vacuole into the host cytoplasm, (*b*) prevention of the fusion of phagosomes and lysosomes, or (*c*) resisting or inactivating lysosomal enzymes after lysosomal fusion (cf. references 14, 16).

Unicellular algae exist as intracellular symbionts in a large number and variety of invertebrates, yet adaptations for sur-

vival of algal symbionts have been investigated only in the *Paramecium bursaria-Chlorella* association (22, 23). Here we examine how *Chlorella* are able to persist within digestive cells of *Hydra viridis*, a fresh water hydra. The results demonstrate that these algal endosymbionts resist host digestion by preventing the fusion of lysosomes with the phagosomes containing them. Similar conclusions have been drawn by Karakashian and Rudzinska (23) to explain the survival of *Chlorella* symbionts in *P. bursaria*. Additionally, the results show that symbiont persistence is highly correlated with the translocation of photosynthate from algal symbiont to host.

We also examine the transport of symbiotic algae from the tip of digestive cells, where phagocytosis occurs, to the base of these cells where algae take up permanent residence. The data

suggest that this transport of algae may also be signaled by symbiont release of photosynthate.

MATERIALS AND METHODS

Experimental Organisms

Symbiotic and aposymbiotic *Hydra viridis*, Florida strain (30), were cultured in M solution (28) at 18°C under a 12-h light/12-h dark photoperiod with an irradiance of 2.1×10^{-5} eisteins $m^{-2} s^{-1}$ provided by fluorescent lamps (Sylvania, Cool White). Animals were fed *Artemia* nauplii daily (25). The symbiotic algae used in this study were separated from 24-h starved *H. viridis* immediately before experimentation.

Preparation of Algae for Phagocytic Challenge

Symbionts were isolated from an homogenate of several thousand hydra in M solution which were disrupted in a tissue grinder. The homogenate was filtered through four layers of bolting silk (35-μm pore size) to reduce the amount of contamination by animal cell debris and then centrifuged. The supernatant fraction was discarded and the resultant algal pellet was washed four times in M solution.

Heat-killed symbionts were prepared by heating isolated symbionts for 5 min at 100°C in M solution. After this treatment heat-killed symbionts routinely were washed four times with M solution.

In phagocytic challenge experiments normal or heat-killed symbionts were incubated in M solution containing either bovine serum albumin, fraction V (BSA), 25 mg/ml; ferric chloride, 10^{-3} M; poly-L-glutamate, 26,000 mol wt, 2.5 mg/ml; protamine sulfate, 2.5 mg/ml; or poly-L-lysine, 70,000 mol wt, 2.5 mg/ml, for 60 min at 20°C with constant shaking. After this treatment the algae were washed four times in M solution. For some experiments the algae, prepared as described above, were incubated for an additional 60 min in either M solution or M solution with BSA, poly-L-glutamate or poly-L-lysine. BSA, protamine sulfate, poly-L-glutamate, and poly-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO).

Injection of Challenge Particles

100 μl of wet packed algae were resuspended in an equal volume of M solution to a final concentration of 10^7 particles/μl and injected into the gut of 3-d starved, aposymbiotic *H. viridis* with a glass micropipette (36). In some experiments excess algae were rinsed from the gut 30 or 60 min later.

Maceration of Hydra

At selected times after injection, the digestive region of each injected animal was excised, macerated on gelatin-coated slides (8), and fixed in 10% formalin. After this treatment, individual phagocytic digestive cells were located by their distinctive morphology, and phagocytized algal cells, easily distinguished from host cell organelles, were visualized with phase-contrast microscopy. Generally, in a given experiment five separate animals were examined. The average number of algae per digestive cell was determined for each animal by counting the algae in the first 25 intact digestive cells obseved, providing observations on a total of 125 digestive cells for each preparation.

Intracellular Movement of Algal Symbionts after Phagocytosis

The ratio of the distance an algal cell moved to the entire length of the digestive cell (R_f) was used as a quantitative measure of the movement of algae at intervals after phagocytosis (7, 31). R_f values were calculated here for the algal symbionts in a total of 125 host digestive cells for each preparation, as described above. To facilitate comparisons between experiments, digestive cells were divided into five zones and the number of algae with R_f values within each zone was recorded. Algae with R_f values were between 0 and 0.19 in zone 1, between 0.20 and 0.39 in zone 2, between 0.40 and 0.59 in zone 3, between 0.60 and 0.79 in zone 4, and between 0.80 and 1.00 in zone 5. The percent of the total algal population within each of these zones was calculated and the results were plotted as frequency histograms.

Assay of ^{14}C-labeled Photosynthate Released In Vitro

Algae incubated with BSA, poly-glutamate, poly-lysine, protamine sulfate, or ferric chloride were divided into aliquots with equal numbers of cells and were resuspended to a final volume of 5.0 ml in citrate-phosphate buffer of pH 7.0 or 4.5, after the addition of 2 μCi NaH^{14}CO$_3$ (10 μg/μCi; New England Nuclear, Boston, MA). These suspensions were incubated for 60 min at 20°C with a constant irradiance of 1.05×10^{-4} einsteins $m^{-2} s^{-1}$ provided by fluorescent lamps (Sylvania, Cool White) and then centrifuged (1,000 g, 4 min). Each algal pellet was resuspended to the original volume with buffer and 0.5-ml aliquots of both the supernatant and resuspended pellet fractions were acidified with an equal volume of 6 N HCl, warmed with an infrared lamp and exposed to a constant stream of air to drive off unfixed ^{14}CO$_2$. After this treatment 10 ml of a scintillation fluor containing Triton X-100, toluene and Omnifluor (New England Nuclear) was added and the samples were counted.

Assay for Host Digestion of ^{14}C-labeled Algal Cells

Algae were resuspended to a final concentration of 10^8 cells/ml in 2 ml of M solution containing 50 μCi NaH ^{14}CO$_3$ (1.7 μg/μCi; New England Nuclear). These cells were incubated for 2 h as described above, washed several times with M solution and incubated under the same conditions for an additional 2 h (cold chase). After these treatments the cells were again washed in M solution and divided into two groups: one group of normal symbionts and the other heat-killed, as described above. Both groups of algae, at concentrations of 10^7 cells/ μl, were loaded into separate glass micropipettes and injected into separate groups of 3-d starved *H. viridis*. Excess algae were rinsed from the hydra coelenteron 30 min after the injections. Labeled algae not used in the injection, both normal and heat-killed symbionts, were resuspended in 10 ml of M solution to a density of 10^4 cells/ml. Injected hydra and resuspended algae were maintained under standard hydra culture conditions.

At selected times after injection, hydra were homogenized and separated into a low-speed centrifugation (100 g, 4 min) pellet and supernatant fraction. The pellet was resuspended to the original volume of the homogenate with M solution, and samples of both fractions were prepared for liquid scintillation counting, as described above. Samples of resuspended labeled algae were similarly treated. The amount of ^{14}C label in the supernatant fraction, expressed as a percent of the total, was used as an estimate of algal degradation.

Perturbation of the Movement of Algae and Algal Persistence with Vinblastine and DCMU

Aposymbiotic animals incubated for 2 h in freshly prepared M solution containing 1 μM vinblastine (Sigma Chemical Co.) were injected with normal or heat-killed symbionts. At selected times after the injection, the digestive region of injected animals was excised and macerated and observations of the algal number and position were recorded.

Algae isolated from symbiotic hydra which were maintained on a normal diel light regime or maintained in the dark for 24 h were incubated in M solution containing 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) for 3 h and then injected into aposymbiotic animals. (Algae incubated for 60 min in this concentration of DCMU did not incorporate ^{14}C when NaH^{14}CO$_3$ was added to the incubation medium.) Injected animals were divided into two groups, one maintained in the light and the other in the dark. At selected times after the injection, the number and intracellular location of phagocytized algae were recorded.

Electron Microscopy

3-d-old starved experimental hydra were relaxed in a 0.5% solution of ethyl carbamate (urethane) for 2 to 3 min at room temperature, before fixation for 1 h at 0°C in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. Animals were postfixed in 1% osmium tetroxide in the same buffer for 1 h at 0°C, dehydrated in ethanol and embedded in epoxy resin (38). Thin sections on Formvar-coated grids were stained with uranyl acetate and examined with a Philips EM 200 electron microscope.

Cytochemistry

The cytochemical localization of acid phosphatase activity was done using β-glycerophosphate as substrate in Tris-maleate buffer (2). Before cytochemical staining, whole animals were incubated in primary fixative containing 10% dimethyl sulfoxide (vol/vol) (3), and the buds, base and hypostome were removed from each animal. The cytochemical incubations were performed at 20°C for 60 min with constant shaking. Control animals were incubated in media lacking substrate or in a complete medium containing 10^{-2} M sodium fluoride. After the incubations the experimental and control animals were rinsed twice in ice cold Tris-maleate buffer, pH 5.0, and twice in 0.05 M cacodylate buffer, pH 7.4, and processed for electron microscopy, as described above.

Labeling Secondary Lysosomes with Ferritin

Secondary lysosomes were labeled with ferritin using a modification of the procedures of Armstrong and Hart (1). Aposymbiotic hydra, starved for 3 d, were injected with ferritin, 100 mg/ml (twice crystallized, cadmium-free; Polyscience, Inc., Warrington, PA). 30 min after this injection, the coelenteron was flushed with M solution. The animals then were incubated for an additional 90 min to allow the ingested, surface-bound ferritin to be incorporated into secondary lysosomes (20).

RESULTS

Algae phagocytized by digestive cells were evaluated for their ability to avoid intracellular digestive attack by host cells. The evaluation was based upon algal persistence at constant numbers within host cells, algal ultrastructural integrity, and the results of a bioassay for digestion of ^{14}C-labeled algae.

Persistence of Phagocytized Algae

Symbiotic algae, if able to avoid intracellular digestion by host cells, should persist in constant or increasing numbers and without loss of structural integrity after phagocytosis. Table I shows that the number of symbiotic algae remained nearly constant over the 24-h observation period after phagocytosis. The number of heat-killed symbionts, on the other hand, declined significantly during this time. Heat-killing dramatically decreases the extent to which these symbiotic algae are phagocytized by hydra digestive cells (cf. reference 26).

Ultrastructural Evidence That Symbionts Avoid Host Digestion

Symbionts phagocytized by digestive cells of aposymbiotic hydra retain all of the ultrastructural features characteristic of these algae before their isolation from green hydra (35) and, more importantly, their ultrastructure is identical with that of isolated symbionts located in the hydra gut (Fig. 1). In contrast, the ultrastructural integrity of heat-killed symbionts is lost within several hours after their phagocytosis (Fig. 2).

Time Course of Digestion of Labeled Symbionts

To determine the time course during which heat-killed symbionts were digested, and to provide additional evidence that normal symbionts were not digested, a bioassay was developed. With this assay, it was expected that if algae were digested, the ^{14}C algal label would be metabolized by, and accumulate in, the animal cells. If algae were not digested the label would remain in the algal cells. After homogenization and centrifugation, the undigested algal cells are recovered in the pellet while the animal cell fragments are recovered in the supernatant fraction. Fig. 3 shows the ^{14}C-label from phagocytized heat-killed symbionts rapidly accumulated in the supernatant
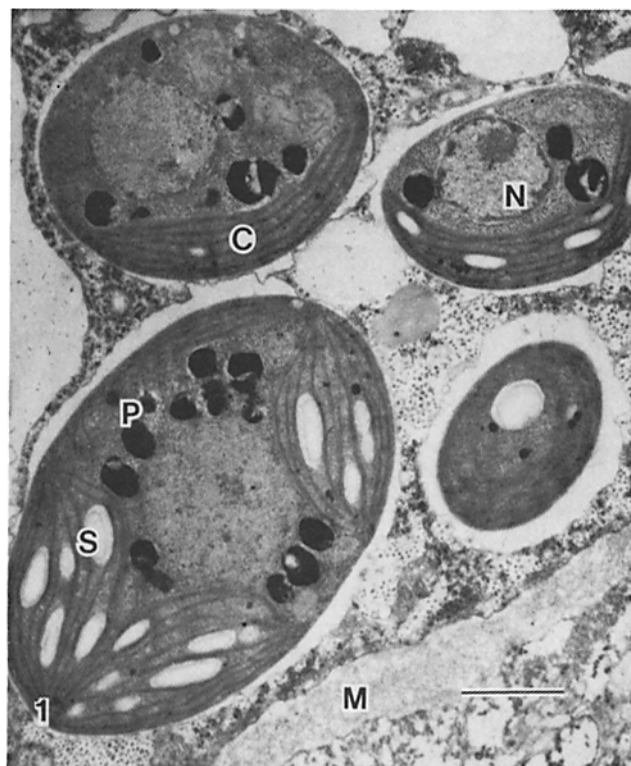

FIGURE 1 Electron micrograph of 3-d starved hydra fixed for microscopy 2 h after an injection with normal symbionts. C, chloroplast; S, starch granules; P, polyphosphate bodies; N, nucleus; M, mesoglea. Bar, 0.5 μm. × 26,000.

fraction while virtually no label from heat-killed symbionts resuspended in culture medium accumulated in the supernatant fraction over the 24-h period examined. This method provided evidence for digestion of heat-killed algae in host cells as early as 30 min after phagocytosis, with nearly 50% of the label appearing in the supernatant fraction by 24 h after phagocytosis.

In contrast, label from phagocytized normal symbionts did not accumulate beyond control levels in the supernatant fraction (Fig. 4). Indeed, after 18 h the level of label released by controls in culture medium was higher than that of phagocytized algae. This result is probably due to slow degradation of control cells maintained in the less favorable in vitro environment. The elevated levels of the total label in the supernatant fraction at the zero time point was probably due to the normal release in vitro or translocation in vivo of photosynthetic products. This is a well-described characteristic of symbiotic *Chlorella* (4, 27, 32). The products released by symbiotic algae are the immediate products of photosynthesis (cf. reference 30). Our attempt to minimize the effect of this release by using a 2-h cold chase was not completely effective.

Cytochemical Evidence That Active Lysosomal Enzymes Are Not Present in Phagosomes Containing Normal Symbionts

To gain insight into the mechanism used by symbionts to avoid host digestion, acid phosphatase cytochemistry was used to determine whether active hydrolytic enzymes were detectable within phagosomes containing heat-killed or normal symbionts. Table II summarizes the cytochemical results. 1 h after

TABLE I

Persistence of Phagocytized Algae in Hydra Digestive Cells

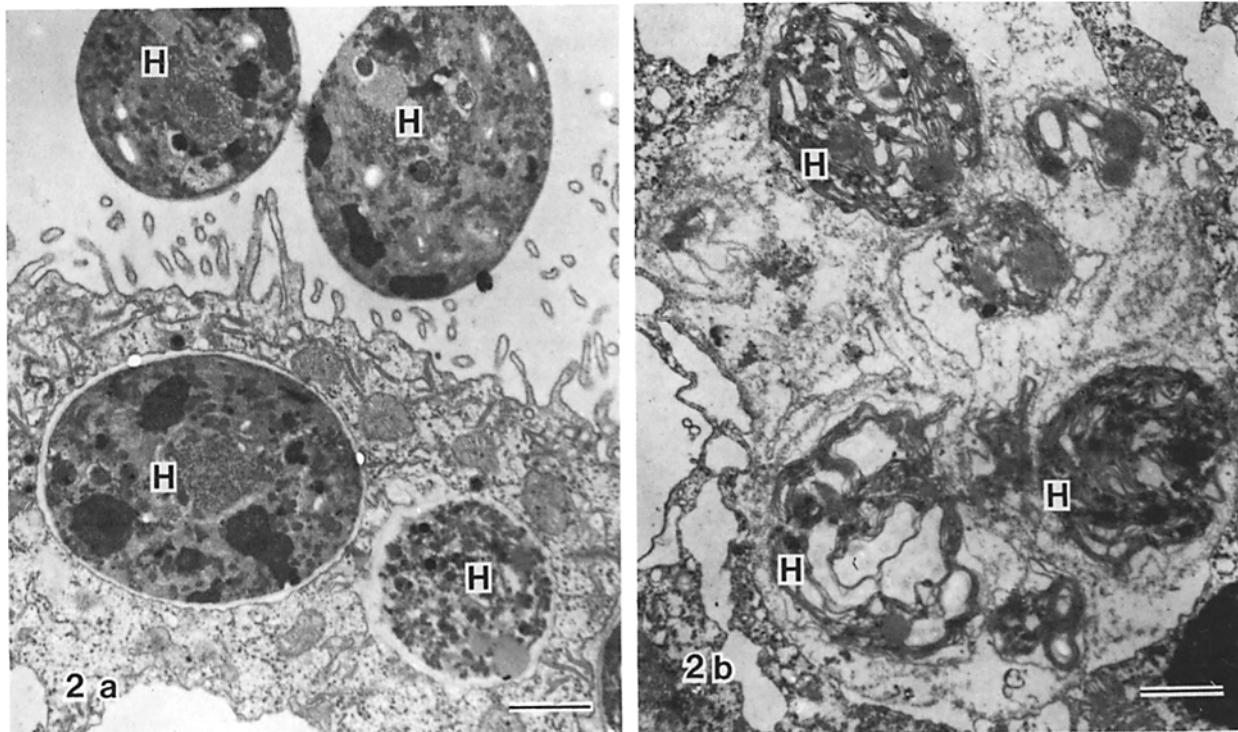| Algae	Mean number of algae in hydra digestive cell at selected times after phagocytosis*	
	2 h	24 h
Normal symbionts	13.48 (0.53)	14.30 (0.34)
Heat-killed symbionts	5.83 (0.35)	0.31 (0.07)

* Values in parentheses are standard errors of the mean.

FIGURE 2 Electron micrograph of 3-d starved hydra fixed for microscopy (a) 5 min or (b) 2 h after an injection with heat-killed symbionts. H, heat-killed symbiont. Bar, 0.5 μm. × 22,000.
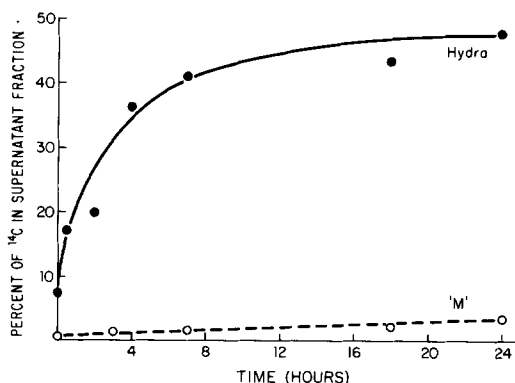

FIGURE 3 Appearance of ^{14}C in the supernatant fraction after injection of heat-killed symbionts into 3-d starved hydra or suspension of heat-killed symbionts in M solution. Symbionts labeled with ^{14}C during photosynthesis and then heat-killed were presented for phagocytosis to hydra digestive cells. At selected intervals after phagocytosis digestive cells were homogenized. With low-speed centrifugation whole algae were separated from algae fragmented by digestion and each fraction was counted for radioactivity. As a control for autolysis labeled algae were suspended in hydra culture medium in the absence of the host, but were otherwise treated as described above for phagocytized algae. Heat-killed symbionts phagocytized by hydra (straight line), heat-killed symbionts resuspended in hydra culture medium (dashed line).

phagocytosis 50% of the phagosomes containing heat-killed symbionts, but only 3% of the phagosomes containing normal symbionts, also contained acid phosphatase reaction product. 5 h after phagocytosis the percent of phagosomes containing heat-killed symbionts and enzyme reaction product increased to 75, while the percent of those containing normal symbionts and reaction product remained unchanged.
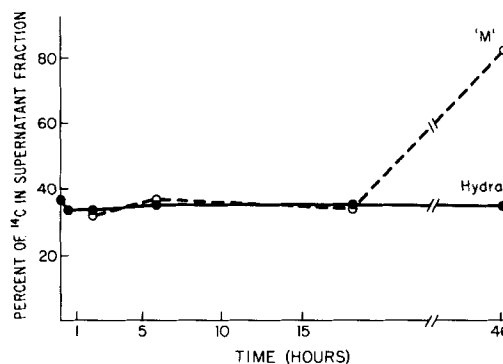
FIGURE 4 Appearance of ^{14}C in the supernatant fraction after injection of normal symbionts into 3-d starved hydra or suspension of normal symbionts in M solution. Normal symbionts labeled with ^{14}C during photosynthesis were presented to hydra for phagocytosis. At selected intervals after phagocytosis digestive cells were homogenized. With low-speed centrifugation whole algae were separated from algae fragmented by digestion and each fraction was counted for radioactivity. As a control for autolysis, labeled algae were suspended in hydra culture medium in the absence of the host, but were otherwise treated as described above for phagocytized algae. Normal symbionts phagocytized by hydra (straight line), normal symbionts resuspended in hydra culture medium (dashed line).

Evidence for the Fusion of Ferritin-labeled Lysosomes with Phagosomes

There are at least two explanations to account for the absence of acid phosphatase activity in phagosomes containing normal symbionts. Either lysosomes did not fuse with such phagosomes or lysosomes did fuse but the acid hydrolyses were inactivated or inhibited by the symbionts. The fusion of secondary lysosomes with phagosomes containing algae can also be detected

TABLE II

Persistence of Algae within Digestive Cells after Phagocytosis: Observation by Electron Microscopy

Challenge particles	Number of algae observed by electron microscopy	Percent in vacuoles with ferritin 1 h	Percent in vacuoles with APase activity 1 h	Percent in vacuoles with APase activity 5 h
Normal symbionts	160 (6)*	3	3	3
Heat-killed symbionts	120 (6)	57	50	75
Normal symbionts treated with poly-lysine	80 (3)	75	—	—

* Numbers in parentheses indicate number of different hydra observed.

in hydra using the electron dense marker ferritin. In digestive cells fixed 90 min after endocytosis of ferritin, but immediately before the time normally chosen for the presentation of algae for phagocytosis, this electron-dense marker was found in a variety of cytoplasmic vacuoles but was not found on digestive cell surfaces where it could have been interiorized along with algae. In experiments where digestive cells were fixed and stained for acid phosphatase activity 90 min after ferritin endocytosis, 100% of the vacuoles with ferritin also contained acid phosphatase reaction product, demonstrating that at this time point ferritin is found within secondary lysosomes. For these latter experiments 105 ferritin vacuoles were observed in sections prepared from three separate animals.

In digestive cells labeled with ferritin and then fixed 1 h after phagocytosis of algae, 57% of the phagosomes containing heat-killed algae also contained ferritin, while only 3% of the phagosomes containing normal symbionts also contained ferritin (Table II). Thus, the fusion of secondary lysosomes with phagosomes appears to be prevented when these phagosomes contain normal living symbionts.

Perturbation of Algal Persistence by Polyions

Polyionic agents have been shown to inhibit or stimulate phagosome-lysosome fusion in other cells (9, 11, 15, 17, 19, 24). Table III shows that the persistence of normal symbionts and heat-killed symbionts within host digestive cells was not affected when these algae were pretreated with polyanionic polypeptides. However, pretreatment of symbionts with poly-cationic polypeptides drastically reduced the number of symbionts remaining in digestive cells 24 h after phagocytosis (Table III). Moreover, poly-lysine pretreatment resulted in the appearance of ferritin in 75% of the phagosomes containing these treated symbionts (Table II). Reduced persistence of poly-lysine pretreated symbionts was abolished after subsequent treatment with poly-glutamic acid, but not after subsequent treatment with BSA (Table III).

Digestion Avoidance Is Not Dependent upon Movement of Symbionts

After phagocytosis normal symbionts, but not other particles, move from phagocytic loci to the base of host digestive cells. To test the hypothesis that avoidance of digestion may be dependent upon the sequestration of symbionts in the base of host cells, we blocked the movement of symbionts (Fig. 5) by treating host cells with vinblastine (cf. reference 7), an inhibitor

of tubulin polymerization. As shown in Table IV, vinblastine treatment inhibited the movement of symbionts but did not affect the ability of normal symbionts to avoid digestion.

Perturbation of Symbiont Movement with Polycations

Normally, within 2 h after phagocytosis, 50% of the phago-cytized symbionts are moved to the base of host digestive cells, leaving <10% in the extreme apical tip (Fig. 6). In contrast, only 10%–15% of the poly-lysine-treated symbionts reach the base of digestive cells with 40% remaining in the apical tip

TABLE III

Effect of Pretreatment with Polyionic Compounds or Photosynthetic Inhibitors on the Mean Number of Algae in Hydra Digestive Cells after Phagocytosis

Pretreatment* Hour 1	Pretreatment* Hour 2	Mean number of algae per digestive cell at selected times after phagocytosis‡ 2 h	Mean number of algae per digestive cell at selected times after phagocytosis‡ 24 h
Normal symbionts			
M	M	13.48 (0.53)	14.30 (0.34)
Poly-G	M	13.02 (0.43)	12.77 (0.52)
BSA	M	14.46 (0.49)	14.16 (0.73)
Poly-L	M	2.85 (0.31)	0.72 (0.10)
Poly-L	Poly-G	17.04 (0.67)	15.70 (0.54)
Poly-L	BSA	13.29 (0.33)	1.09 (0.18)
FeCl₃	M	1.72 (0.17)	2.31 (0.22)
Prot	M	7.36 (0.35)	4.26 (0.28)
DCMU		14.16 (0.59)	12.98 (0.52)
DCMU§		14.87 (0.56)	7.32 (0.36)
Heat-killed symbionts			
M	M	5.83 (0.35)	0.31 (0.07)
BSA	M	14.80 (0.40)	0.30 (0.07)

* Concentration of agents used in the treatments: poly-lysine (Poly-L), 2.5 mg/ml; poly-glutamate (Poly-G), 2.5 mg/ml; BSA, 25 mg/ml; Protamine sulfate (Prot), 2.5 mg/ml; DCMU, 5 × 10⁻⁶ M; FeCl₃, 10⁻³ M.

‡ Values in parentheses are standard errors.

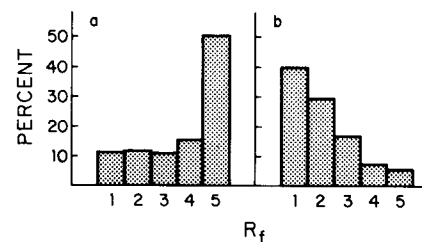§ Animals were kept in the dark after algal injection.

FIGURE 5   Histogram showing the effect of vinblastine on the intracellular location of symbionts 2 h after their phagocytosis by host digestive cells. Normal symbionts were injected into control hydra or hydra pretreated for 2 h with 10⁻⁶ M vinblastine. 2 h after the algal injection hydra were macerated and the R_f values (see Material and Methods) were measured for the algal symbionts within 25 digestive cells from each of five separate animals. The digestive cells were divided into five zones and the number of algae located in each zone expressed as a percent of the total number of algae phagocytized. (a) Normal symbionts injected into control hydra; (b) normal symbionts injected into vinblastine, treated hydra. R_f zones (1 = R values 0.0–0.19, 2 = 0.20 to 0.39, 3 = 0.40 to 0.59, 4 = 0.60 to 0.79 and 5 = 0.80 to 1.00).

(Fig. 6). Heat-killing of symbionts similarly prevents their movement (Fig. 6). Protamine sulfate also inhibits algal movement but not to the same extent as poly-lysine or heat-killing. The poly-lysine inhibition of transport could be removed by subsequent treatment with poly-glutamate, but not with subsequent treatment with BSA. Poly-glutamate treatment alone did not affect the transport of symbionts nor did it restore transport to heat-killed symbionts (Figs. 6 and 7). Ferric chloride treatment also was without effect on transport of symbionts (Fig. 6). These results demonstrate that treatments which perturb algal persistence also interfere with the movement of algae.

TABLE IV

Effect of Vinblastine Treatment of Hydra on the Mean Number of Algae in Hydra Digestive Cells after Phagocytosis

Treatment	Mean number of algae per digestive cell at selected times after phagocytosis*	
	2 h	24 h
Normal symbionts	15.73 (0.58)	15.54 (0.44)
Normal‡ symbionts + vinblastine	9.98 (0.36)	11.09 (0.28)
Heat-killed symbionts	5.83 (0.28)	0.31 (0.07)
Heat-killed symbionts + vinblastine	4.48 (0.23)	0.51 (0.08)

* Values in parentheses are standard errors.
‡ Before algal injection aposymbiotic animals were incubated for 2 h in 10^{-6} M vinblastine.
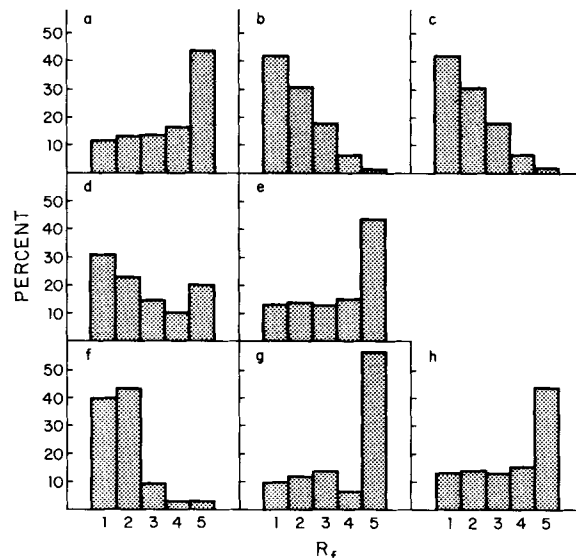
FIGURE 6 Histograms showing the effect of polyions on the intracellular location of symbionts 2 h after their phagocytosis by host digestive cells. Normal or heat-killed symbionts treated with or without several different polyions were injected into 3-d starved hydra. 2 h after the injection, hydra were macerated and the R_f values for algal symbionts within 25 digestive cells of five separate animals were calculated and expressed as in Fig. 5. (a) Normal symbionts, (b) poly-lysine-treated symbionts, (c) heat-killed symbionts, (d) Protamine sulfate-treated symbionts, (e) symbionts treated sequentially with poly-lysine and poly-glutamate, (f) symbionts treated sequentially with poly-lysine and BSA, (g) poly-glutamate-treated symbionts, and (h) ferric chloride-treated symbionts.
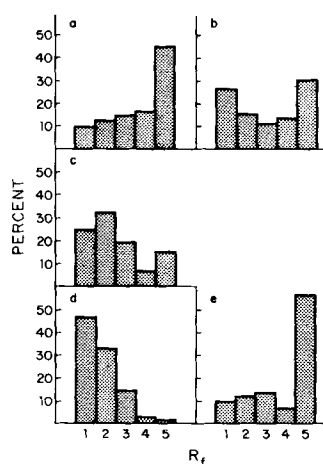
FIGURE 7 Histograms showing the effect of DCMU and darkness on the intracellular location of symbionts 2 h after their phagocytosis by host digestive cells. Normal symbionts or symbionts treated with DCMU and/or prolonged darkness were injected into 3-d starved hydra. These injected animals were maintained either in the light or in dark. 2 h after algal phagocytosis, hydra were macerated and R_f values were calculated and expressed as in Fig. 5. (a) Control symbionts, (b) symbionts isolated under dim light from green hydra maintained in the dark 20 h before algal isolation, (c) symbionts isolated as described in (b) and incubated in DCMU 5×10^{-6} M for 3 h before host injection, (d) symbionts isolated and treated as described in (c) and injected into animals which were then kept in dark after algal injection, (e) symbionts isolated and treated as described in (c) and injected into animals which were then kept in light.

Correlation between Perturbed Symbiont Release of Photosynthate, Persistence, and Movement

One unique physiological characteristic of symbiotic *Chlorella* which may be required for their "recognition" by the host is their ability to selectively release photosynthetic products. To determine if treatments which perturbed algal movement and algal persistence also perturbed release of photosynthetic products in vitro we treated algae with polyions and then measured their in vitro release of photosynthate at pH 7.0 and 4.5. The data, expressed as a ratio of release at pH 7.0 to 4.5, show that polycations interfered with the release of photosynthate (Table V). Perturbation of release of photosynthate by poly-lysine treatment, like the effects of poly-lysine on algal movement and persistence, could be removed if, subsequent to the poly-lysine treatment, symbionts were treated with poly-glutamate. Subsequent treatment with BSA was not effective in offsetting the poly-lysine treatment.

These results suggest that release of photosynthetic products may initiate algal movement and inhibit the fusion of lysosomes with phagosomes containing symbionts. We further tested this hypothesis by perturbing photosynthesis. Fig. 7 shows that symbionts which were isolated from animals maintained in the dark for 24 h and symbionts which were treated with DCMU and darkness were not moved nor did they persist to the same extent as controls. When DCMU and dark-treated symbionts were injected into animals maintained thereafter in the dark, <5% of these algae were moved to the base of host cells within 2 h of phagocytosis, and only 50% of the phagocytized algae were present in the digestive cells 24 h after phagocytosis (Fig. 7 and Table III). If animals were kept in the light after algal injection, the symbionts were moved to the base of host cells and persisted to the same extent as control symbionts that were not exposed to long periods of darkness or DCMU (Fig. 7 and Table III). These latter observations demonstrate that the treatments used to inhibit photosynthesis did not permanently damage the symbionts.

TABLE V

Release of Photosynthate by Algae In Vitro after Treatment with Polyionic Compounds

Treatment*		Incubation pH	^{14}C release into medium as percent of the total fixed‡	Release ratio
Hour 1	Hour 2			
M	M	4.5	75.4 (8.7)	0.10
		7.0	7.8 (2.4)	
BSA	M	4.5	66.5 (3.2)	0.07
		7.0	5.9 (2.1)	
Poly-G	M	4.5	82.2 (7.4)	0.11
		7.0	8.6 (3.6)	
FeCl₃	M	4.5	62.7 (4.5)	0.11
		7.0	6.8 (2.1)	
Prot	M	4.5	41.6 (6.9)	0.25
		7.0	10.5 (3.5)	
Poly-L	M	4.5	36.5 (11.7)	0.53
		7.0	19.2 (9.1)	
Poly-L	Poly-G	4.5	53.4 (5.9)	0.08
		7.0	5.4 (2.9)	
Poly-L	BSA	4.5	46.3 (4.5)	0.24
		7.0	11.5 (3.5)	

* Concentration of the compounds used in the treatments: Poly-L, 2.5 mg/ml; Poly-G, 2.5 mg/ml; protamine sulfate, 2.5 mg/ml; BSA, 25 mg/ml; FeCl₃, 10^{-3} M.

‡ Values are calculated from the means of four separate experiments, each sampled in triplicate. The values in parentheses are standard deviations.

DISCUSSION

Host Digestion of Symbionts

Light microscope examinations of macerated hydra have revealed that, whereas normal symbionts persist within host digestive cells, heat-killed and other effete symbionts do not (33, 34). Two lines of evidence in this study, one from radiolabel and the other from morphological experiments, strongly suggest that effete symbionts, such as heat-killed symbionts, do not persist because they are digested within host cells. Thus, ^{14}C-labeled heat-killed symbionts, which have been phagocytized by hydra digestive cells, were rapidly degraded into smaller nonsedimentable fragments. In contrast, phagocytized normal symbionts, as well as normal symbionts and heat-killed symbionts resuspended in hydra culture medium, did not yield such ^{14}C-labeled fragments. Electron microscopy revealed a dramatic loss of the cellular integrity of phagocytized heat-killed symbionts with a time course that paralleled digestion measured in the radiolabel experiments. All of these results are consistent with the interpretation that the maintenance of normal symbionts within host digestive cells at relatively constant numbers is due to their avoidance of host digestion.

How do algal symbionts avoid host digestion? Electron micrographs, presented in this and other studies (6, 35), clearly show that normal symbionts are enclosed by intact phagosomal membranes at all times, making it unlikely that normal symbionts resist host digestion by escaping from the phagosomal vacuole (cf. references 14, 16). Nor can persistence result from rapid symbiont multiplication overwhelming the host lysosomal system, since algal persistence is not correlated with symbiont numbers per digestive cell. Three other possibilities remain: (a) inhibition of or (b) resistance to host lysosomal enzymes, and (c) prevention of phagosome-lysosome fusion. Together, the cytochemical results and the results of experiments in which ferritin was used to label secondary lysosomes

suggest that algal symbionts use the latter of these methods. It should be noted that this method for avoiding host digestion is not unique to hydra symbionts. Prevention of phagosome-lysosome fusion is also seen in mammalian cells infected with one of a variety of parasites, including *Toxoplasma gondii*, *Mycobacterium tuberculosis*, *Mycobacterium microti*, *Chlamydia psittaci*, *Encephalitozoon cuniculi* (cf. 14, 16), as well as *Paramecium bursaria* infected with symbiotic *Chlorella* (22, 23).

Polyion Interference with Symbiont Persistence

Little is known about the mechanisms these symbionts or mammalian parasites use to prevent phagosome-lysosome fusion. However, treating macrophages for several days with certain polyanions, including suramin, dextran sulfate, polyglutamate, and chlorite-oxidized amylose, produces a result which mimics the symbiont perturbation of phagosome-lysosome fusion (9, 11, 15, 17, 19, 24). For example, yeast cells that are readily digested in untreated host cells resist digestion in host cells treated with these polyanions. This resistance can be removed when host cells are subsequently treated with the lipophilic cations chloroquine and tributylamine (18, 19).

These observations of polyanion inhibition of phagosome-lysosome fusion and the antagonistic activity of lipophilic cations suggested to us that the effects of these drugs might be related to their charge, and that treating digestive cells with polycations might stimulate phagosome-lysosome fusion and eliminate the ability of symbionts to prevent phagosome-lysosome fusion. Since polycations can damage cells (5), we attempted to limit the effect of the polycations to the vacuoles containing symbionts and to reduce the concentration of polycations to which the digestive cells were exposed by treating the algae with polycations and then washing the algae several times in M solution before they were injected into hydra. This procedure drastically reduced the persistence of normal symbionts and greatly enhanced phagosome-lysosome fusion. The poly-lysine treatment did not permanently damage algae, since poly-lysine-treated algae subsequently treated with poly-glutamate persisted in host digestive cells to the same extent as controls. The polycation interference with algal persistence can be interpreted in several ways. One interpretation is that polycations affect the fate of phagocytized particles by altering or masking some of their surface features which are responsible for symbiont recognition by the host. One surface feature important in host recognition of symbionts which is altered by poly-lysine treatment and which returns to normal with subsequent poly-glutamate treatment is surface charge. In a companion study we were able to quantify changes in symbiont cell surface charge after polyion treatment and observed that ferric chloride and poly-lysine treatment of symbionts produced quantitatively similar changes in cell surface charge (26). Ferric chloride treatment, however, does not affect symbiont persistence, demonstrating that polycation alteration of symbiont surface charge alone cannot account for the effects these agents have on persistence. Another interpretation of the polycation interference with algal persistence is that polycation treatment interferes with the symbionts's production and release of a metabolite or biosynthetic product which inhibits phagosome-lysosome fusion. The observations that living, but not heat-killed, *T. gondii* (21), *M. tuberculosis* (1), and *Chlorella* (22; Table II), are able to prevent phagosome-lysosome fusion, together with the recent observation that the ammonia production by *M. tuberculosis* perturbs phagosome-lysosome fusion (13), support this latter interpretation.

Intracellular Movement of Symbionts

Symbiotic algae not only avoid digestion but also are moved within host cells from the apical site of phagocytosis to a permanent basal residence. Several investigators have suggested that the sequestration of algae in the basal region of host digestive cells may protect them from expulsion or host digestion (6, 31). Experiments, demonstrating that polycationic treatments which reduce algal persistence also block algal transport, appear to support this hypothesis. However, experiments demonstrating that vinblastine treatment blocked the transport of algae, but did not affect the persistence of normal or heat-killed symbionts, show that the sequestration of algae is not a prerequisite for symbiont avoidance of host digestion.

Symbiont Translocation of Photosynthate

As a measure of cell viability after treatment with polyanionic and polycationic polypeptides, we recorded the ability of symbionts to fix and release labeled carbon. Isolated symbionts fix labeled carbon at rates similar to those of free-living algae but, unlike free-living algae, release into their medium as much as 80% of their recent photosynthetic products (4, 32). This in vitro release of photosynthate is pH-dependent, i.e., the amount of fixed labeled carbon released into the medium decreases as the pH of this medium is increased (4, 29, 32). *In situ*, hydra symbionts release and translocate to the host about 40% of their photosynthate (10, 37).

While the polyion treatment of symbionts did not affect their rate of photosynthesis (data not shown), the poly-lysine and protamine sulfate treatments which blocked the transport of algae and reduced algal persistence also interfered with algal translocation. The poly-lysine perturbation of photosynthate release was partially removed when poly-lysine-treated symbionts were subsequently treated with poly-glutamate, but not when they were treated with BSA. Likewise, the subsequent treatment of poly-lysine-treated symbionts with poly-glutamate, but not BSA, removed the poly-lysine block of the intracellular transport of algae and reduction of algal persistence. These results suggest that translocation of photosynthate may influence both phagosome-lysosome fusion and the intracellular transport of algae. If this is true, then other known inhibitors of photosynthesis and translocation should also disrupt the transport of algae and enhance host digestion of symbionts.

When symbionts treated with DCMU and darkness in combination were injected into hosts, the symbionts did not persist, nor were they transported when host animals were thereafter kept in the dark. They did persist and were transported when host animals were kept in the light. These observations are consistent with the interpretation that photosynthesis or one of its concomitant processes plays a role in both algal transport and avoidance of host digestion by hydra symbionts. Recent work of McAuley and Smith (personal communication) supports the interpretation that release of photosynthate influences the transport of algae.

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