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Assimilation of Inorganic Nitrogen by Marine Invertebrates and Their Chemoautotrophic and Methanotrophic Symbionts

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Symbioses between marine invertebrates and their chemoautotrophic and methanotrophic symbionts are now known to exist in a variety of habitats where reduced chemical species are present. The utilization of chemical energy and reliance on C_1 compounds by these symbioses are well documented. Much less is known about their metabolism of nitrogen. Earlier work has shown that the tissues of organisms in these associations are depleted of ^{15}N compared with those of other marine organisms, indicating that local sources of nitrogen are assimilated and that novel mechanisms of nitrogen metabolism may be involved. Although these symbioses have access to rich sources of ammonium (NH_4^+ and NH_3) and/or nitrate, several investigators have proposed that N_2 fixation may account for some of these isotope values. Here we report that [^{15}N]ammonium and, to a lesser degree, [^{15}N]nitrate are assimilated into organic compounds by *Solemya reidi*, a gutless clam containing S-oxidizing bacteria, and seep mussel Ia, an undescribed mytilid containing methanotrophic bacteria. In contrast, *Riftia pachyptila*, the giant hydrothermal vent tube worm symbiotic with S-oxidizing bacteria, assimilated nitrate but not exogenous ammonium. The rates of assimilation of these sources are sufficient to at least partially support C_1 compound metabolism. N_2 assimilation was not exhibited by the symbionts tested.

The finding that the dominant marine invertebrates living in association with deep-sea hydrothermal vents are supported by chemolithoautotrophic sulfur-oxidizing bacterial endosymbionts (5, 15) led to the investigation of similar symbioses in other habitats where reduced chemical species are present. Such symbioses are now known to occur in sulfidic environments, such as sewage sludge outfalls, eelgrass beds, anoxic basins, and pulp-mill effluent sites. In addition, symbioses between deep-sea mussels and methanotrophic endosymbiotic bacteria are found on the Louisiana Slope of the Gulf of Mexico (9, 38) and on the Florida Escarpment (6). The physiology of the organisms involved in this association, which will be referred to hereafter as symbioses, has been the subject of two recent reviews (8, 17). In the present study, we investigated assimilation of dissolved nitrogen compounds by three such symbioses: *Solemya reidi*, *Riftia pachyptila*, and an undescribed deep-sea mussel containing methanotrophic symbionts (seep mussel Ia).

S. reidi is a gutless protobranch bivalve (45) that inhabits burrows at sewage sludge outfalls and pulp-mill effluent sites. The sulfur-oxidizing symbionts are housed within vacuoles in bacteriocyte cells of the gill filaments. These bacteria are rod-shaped and have the cell wall ultrastructure typical of gram-negative bacteria (4, 16, 23). Thiosulfate and hydrogen sulfide are oxidized by the symbionts, resulting in net fixation of CO_2 by the intact association (1). *R. pachyptila*, the first chemoautotrophic symbiosis discovered, is a large vestimentiferan tube worm found around deep-sea hydrothermal vents. The symbionts are sulfide oxidizers (21, 53) and are densely packed in an organ called the trophosome which makes up on average 15% of the association's wet weight. Metabolites are supplied to the trophosome by the host's circulatory system (7). Net fixation of CO_2 has been demonstrated (10), and a high pH inside the host relative to that of the environment acts to concentrate inorganic carbon around the symbionts (11). Seep mussel Ia, the best-studied methanotrophic symbiosis, is found around hydrocarbon seeps on the Louisiana Slope of the Gulf of Mexico (9). The endosymbionts are housed within the mussel gill and contain the stacked internal membranes typical of type I methanotrophs (9, 22). Net assimilation of C based solely on methane uptake by the intact association and shell growth with methane as the sole carbon source have been demonstrated (3, 9, 32).

Although C_1 compound metabolism by these associations has been much studied, little is known about nitrogen sources. The environment that is encountered by the symbionts reflects the surrounding seawater but is also governed by the physiology of the host. The waters inhabited by these symbioses are generally rich in inorganic nitrogen sources. Many of these symbioses are found in the deep ocean where nitrate concentrations are usually around 40 μM (28). In addition, high concentrations of ammonium ranging up to millimolar concentrations have been observed in sediment pore water (30, 35) and vent or seep effluent (37, 47). Nutrients must first pass through the tissue of the host before they can be assimilated. In the case of nitrate, an inability for the host to transport nitrate, since it is not metabolized by heterotrophic metazoans, may restrict its availability to the symbionts. Ammonium could be acquired both from the environment and host amino acid catabolism and may be abundant within host tissues, increasing the availability to symbionts over that of their free-living counterparts. Other than stable nitrogen isotope measurements demonstrating that these organisms are anomalously depleted of ^{15}N compared with organisms found elsewhere in the marine environment (2, 12, 13, 17, 20, 31, 44), only a few studies have attempted to assess nitrogen sources directly (35, 36, 43). One belief that has emerged from the stable nitrogen isotope data is that the symbionts may be capable of nitrogen fixation (2, 17, 31). Although consistent with the isotope data, it is not a reasonable assertion a priori, since these associations are likely to preferentially utilize the high concentrations of ammonium and nitrate present in their environment. In the present study, using ^{15}N-labelled tracers, we also found that ammonium and nitrate can be assimilated and that nitrogen

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fixation is not an important source of nitrogen to these associations. This is of particular interest, given the novel environment with respect to ammonium and nitrate that is encountered by chemotrophic symbionts in association with invertebrates and the potentially rich environments encountered by the associations themselves.

MATERIALS AND METHODS

Water samples. Sediment pore water from sludge in which *S. reidi* were collected was placed in a cylindrical polyvinyl chloride vessel. The vessel was then pressurized with N_2 gas (10 lb/in^2), which forced the pore water through a filter plate constructed of coarse-mesh Nitex and an ashed-glass-fiber filter supported by a perforated acrylic disk (35). The sample was then filtered (pore size, 0.22 μm) and frozen at −30°C until analysis. Water samples around *R. pachyptila* were collected with titanium samplers either from the DSRV *Alvin* or *Nautile* submarine and analyzed immediately on board. Water samples around seep mussels were collected by using the sampling port of the DSRV *Sealink* submarine. The sampler, developed by C. R. Fisher, has a small dead volume and an inlet consisting of sintered metal. Following flushing of the sampler, a few milliliters of sample was collected into a syringe and analyzed immediately on board. Analyses of ammonium and nitrate were performed by standard colorimetric methods adapted to flow injection analysis (29, 52). Routine sensitivity of less than 0.5 μM and precision of 1 to 3% were obtained.

Organisms. *S. reidi* were collected by Van Veen grab near the Hyperion sewage sludge outfall in Santa Monica Bay, Calif., at a depth of 90 to 110 m during March 1991. Clams were maintained in the laboratory in tanks containing sewage sludge and irrigated with seawater at 5 to 9°C as described previously (1). *R. pachyptila* were collected from the Genesis (12°48.68′N, 103°56.39′W), Parigo (12°48.60′N, 103°56.38′W), and Elsa (12°48.18′N, 103°56.24′W) sites on the East Pacific Rise from depths of around 2,600 m by submarine during November 1991 and April 1992. Worms were kept in a thermally insulated box during recovery and then maintained on board at 12.2 or 21.4 MPa in pressurized flowing-water aquaria at 8°C. Seep mussels were collected on the Louisiana Slope of the Gulf of Mexico (27°41′N, 91°32′W) by submarine during August 1991. Mussels were kept in a thermally insulated box during recovery, maintained on board at 5 to 7°C, and periodically provided with methane and oxygen. Mussels were maintained in the laboratory in flowing-seawater tanks at 5 to 7°C bubbled with methane. *Mytilus edulis* were collected from pilings on the Goleta Pier in Santa Barbara, Calif., and maintained in flowing-seawater tanks.

Experiments. Incubation conditions for tracer experiments were as follows. The ^{15}N substrate concentrations for $^{15}NH_4^+$ or $^{15}NO_3^-$ experiments were 50 μM. *S. reidi* were incubated for 2 to 48 h in flowing-water aquaria under conditions of pH 7.5 to 8.0 and 5 to 7°C. *R. pachyptila* were incubated in a pressurized aquarium with recirculating flowing water under the following conditions: 3 g of MOPS (morpholinepropanesulfonic acid) buffer liter^{-1}, pH 6.8 to 8.0, 5 to 10°C, and 12.2 or 21.4 MPa for 12 h. *Riftia* and *S. reidi* incubations were both conducted under conditions of 2.5 mM ΣCO_2 (20% $^{13}CO_2$), 100 μM H_2S, and 100 μM O_2. Seep mussels were maintained in static-water aquaria under conditions of 100 to 200 μM $^{13}CH_4$, 100 μM O_2, pH 8.0, and 5 to 7°C for 3 to 24 h. *M. edulis* were incubated in static-water aquaria under conditions of pH 8.2 and 10°C for 5 h. $^{15}N_2$ incubations of *S. reidi* or seep mussels were performed in respirometers containing 600 ml of helium-purged seawater enriched with 500 μM thiosulfate or 300 μM $^{12}CH_4$, respectively, and equilibrated with 100 ml of >99% doubly labelled $^{15}N_2$. Oxygen was added as needed by admitting O_2 gas through a septum with a syringe. The concentrations of ammonium and nitrate were determined by flow injection analysis, and the concentrations of dissolved gases were determined by gas chromatography (7).

Isotope analysis. Samples were dried at 60°C and ground to a fine powder. To remove ammonium and ΣCO_2, samples were treated with 2 N NaOH, kept at 20 to 25°C for 24 to 48 h, treated with 2 N HCl, and then dried at 60°C. In Results, assimilation refers to incorporation of ^{15}N and ^{13}C into this fraction. Various quantities (1 to 2 mg) of treated sample were placed in tin capsules and weighed to the nearest 0.001 mg with a Cahn electrobalance. An automated CHN analyzer (Roboprep-CN; Europa Scientific) interfaced with an isotope ratio mass spectrometer (Tracermass; Europa Scientific) was used to determine $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios as well as the percentages of N and C present in these samples. The encapsulated samples were flash-combusted at 900°C and reduced over copper metal at 550°C. The N_2 and CO_2 produced were separated by gas chromatography and then admitted directly to the isotope ratio mass spectrometer for determination of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios. We routinely obtained precision of less than 0.01 atom% [(^{15}N/total N) × 100] for ^{15}N and 0.001 atom% for ^{13}C and ±0.1% for the percentages of C and N with reference materials (NBS 1572, citrus leaves). The rates of assimilation are given as micromoles gram (wet weight) of tissue^{-1} h^{-1}. The difference in percent of heavy isotope present in samples from tracer experiments and from animals not exposed to labelled compounds was determined and then converted to micromoles gram (wet weight) of tissue^{-1} from percent water and percent C or percent N results.

RESULTS

Water samples. Unlike many other marine environments, the environments inhabited by these symbioses were found to be rich in ammonium and/or nitrate. Pore water from sediment where *S. reidi* were collected contains 54 to 64 μM ammonium (35), and in the present study, we found that nitrate is also present (Table 1). At 13°N on the East Pacific Rise, negligible amounts of ammonium were found around *R. pachyptila* clumps, but nitrate was always present and as high as 37.5 μM. The sediment pore water at the hypersaline cold seeps where seep mussel Ia is found is rich in ammonium (30), as was hypersaline effluent collected at the brine pool, a brine-filled pockmark surrounded by mussels (40) (Table 1). The ammonium concentrations in water overlying mussels were variable, since seep effluent and pore water mix with ambient bottom water around the mussels. We observed concentrations ranging from 1.6 to 13 μM. Nitrate was also present in these samples and ranged from 9.4 to 41 μM.

Tracer experiments. The incorporation rates of ^{15}N and ^{13}C presented may be problematic if incorporation during the incubation period were not linear. Linearity of incorporation for up to 48 h was observed in preliminary tests for both *S. reidi* and seep mussel Ia. Therefore, the increases observed are valid as rates. For *R. pachyptila*, we did not attempt a time course experiment, primarily because of technical considerations. Although we maintained a consistent protocol for all incubations that allowed the worms several hours under experimental conditions before the addition of labelled substrates, we do not know what sort of physiological and behavioral inconsistencies there were over the course of the 12-h incubation period. The rates of ^{15}N and ^{13}C incorporation were measured from tissue samples treated with NaOH and HCl to remove ammonium

TABLE 1. Ammonium and nitrate concentration ranges in environments of chemoautotrophic and methanotrophic symbioses

Species and sampling location	Concn (μM) range[a]		Reference(s)
	Ammonium	Nitrate	
S. reidi			
Hyperion sewage sludge outfall, Calif.			
Pore water	54–64	1.5–10.8	35; this study
Seep mussel Ia			
Louisiana Slope, Gulf of Mexico			
Pore water	33–1,414	ND	39
Over mussels	1.6–13	9.4–41.0	This study
Brine from brine pool	9,350, 6,883	6.7	This study
R. pachyptila			
Rose Garden	0–2	8–40	28
13°N East Pacific Rise	<0.1–2.7	18.3–37.5	This study

[a] Values shown are ranges, except for brine pool values, for which only the measurement(s) shown was made. ND, not determined.

and CO_2 and are referred to as assimilation. If samples contain unassimilated $^{15}NO_3^-$, this would result in overestimation of assimilation rate, particularly in $^{15}NO_3^-$ incubations. However, the amount of $^{15}NO_3^-$ present was likely negligible. For $^{15}NO_3^-$ incubations, assuming that $^{15}NO_3^-$ concentrations in the tissue were in equilibrium with the medium, the presence of $^{15}NO_3^-$ would have resulted in overestimates of only 0.001 to 0.025 μmol g^{-1} h^{-1}. Dialysis (100-molecular-weight cutoff) of a subset of samples for 48 h against distilled water resulted in 22 to 50% loss of both carbon and nitrogen label, indicating that low-molecular-weight organic compounds were removed in addition to label present as $^{15}NO_3^-$. These results do demonstrate that ^{15}N incorporation observed in $^{15}NO_3^-$ incubations was not primarily in the form of unassimilated $^{15}NO_3^-$.

Both $^{15}NH_3$ and $^{15}NO_3^-$ were assimilated by *S. reidi* and seep mussel Ia, whereas $^{15}NO_3^-$ and negligible $^{15}NH_3$ were assimilated by *R. pachyptila* (Table 2). Because of the difficulties involved in maintaining *R. pachyptila* under high hydrostatic pressure, not all worms exhibited high rates of $^{13}CO_2$ assimilation. Data given in Table 2 are from worms that assimilated >1 μmol of C g^{-1} h^{-1}. Negligible assimilation rates of $^{15}NH_3$ and $^{15}NO_3^-$ were exhibited by a nonsymbiotic mussel, *M. edulis*. The rates of $^{15}NO_3^-$ assimilation exhibited by *S. reidi* and seep mussel Ia were generally lower than the rates exhibited for $^{15}NH_3$ assimilation. The rates of $^{15}NO_3^-$ assimilation were particularly low in seep mussel Ia; however, higher assimilation rates have been observed in later studies and appear to be dependent on collection site (34). Assimilation of N_2 was not observed in either *S. reidi* gill or seep mussel Ia gill or mantle. N_2 assimilation was not analyzed for *R. pachyptila* or *M. edulis*. When assimilation of $^{15}NH_3$ or $^{15}NO_3^-$ was observed, it was primarily in the symbiont-containing tissue.

Strong correlations were observed between carbon and nitrogen assimilation when significant assimilation was observed (Fig. 1). These findings indicate that carbon and nitrogen assimilation are dependent on each other or that they covary. Comparison of C/N assimilation ratios with C/N ratios of the tissues of organisms in the associations gives an indication of whether these sources are sufficient to meet the nitrogen demand in support of C_1 compound metabolism. For *R. pachyptila*, the C/N assimilation ratio observed for ammonium was around 40 and no significant correlation between C and N assimilation was observed (Table 3). The nitrate C/N assimilation ratio was 11.73 compared with a C/N ratio of 3.92, indicating that nitrate may be the more important source of nitrogen that can at least partially meet nitrogen needs in support of ΣCO_2 assimilation. For *S. reidi*, the ammonium C/N assimilation ratio was 3.95 compared with 12.65 for nitrate. Thus, ammonium assimilation may be sufficient to support ΣCO_2 assimilation and nitrate can partially support nitrogen needs. For seep mussel Ia, the ammonium C/N assimilation ratio was 12.70, indicating that ammonium can support a substantial portion of nitrogen needs. The nitrate C/N assimilation ratio was around 200, with no significant correlation between C and N assimilation observed, indicating that for mussels in the present study, nitrate assimilation contributed negligibly to the nitrogen needs of the association.

DISCUSSION

One characteristic of communities based on symbiotic chemosynthesis is high biomass compared with elsewhere in the

TABLE 2. Assimilation of ^{15}N-labelled inorganic nitrogen sources by chemoautotrophic and methanotrophic symbioses

Species and tissue	Symbiont type	^{15}N assimilation rate (μmol of N g^{-1} h^{-1}) [mean ± SD (*n*)][a]		
		$^{15}NH_3$ incubation	$^{15}NO_3^-$ incubation	$^{15}N_2$ incubation
S. reidi	Thiotrophic			
Gill		0.505 ± 0.252 (18)	0.078 ± 0.066 (13)	0.0004 ± 0.0003 (3)
Mantle		0.059 ± 0.023 (19)	−0.011 ± 0.015 (12)	—
R. pachyptila	Thiotrophic			
Trophosome		0.032, 0.051 (2)	0.359 ± 0.388 (8)	ND
Plume		−0.012, 0.037 (2)	0.048 ± 0.019 (8)	ND
Seep mussel Ia	Methanotrophic			
Gill		0.361 ± 0.161 (16)	0.061 ± 0.047 (17)	0.001 ± 0.001 (3)
Mantle		0.046 ± 0.042 (18)	0.045 ± 0.040 (19)	0.001 ± 0.001 (3)
M. edulis	Nonsymbiotic			
Gill		0.022 ± 0.018 (5)	0.006 ± 0.003 (5)	ND
Mantle		0.012 ± 0.011 (5)	0.005 ± 0.005 (5)	ND

[a] Values are means ± standard deviations, except for the values for $^{15}NH_4^+$ incubation of *R. pachyptila* for which only the two measurements are shown. —, not detected; ND, not determined.

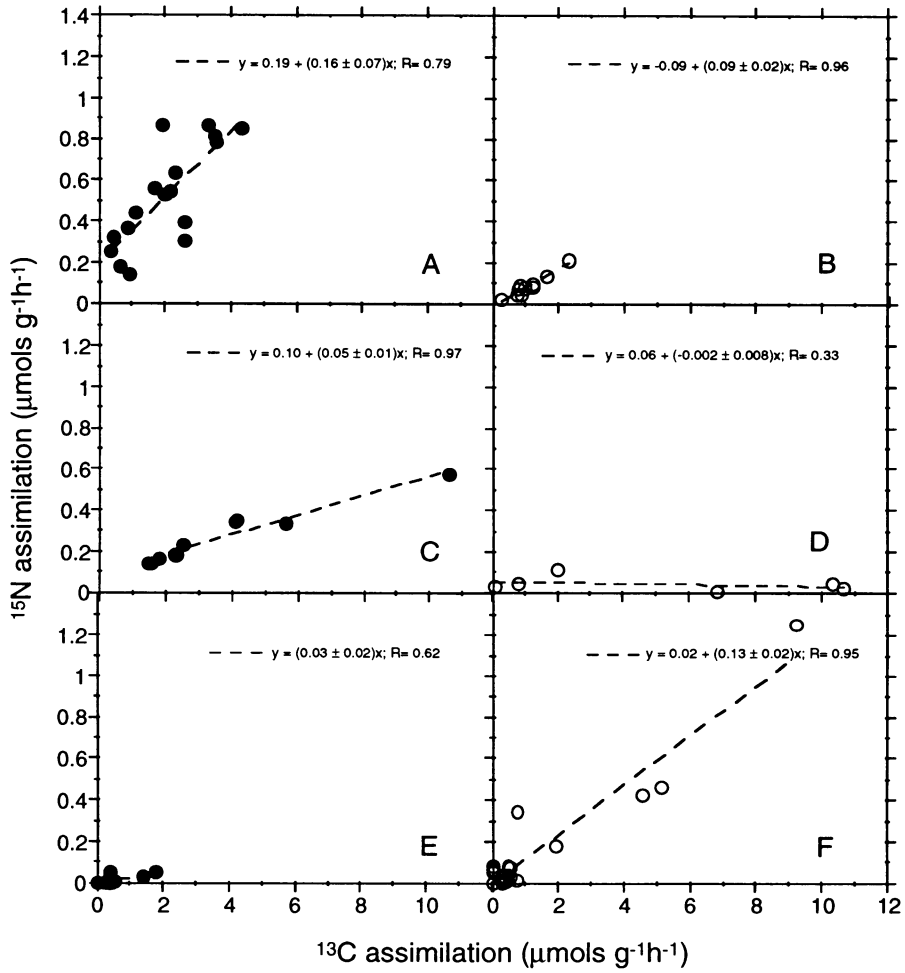

FIG. 1. Relation between ^{13}C and ^{15}N assimilation rates in symbiont-containing tissue for different individuals. (A) *S. reidi* ammonium incubations; (B) *S. reidi* nitrate incubations; (C) seep mussel Ia ammonium incubations; (D) seep mussel Ia nitrate incubations; (E) *R. pachyptila* ammonium incubations; (F) *R. pachyptila* nitrate incubations. Slopes of regression lines are given with 95% confidence intervals.

deep ocean. Reduced compounds such as sulfide and methane provide the energetic basis for this high productivity. Mechanisms for assimilation of inorganic nutrients necessary for growth on C_1 compounds have not been extensively investigated, and the role of nutrient availability in regulating biomass has not been assessed. From the results of the present study and previous findings, it is clear that the environments inhabited by symbiotic associations between chemosynthetic bacteria and marine invertebrates are often rich in ammonium and/or nitrate and that these sources can be assimilated. The abundance of nitrate and ammonium at these sites may be a factor that enables high productivity, and spatial variation in availability may affect the distribution of these organisms.

TABLE 3. C/N assimilation ratios and tissue C/N ratios

Species	C/N assimilation ratio [mean ± SD (*n*)][a]		Tissue C/N ratio [mean ± SD (*n*)]
	Ammonium	Nitrate	
S. reidi	3.95 ± 1.93 (17)	12.65 ± 2.70 (10)	4.62 ± 0.50 (26)
R. pachyptila	43.27, 35.07	11.73 ± 3.62 (8)	3.92 ± 0.51 (6)
Seep mussel Ia	12.70 ± 2.85 (9)	204 ± 277 (7)	4.23 ± 0.56 (27)

[a] Values shown are means ± standard deviations, except for ammonium assimilation ratios for *R. pachyptila* for which only the two measurements are shown.

The physiological capabilities with respect to ammonium and nitrate incorporation of these symbioses differ and, interestingly, correspond to the availability of ammonium and nitrate in their environments. For *S. reidi*, ammonium is more abundant in pore water than nitrate is (Table 1). In terms of assimilation, the capability to assimilate nitrate was lower than that for ammonium. At 13°N on the East Pacific Rise where we collected *R. pachyptila*, negligible amounts of ammonium are present, and unlike any other symbiosis tested, these worms did not exhibit ammonium assimilation. Comparative studies on *R. pachyptila* collected from hydrothermal vents where ammonium is abundant, e.g., the Endeavor Segment of the Juan de Fuca Ridge (37) or the Guaymas Basin (47), are necessary to assess whether the inabilities to take up and assimilate ammonium are general features of these worms. At the cold seeps on the Louisiana Slope of the Gulf of Mexico, both ammonium and nitrate can be abundant. Seep mussels exhibited high rates of ammonium assimilation, but in the present study, rates of nitrate incorporation were low.

These symbionts did not assimilate $^{15}N_2$. Previous findings of stable nitrogen isotopic compositions in tissue similar to that of dissolved N_2 (31), as observed in aquatic diazotrophs (41, 48), led to the belief that these symbioses may assimilate N_2. This is difficult to reconcile with the finding that ammonium and/or nitrate are assimilated and abundant in these environments. Furthermore, for symbiont nitrogen fixation to occur within an ammoniotelic aerobic host, mechanisms for maintenance of low levels of oxygen and ammonium around the symbionts are likely required. In the present study, we found that $^{15}N_2$ was not assimilated by *S. reidi* and seep mussel Ia. Although we did not test for N_2 assimilation in *R. pachyptila*, the absence of acetylene reduction by trophosome tissue has been observed (19). We conclude that combined sources and not N_2 are the forms of inorganic nitrogen assimilated by these symbioses in support of C_1 compound assimilation.

The entire range of $\delta^{15}N$ values exhibited by chemoautotrophic and methanotrophic symbionts can be accounted for by the assimilation of nitrate and ammonium. The $^{15}N/^{14}N$ ratio (expressed in δ notation and where $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where R is the $^{15}N/^{14}N$ ratio and standard refers to atmospheric N_2) of an organism reflects its sources of nitrogen and the mechanisms involved in assimilation. A distinguishing feature of chemosynthetic symbioses is that they are depleted of ^{15}N, with $\delta^{15}N$ values ranging from -13 to $+5.0‰$ compared with the values of ≈ 5 to $15‰$ observed for many other marine organisms (2, 12, 13, 17, 20, 31, 44). These low $\delta^{15}N$ values may be the result of assimilation under conditions in which combined nitrogen concentrations are high. Combined nitrogen is abundant in situ, and it is reasonable to speculate that it is also abundant within the association. Since biochemical conversions generally favor ^{14}N substrates over ^{15}N substrates, organisms may exhibit low $\delta^{15}N$ values when substrate pools act as an infinite reservoir. Isotopic discrimination is expressed by the symbol α, which is related to the $\delta^{15}N$ values by the relation $\alpha = \Delta + 1$, where $\Delta = (\delta^{15}N_{source} - \delta^{15}N_{product})/1{,}000$. The ammonium assimilation enzymes glutamine synthetase and glutamate dehydrogenase discriminate against ^{15}N and exhibit α values (for NH_4^+) of 1.008 to 1.013 and 1.002 to 1.010, respectively (27, 50). This explains in part the maximum in vivo Δ values of 0.012 to 0.027 (27, 41, 49) and 0.013 to 0.023 (41, 49) that are observed for ammonium and nitrate uptake and assimilation by aquatic microbes cultured under conditions of high nitrogen availability. Such values for Δ are in the range of values observed for *Solemya* clams (12, 13, 33, 46) and are likely for seep mussel Ia. Provided that the isotopic compositions of the sources are not anomalously depleted of ^{15}N, the negative values may reflect a large Δ and assimilation of nitrogen sources at high concentration.

$\delta^{15}N$ values close to $0‰$ may reflect ammonium or nitrate assimilation with a low Δ. The degree to which nitrogen sources at the site of assimilation act as an infinite reservoir is also dependent on host physiology. If substrate does not exchange freely between the environment and site of assimilation, the isotopic composition of the source at the assimilation site can differ from that in the environment, altering the relation between Δ and α. Assuming that there is no discrimination during transport, $\Delta = (F_3/F_1) \times (\alpha - 1)$ (24), where F_1 is the influx rate, F_2 is the assimilation rate, F_3 is the efflux rate, and $F_3 = F_1 - F_2$. At higher substrate concentrations where efflux is greater, F_3/F_1 approaches 1, resulting in the relation of $\Delta = \alpha - 1$ given above. Thus, the finding that *S. reidi* and some seep mussels exhibit Δ values close to $\alpha - 1$ suggests that the sources of nitrogen exchange freely between the assimilation site and the environment. Assuming that nitrate is the primary nitrogen source of *R. pachyptila* results in a Δ close to 0, since the stable isotope compositions of its tissues range from -2.9 to $+5.0‰$ (18). Such a small value for Δ may reflect nitrate limitation at the site of assimilation or be due to the absence of nitrate leakage (i.e., a low value for F_3/F_1) from the host tissue because of active uptake.

From the present study, it is clear that combined inorganic nitrogen is abundant in the environments inhabited by these symbioses and that it can be assimilated. Therefore, it is possible that growth based on C_1 compound metabolism by these associations is not limited by nitrogen availability. However, acquisition of nutrients by the host and subsequent supply to the symbionts is paramount in governing the degree to which nitrogen is limiting, if at all. Evidence from stable isotopes indicates that in some cases (i) high concentrations of these sources are present at the site of assimilation and (ii) sources exchange freely between the assimilation site and the environment. Both conditions may reflect the absence of nitrogen limitation. The variability in the isotope data that is observed may result from the degree to which conditions i and ii hold true. To address the issue of nitrogen limitation, it is necessary to characterize the conditions with respect to ammonium and nitrate within these associations as well as the mechanisms of uptake and assimilation. Even though sources are rich in the environment, supply to the assimilation site may limit the assimilation rate. The role of the host in nutrient acquisition by symbiotic associations between algae and cnidarians has been investigated and the prevailing model is one in which the host does not participate in uptake or assimilation (14, 42). If this is the case, then the concentration of ammonium within the host tissues will be governed by internal and external pH and the ammonium concentration in the environment. Since an internal pH lower than that of the environment favors the concentration of ammonium from the medium, the finding that *R. pachyptila* exhibits a hemolymph pH of 7.5 compared with pH 6 in the surrounding water (11) may account for its inability to assimilate ammonium. The different abilities to assimilate nitrate observed in the present study may be a function of host uptake capability. NO_3^- is an anion and may not readily diffuse across the host epidermis. As a result, active transport of NO_3^- by the host may be required but is without precedent in marine invertebrates. In the present study, C/N assimilation ratios equal to or below the C/N ratio of the organism itself were observed only for ammonium assimilation by *S. reidi* (Table 3). Thus, nitrogen limitation caused by the rate of supply to the site of assimilation may be possible.

The substantial rates of nitrate incorporation exhibited by two of the symbioses tested is of interest from the physiological perspective. In addition to the problem of nitrate entry into the host, there are also the problems of inhibition of nitrate uptake and assimilatory reduction by host excretory ammonium. Inhibition by excretory ammonium has been postulated as an explanation for why symbioses between algae and marine invertebrates, which all take up exogenous ammonium, do not always take up nitrate (51). Substantial concentrations of ammonium are present in the hemolymph of *S. reidi* (30 to 170 μM [35]), *R. pachyptila* (60 to 1,725 μM [34]), and seep mussel Ia (10 to 140 μM [36]). Thus, it is not clear how nitrate assimilation is possible. One explanation is that the symbionts use dissimilatory pathways to produce ammonium, which is then assimilated. Dissimilatory nitrate reduction has been demonstrated in *S. reidi* (54) as well as in symbionts of *R. pachyptila* (25, 26). The possibility that these associations use nitrate both as a source of nitrogen and for respiration

increases the potential importance of nitrate acquisition to these associations.

In the present study, assimilation of inorganic sources of nitrogen, which is vital for autotrophic functioning of these symbioses, was demonstrated. N_2 assimilation was not observed. Given the finding that ammonium and/or nitrate are abundant where these associations are found, it is tempting to believe that they are not limited by the availability of nitrogen. In some cases, we have strong evidence for the absence of nitrogen limitation: low $\delta^{15}N$ values, that we believe reflect large values for Δ, are observed in *Solemya* clams and some seep mussels, and a C/N assimilation rate for ammonium lower than the tissue C/N ratio in *S. reidi* was found. In other cases, it is possible that although the source concentration is high in the environment, the capability to supply these sources to the site of assimilation may limit the rate of nitrogen assimilation and result in nitrogen limitation. Future study will need to address the mechanisms involved in nutrient acquisition and assimilation. Of particular interest is the role of host physiology in uptake and assimilation, nitrate transport into the host tissues, and the role of dissimilatory nitrate reduction in nitrate assimilation.

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