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Organic Carbon Transfer from Methanotrophic Symbionts to the Host Hydrocarbon-seep Mussel

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Abstract

The mechanism of transfer of fixed carbon from methanotrophic symbionts to their host (an undescribed mussel found in association with hydrocarbon seeps in the Gulf of Mexico) was investigated through ultrastructural observations and incubations with ^{14}C-methane. Unlike other autotrophic symbioses studied with labeled inorganic carbon, there was no appreciable transfer of labeled organic carbon from the symbiont-containing tissues to symbiont-free tissues during a 2 hour pulse and two hour chase period. However, after 1 and 5-day chase periods, quantitative transfer of labeled organic carbon into symbiont-free tissues was documented. We propose that the longer time course of transfer of label in this association is due to host digestion of symbionts, rather than translocation of small molecular weight compounds from symbiont to host. Additional evidence for this was found in the presence of abundant secondary lysosomes in the host bacteriocytes, which often contain easily recognized remnants of the symbionts.

Keywords: methanotrophy, translocation, symbiosis, cold seep, nutrition

1. Introduction

Since the initial discovery of symbioses between chemoautotrophic bacteria and marine invertebrates, investigators have hypothesized that the symbiotic bacteria were contributing to the nutrition of their hosts. Ultrastructural studies of the symbiont-containing tissues of a variety of hosts suggested that some of the symbionts were being digested in the host cells (Bosch and Grasse, 1984; Fiala-Médioni et al., 1986a; 1990; Fiala-Médioni and Le Pennec, 1987; Herry et al., 1989). However, the static nature of such studies does not allow any conclusions to be reached concerning either the magnitude or the importance of nutritional carbon transfer effected by this process (Fisher, 1990). In the well-studied algal-invertebrate symbioses it is accepted that in most associations the symbionts translocate a portion of the carbon they fix directly to their host (Muscatine, 1980; Muscatine et al., 1984). Although the compounds translocated vary in different associations, a common theme is release of small molecular weight compounds (usually sugars and/or amino acids) concomitant with fixation. In other words, the symbiotic algae release early fixation products at the time of photosynthetic carbon fixation. In one chemoautotrophic symbiosis, *Solemya reidi*, it was found that the bacteria apparently release chemosynthate on a similar time scale and magnitude to many algal-invertebrate associations (Fisher and Childress, 1986).

The undescribed hydrocarbon-seep mussel from the Gulf of Mexico contains abundant symbiotic methanotrophic bacteria in gill cells (Childress et al., 1986; Fisher et al., 1987) as does a deeper-living mussel collected from the Florida Escarpment (Paull et al., 1985; Cavanaugh et al., 1987). The hydrocarbon-seep mussel is capable of filter feeding (Page et al., 1990); however, a variety of evidence indicates that the symbionts are a major source of nutritional carbon for their host. The strong correlation between the stable carbon isotopic content ($\delta^{13}C$) of the mussels' tissue and of the methane in their environment, and the similarity between the $\delta^{13}C$ values of gill and non-gill tissue indicates that methane carbon is incorporated into the hosts' tissues and substantiates the nutritional role of the symbionts in this species (Childress et al., 1986; Brooks et al., 1987). Similarly, the $\delta^{13}C$ value of the Florida Escarpment mussel reflects the biogenic methane used as a carbon source by its symbionts (Paull et al., 1985; Cary et al., 1989; Martens et al., 1991). The demonstration of shell growth of the hydrocarbon-seep mussel, with methane as sole carbon and energy source, also indicates that the symbionts are meeting at least some of the nutritional needs of their host in this association (Cary et al., 1988). However, direct evidence of nutritional transfer between host and symbiont

has not been provided, nor has the mechanism of carbon exchange between the partners been investigated.

Since the symbionts in this association use methane as a carbon source, it provides a unique opportunity for an unambiguous investigation of carbon transfer using ^{14}C. In all of the other autotrophic associations similarly investigated the symbionts use CO_2 (or bicarbonate) as a carbon source, and since invertebrates also incorporate inorganic carbon directly, these studies are somewhat ambiguous with respect to the source of the carbon fixation. No metazoans incorporate methane directly so this is not a factor in this association. We addressed the question of nutritional carbon transfer in the Gulf of Mexico hydrocarbon-seep mussel symbiosis.

2. Materials and Methods

Collection and maintenance of mussels

The undescribed mytilids (seep mussels) were collected using the *Johnson Sea Link* from 600 to 700 m of water on the Louisiana Slope in the Gulf of Mexico (27° 41′N; 91° 32′W) on September 19, 1989. They were brought to the surface in an insulated container on the front of the submersible and placed in fresh chilled sea water (~7°C) immediately upon recovery. The mussels were maintained on board ship in 20 l plastic buckets at 7–9°C. The sea water in the buckets was bubbled with methane and air twice daily and changed every 36 hr. The animals were air-freighted back to the University of California, Santa Barbara where they were maintained in flowing sea water (7.5°C) bubbled with natural gas before use in the experiments initiated on October 17, 1989. The seep mussels in these experiments ranged in shell length from 34 to 42.5 mm and 1.6 to 4.4 g wet weight.

Several species of "seep mussels" have been found at different sites in the Gulf of Mexico (Paull et al., 1984; Brooks et al., 1987; Brooks et al., 1990; Fisher, submitted; McDonald et al., submitted) and are currently being described. Taxonomists and geneticists working on the symbiont-containing mytilids in the Gulf of Mexico have assigned them temporary designations (C. Craddock, R. Vrijenhoek, R. Lutz, and R. Turner, pers. commun.). Two species of seep mytilids have been collected from the shallow hydrocarbon/salt seep sites. One of these is very rare, and was only recently noted in collections of mussels from these sites (JJC, pers. obs.). The mussels used in this study belong to the common species from the shallow hydrocarbon seep sites, and have been given the species designation, Seep Mytilid Ia. Other publications concerning this same species of mussel are: (Childress et al., 1986; Brooks et al., 1987; Fisher et al., 1987; Kennicutt et al., 1988a,b; MacDonald et al., 1989;

MacDonald et al., 1990a,b; Page et al., 1990; Kochevar et al., 1992; Lee et al., 1992).

Histology

Pieces of gill tissue from freshly collected individuals were fixed in 3% glutaraldehyde in 0.1 M phosphate-buffered 0.35 M sucrose (pH 7.3) and stored in this fixative at 4°C for up to 2 weeks. The tissues were then washed in buffered sucrose, post-fixed in 1% osmium (on ice) for 1 hr, dehydrated through a graded ethanol series, and embedded in Spurr's embedding medium. Thin sections were cut using a Sorvall MT2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips 300 transmission electron microscope.

^{14}C-methane incubations

Twenty-five seep mussels and two intertidal mussels (*Mytilus edulis*) were incubated in sea water containing ^{14}C-methane. The mussels were placed in 900 ml of chilled 0.22 μm filtered sea water (7.5°C) containing 80 μM cold methane and 230 μM oxygen for 30 min before the addition of labeled methane. ^{14}C-methane dissolved in 140 ml of sea water was added to the incubation vessel bringing the total incubation volume to 1040 ml and the final activity of labeled methane to 0.146 μCi ml^{-1}. A fitted piece of "parafilm" was floated on the surface of the incubation vessel in order to minimize exchange with the atmosphere. The dissolved oxygen concentration was 100 μM after 90 min (1 hr after addition of labeled methane). Two hours after addition of the labeled methane all mussels were removed. The "pulse" seep mussels and controls (*M. edulis*) were placed in 1 l of sea water on ice for immediate dissection. The "chase" animals were placed in 4 l of sea water containing about 100 μM dissolved methane. After three changes of sea water (2 hr each) the "chase" animals were transferred to flowing seawater aquaria at 7.5°C for the remainder of the chase periods. Concentrations of total methane and oxygen in the incubation medium were determined by gas chromatography (Childress et al., 1984), and the activity of labeled methane in the incubation medium was determined by scintillation counting of replicate samples.

Dissection, sample preparation, and scintillation counting

Each mussel required 4–6 min for dissection. The exact times of dissection were noted for each "pulse" mussel and these times were used to calculate the rates of methane incorporation h^{-1}. The rates of incorporation by the "chase"

mussels are based on 2 hr exposure to labeled methane and have also been standardized to h^{-1}. All of the measured methane incorporation rates were further standardized to g wet weight of mussel soft tissue. The mussels were dried and drained of sea water by gently prying them open 2–3 mm. The adductors were severed and the gills separated from the remaining tissue. All fluid produced after the adductors were severed was collected and 0.05 ml aliquots were assayed for radioactivity using the same methods described below for tissue homogenates. Gill and non-gill tissues were processed separately using the same technique. The tissues were homogenized in a 9×volume of distilled, deionized water and replicate 0.1 ml samples were placed in scintillation vials. Samples were acidified with 0.1 ml of 2N HCl, allowed to dry overnight, then degassed twice with N_2 (5–10 sec each time) to assure the removal of residual volatile label (mostly $^{14}CH_4$ and $^{14}CO_2$). Samples were then digested with a quaternary ammonium hydroxide (TS-1, National Diagnostics) for 24 hr, neutralized with glacial acetic acid, and dissolved in 10 ml of scintillation cocktail (3a40, National Diagnostics). Disintegrations per minute were determined by scintillation counting on a Beckman LS 6800 counter, corrections being made for background and counting efficiency ($\sim$ 92%). Rates of methane fixation into organic carbon were calculated based on the specific activity of methane in the incubation media and the levels of acid stable, non-volatile radioactivity in the tissues and fluid.

3. Results

The results of the incubations of live seep mussels in sea water containing labeled methane are summarized in Table 1 and Fig. 1. It should be noted that these rates represent rates of accumulation of methane into organic material and not the total methane oxidation rates, which would be roughly twice these

Table 1. Results of pulse-chase incubation of seep mussel species Ia in ^{14}C-methane

Treatment	DPM g^{-1} (10^4)		μ mol CH_4 (incorp·$g^{-1}h^{-1}$)	(n)
	Total	non-gill		
Pulse (2–2.4 hr)	133 ±109	5.1 ±3.3	0.3 ±0.26	(6)
Chase (2 hr)	99 ±61	2.9 ±1.4	0.22 ±0.14	(6)
Chase (24 hr)	109 ±104	13.7 ±15.6	0.24 ±0.23	(6)
Chase (115 hr)	66 ±39	27.4 ±8.6	0.15 ±0.09	(7)

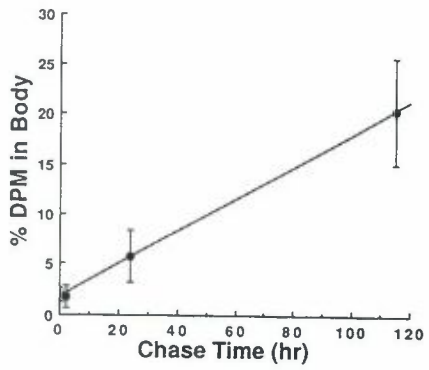

Figure 1. The percent of labeled organic carbon found in the symbiont free tissues (non-gill tissue) of seep mussel species Ia after chase periods in unlabeled methane ranging from 0 to 115 hr. The error bars represent ±1 standard deviation of the mean. n=6 for 0, 2, and 24 hr chase and 7 for 120 hr chase periods. The line shown is a linear regression of the full data set and the equation of the line is $y = 0.161x + 1.81, (r^2 = 0.89)$.

values (Fisher et al., 1987; Kochevar et al., 1992) due to oxidation of methane to CO_2. The absolute rates of incorporation varied by about one order of magnitude among individuals in all treatments, which was most likely due to a combination of behavioral differences among the mussels during the 2 hr pulse incubation period and possibly to differences in the symbiont complement among the mussels. Animals which remained closed for a significant portion of the pulse incubation were not exposed to the ^{14}C-methane for as long as mussels that remained open. Although metabolic rates vary allometrically with animals size, this was not a significant factor in this study for three reasons: (1) the experimental mussels were of fairly uniform size; (2) different sized mussels were distributed equally among the time points; and (3) there was no significant correlation between wet weight and methane incorporation rate.

The wet weights of gill and non-gill tissues and fluids were determined for each of the experimental mussels. Gill tissue accounted for 29.1±2.4% (mean ±SD, n=25) of the soft tissue wet weight in these 1.6 to 4.4 g seep mussels, determined as the difference between the gill wet weight and the sum of the wet weights of the tissues and fluids removed from the shell.

Over 97% of the acid-stable, non-volatile label was found in the symbiont-containing gill tissue after both the two-hour chase periods. About 94% of the label remained in this tissue after a 24 hr chase period, and the relative amount dropped to about 80% after 5 d. The percent of total counts found in

the fluid generated during dissection of the mussels averaged between 0.5 and 1.1% in each treatment and the amount was not significantly different between time points. The percent of total counts found in the non-gill tissue is shown in Fig. 1. This increase with time is not due to differential loss between the tissues, but rather to accumulation of label in the non-gill tissues (Table 1).

Two intertidal mussels (*Mytilus edulis*) were incubated with the seep mussels in the labeled methane. Radioactivity found in their tissues after 2 hr represented a rate of 0.0012 $\mu M \cdot g^{-1} \cdot h^{-1}$ of incorporation (more than 2 orders of magnitude less than the average rate of the pulse seep mussels) and was probably due either to free-living methanotrophs on their gills or in their mantle cavity (the *M. edulis* were held with the experimental animals in the presence of methane for several days prior to the experiment), or to uptake of labeled dissolved organic material released by the seep mussels.

Examination of transmission electron micrographs provided several lines of supporting evidence for the digestion of symbionts by the host mussels. The symbionts are localized at the apical end of the bacteriocytes (near the filament surface) (Fig. 2a). A significant fraction of the basal portion of the host bacteriocytes is occupied by what has been described by other authors as "myelin-like" figures (Fig. 2a), which are apparently secondary lysosomes containing the remnants of digested symbionts (Bosch and Grasse, 1984; Fiala-Médioni et al., 1986, 1990). In this association, the distinctive stacked internal membranes and electron translucent accumulation bodies of the symbionts make the symbionts very easy to recognize, even in the latter stages of digestion (Fig. 2a,b). These partially digested symbionts were visible in micrographs of virtually every bacteriocyte examined, and are found associated with the secondary lysosomes. Degeneration of the symbionts seems to originate at the outer surface of the bacterial cells, suggesting that it is a result of forces external to, and not inside of, the symbionts.

4. Discussion

Pulse-chase incubations of autotrophic symbioses with ^{14}C-inorganic carbon have been used by many investigators to demonstrate translocation of fixed carbon from symbiont to host tissues. Although this technique underestimates the magnitude of translocation (Muscatine et al., 1984), when applied correctly it has proven adequate to the demonstration of the phenomenon of translocation. Typical experiments using this methodology with zooxanthellae-invertebrate symbioses have found around 40% (with a range of 20 to 60%) of the fixed carbon translocated to host tissues during pulse incubations in sea water with added $NaH^{14}CO_3$ (Muscatine et al., 1984; Trench,

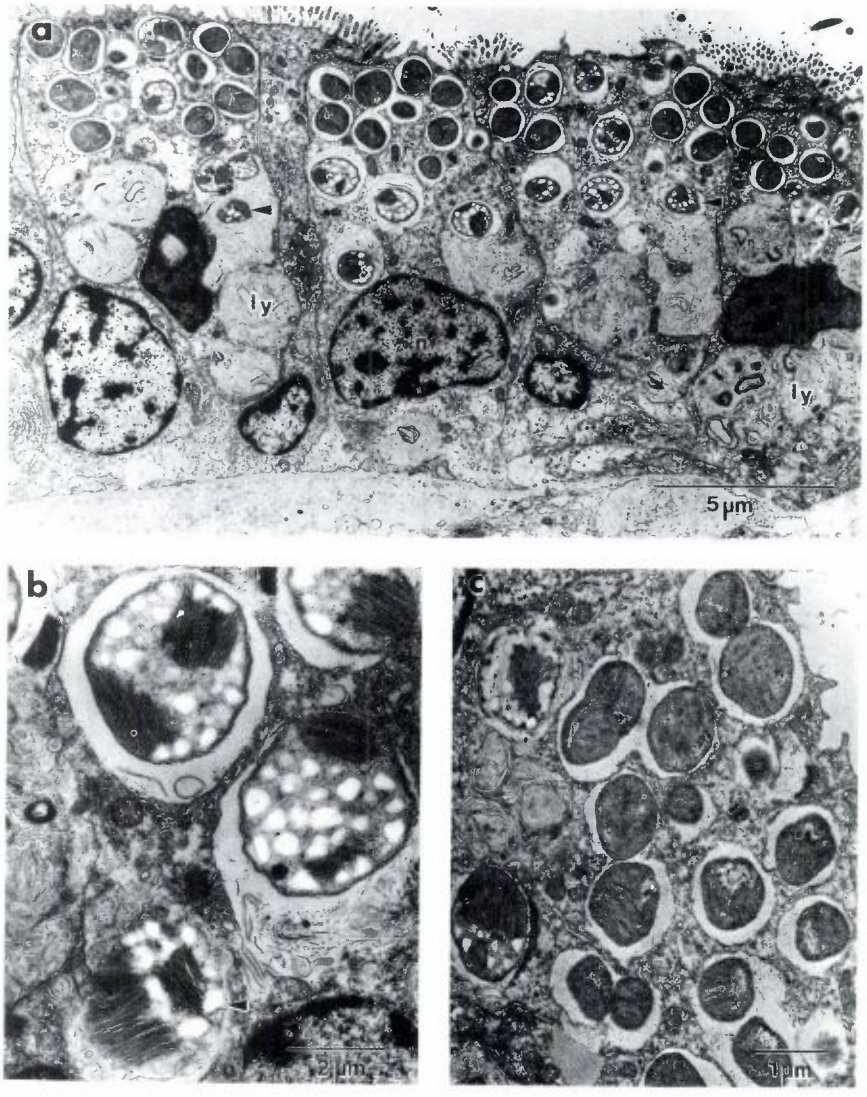

Figure 2. Electron micrographs of sections through gill filaments of seep mussel species Ia.
(a) Bacteriocytes and interstitial cells along one surface of a gill filament. The external surface (with cilia and microvilli on the interstitial cells) is at the top of the micrograph and the blood space between the two surfaces of the filament is at the bottom. A few of the partially digested symbionts are indicated by arrows. ly-lysosome, n-bacteriocyte nucleus (×7300), scale bar = 5 μm.
(b) A higher magnification view of a few symbionts in various stages of lysosomal fusion and digestion. Note the absence of a cell wall on the symbiont indicated by the arrow (×20000). Scale bar = 2 μm.
(c) View of a portion of a bacteriocyte containing several symbionts in the process of division. (×14000). Scale bar = 1 μm.

1974) reviewed by Trench (1979), Muscatine (1980), Cook (1983). Fisher and Childress (1986) found over 40% of the labeled organic carbon in symbiont-free tissues of *Solemya reidi* (a gutless clam with chemoautotrophic bacterial symbionts) after only a 10 min pulse incubation in $NaH^{14}CO_3$, and calculated that a minimum of 45% of the carbon fixed by the symbionts is promptly translocated to the host. Herry et al. (1989) investigated translocation of fixed carbon from symbionts to host in a lucinid clam with chemoautotrophic symbionts, *Loripes lucinalis*. However, because the investigators did not supply the symbionts with an energy source, used non-quantitative methods (tissue autoradiography), and obtained data from only a single chase period (4 day), one cannot draw conclusions concerning either the mode or magnitude of carbon transfer in this association. Distel and Felbeck (1988), examined the release of labeled carbon products by freshly isolated symbionts from the lucinid clam, *Lucinoma aequizonata*. Although their results should be considered preliminary because the symbionts were not supplied an energy source nor were the potential effects of a host factor tested (Fisher, 1990), these investigators found that less than 5% of the fixed carbon was released into the media by the isolated chemoautotrophic symbionts.

Fiala-Médioni et al. (1986b) conducted *in situ* incubations with the hydrothermal-vent mytilid, *Bathymodiolus thermophilus*, in labeled bicarbonate. After a 23.5 hr *in situ* incubation in a sealed container, between 77 and 93% (n=4) of the label was found in the gill tissue, a finding similar to ours, in a related mussel. As the authors acknowledge, heterotrophic incorporation of label was likely a partial contributor to the measured incorporation, a factor which could be substantial if the oxygen tensions dropped significantly during the incubations. However, autoradiography of gill pieces indicated that most of the non-soluble label resided in the portion of the bacteriocytes where the symbionts reside, suggesting substantial autotrophic carbon fixation.

The results of the pulse-chase incubations of the seep mussel in ^{14}C-methane (Table 1 and Fig. 1) were dramatically different from similar previous studies. After both the two hour pulse incubation and the two hour chase period, over 95% of the labeled organic carbon was found in the gill tissues of all animals and the average value in both groups was over 97%. Even after a 24 hour chase period, 93.6±3.2% (av±SD, n=6) of the label was still in the gill tissue. These data indicate that organic carbon compounds derived from the oxidation of methane were not translocated in significant quantities from the symbiotic bacteria to the host tissues in the same manner as in numerous other autotrophic symbioses. This difference in the time course of transfer of labeled organic carbon is too great to be attributed simply to differences in the carbon fixation pathways or to translocation of more complex molecules in the case of

the methanotrophic symbiosis. Rather, it is a reflection of a basic difference in the mode of carbon exchange in this symbiosis. It should be noted that this study is unlike other similar experiments which used $NaH^{14}CO_3$ as a tracer, in which a lower specific activity of labeled CO_2 inside host tissues would lead to an underestimate of fixation rates. Since methane is not a respiratory end product of animals, there should be no difference in the specific activity of the methane inside or outside of the mussels.

Although we made no attempt to separate the symbionts from the host tissues in this study (and therefore cannot distinguish between label in the symbionts and label in host tissues) it was clear that appreciable label was not translocated to the symbiont-free tissues in either the pulse or 2 hour chase period. However, $\delta^{13}C$ analyses of paired tissues (gill and either foot or mantle) from individual seep mussels indicated that the bulk (if not all) of the organic carbon in the symbiont-free tissues was derived from methane (Brooks et al., 1987, and J.M. Brooks, pers. commun.). The mechanism for this transfer is suggested by the results from the longer chase periods. After a 5 day chase period, an average of 20.9±5.4% (±SD, n=7) of the labeled organic carbon was in symbiont-free tissues (non-gill) of the experimental animals. Furthermore, because the absolute amount of label in non-gill tissues was significantly higher after both 24 and 115 hr chase periods ($p = 0.009$ and 0.0001 respectively in a comparison of means by a one-tailed t-test), one can conclude that the change in the relative distribution of the label was not due simply to differential loss of label from the different tissue classes, but rather to quantitative transfer of label from the gills to non-gill tissues (Table 1). This long delay in the transfer of label from the gills to symbiont-free tissues supports the hypothesis that the host mussels are obtaining nutritional carbon from their symbionts by digesting the symbionts and not through the process of translocation.

Ultrastructural studies have been interpreted by many investigators as providing evidence of intracellular digestion of symbiotic chemoautotrophic bacteria by hosts ranging from vestimentiferan tube worms (Bosch and Grasse, 1984), pogonophoran tube worms (Southward, 1982), and oligochaetes (Giere, 1985; Giere and Langheld, 1987) to a variety of bivalve mollusks (Fiala-Médioni, 1984; Vetter, 1985; Distel and Felbeck, 1987; Fiala-Médioni and Le Pennec, 1987, 1988; Southward, 1987; Fiala-Médioni et al., 1990). Most of these investigators note that such studies must be interpreted with caution because their static nature yields no information on the magnitude or time course (and therefore importance with respect to transfer of chemosynthate). Several of the investigators suggest that symbiont digestion in those associations may be a reflection of normal "cellular housekeeping" (Distel and Felbeck, 1987; de Burgh et al., 1989) and not the main pathway of organic carbon transfer

between symbiont and host. Basically, three types of ultrastructural evidence are cited as supporting bacterial digestion by the hosts: (1) presence of symbionts in "various stages of digestion" in host cells (Bosch and Grasse, 1984; Giere, 1985; Southward et al., 1986; Giere and Langheld, 1987; de Burgh et al., 1989); (2) merging of host lysosomes with vacuoles containing senescent bacteria or "lysosome-like" bodies containing bacteria (Vetter, 1985; Fiala-Médioni and Le Pennec, 1988; Fiala-Médioni et al., 1990); and (3) vacuoles containing abundant membrane whorls or "myelin-like figures" (Bosch and Grasse, 1984; Distel and Felbeck, 1987), that resemble figures typically seen in lysosomal residual bodies. In light of the results of the ^{14}C-methane incubations described above, micrographs of gill tissues from the seep mussel were examined and all three of these types of ultrastructural evidence for bacterial digestion were identified.

Senescent bacteria and bacteria in various stages of disintegration in host lysosomes were present in every gill cell examined (some examples are found in Fig. 2). Most of these bacteria contained internal electron lucent bodies which are most likely bacterial reserve substances such as polyhydroxybutyrate (Anthony, 1982). That the myelin-like figures are in fact secondary lysosomes is supported by the fact that bacteria in final stages of digestion are often clearly inside these organelles (Fig. 2a,b). As previously noted, the distinctive symbiont morphology, with stacked internal membranes and electron lucent accumulation bodies, renders them easily recognizable, even in the later stages of digestion. Trench (1974) argues convincingly that the senescent or "pycnotic" zooxanthellae visible in many micrographs of *Zoanthus sociatus* (an anemone which harbors symbiotic algae) are a result of autolysis and not host digestion, based on the fact that internal algal structures degrade before the cell wall. In contrast, the remains of bacterial cells with clearly recognizable internal membranes, and no cell walls, were often visible inside host lysosomes (Fig. 2a,b). Perhaps the most striking piece of ultrastructural evidence was the shear volume of host cells occupied by lysosomes showing myelin-like figures. In micrographs of many cells (such as Fig. 2a) these organelles occupied almost one-third of the cross sectional area of the cell. Numerous dividing bacteria were also seen in many host gill cells (Fig. 2c). We made no effort to quantify the numbers of dividing symbionts because only cells dividing in the plane of the section would be counted "as undergoing division" and thus our counts would significantly underestimate the number of dividing cells. However, it was obvious from examination of the micrographs that the symbionts were actively dividing in the host tissues.

These ultrastructural and physiological data support the hypothesis that transfer of nutritional carbon between symbiont and host in this symbiosis is

primarily through digestion of the symbionts in gill bacteriocytes. The abundance of similar ultrastructural evidence in other chemoautotrophic symbioses suggests that this mechanism may be important in other associations as well. However, the demonstration of the importance of translocation concomitant with fixation in *Solemya reidi*, an association in which some similar ultrastructural evidence of digestion is also evident, indicates that additional physiological data is necessary before any conclusions can be reached concerning the relative importance of these processes.

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