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THE NUTRITIONAL ROLE OF ENDOSYMBIOTIC BACTERIA IN , C65
ANIMAL-BACTERIA SYMBIOSES: *SOLEMYA VELUM*, A CASE 1990
STUDY

By

Noellette Mary Conway

B. A. (Mod.) Hons., Trinity College Dublin, Ireland
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Submitted in partial fulfillment of the requirements for the degree of

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at the

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and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION

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Signature of Author.....

Joint Program in Biological Oceanography, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution

Certified by.....

Judith McDowell Capuzzo, Thesis Supervisor

Accepted by.....

Donald Anderson, Chairman, Joint Committee on Biological Oceanography, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution

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THE NATIONAL BUREAU OF STANDARDS
 1000 COLLEGE AVENUE
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For Joe, Bettie, and Hagen.

THE NUTRITIONAL ROLE OF ENDOSYMBIOTIC BACTERIA IN ANIMAL-BACTERIA SYMBIOSES: *SOLEMYA VELUM*, A CASE STUDY

By

Noellette Mary Conway

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution.

ABSTRACT

The trophic interactions occurring between endosymbiotic bacteria and the host in animal-bacteria symbioses were investigated using the endosymbiont-containing protobranch clam, *Solemya velum*, as a general model. C, N, and S stable isotope compositions were investigated in tissues of *S. velum*; C and N isotope values were also examined in an enriched bacterial fraction, separated from intact gills by differential centrifugation. The bacterial fraction and host tissues had similar $\delta^{13}\text{C}$ values which were different to those of organisms utilizing a phytoplankton-based food chain. The $\delta^{15}\text{N}$ values of both *S. velum* and the endosymbiont fraction were comparable and considerably lower than those of bivalve controls that do not harbor endosymbionts. The $\delta^{34}\text{S}$ values of *S. velum* were very negative and suggest the use of biogenically produced H_2S as a S source. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotope ratios of *S. velum* suggest that the endosymbiotic bacteria may provide almost 100% of the hosts C and N budgets.

The lipid composition of most organisms is related to dietary intake. Consequently, a detailed analysis of the lipid composition of *S. velum* was undertaken to verify the nutritional importance of the symbiotic bacteria. The $\delta^{13}\text{C}$ ratios of the lipids were also measured in order to determine potential carbon sources for the lipids of *S. velum*. The lipids of *S. velum* were characterized by large amounts of 18:1 ω 7 (*cis*-vaccenic acid, a lipid found in many species of bacteria), 16:0, 16:1 ω 7 and low concentrations of the highly unsaturated fatty acids characteristic of most marine bivalves. The predominant sterol found in *S. velum* was cholesterol. The *cis*-vaccenic acid found in *S. velum* is almost certainly symbiont-derived and could serve as a biomarker for symbiont-lipid

incorporation by the animal host. The high concentrations of *cis*-vaccenic acid in the tissues of *S. velum* suggest an important role for the endosymbionts in the lipid metabolism of this bivalve. In addition, the presence of *cis*-vaccenic acid in all the major lipid classes of *S. velum* demonstrates both incorporation and utilization of this fatty acid. The reduced amounts of polyunsaturated fatty acids found in *S. velum*, along with the absence of sterols of plant origin, provide further evidence to suggest that this symbiosis relies on endosymbiont chemoautotrophy to fulfill the majority of its nutritional requirements. The $\delta^{13}\text{C}$ ratios of the fatty acids and sterols of *S. velum* were very negative and similar to the values found for the fatty acids of *Thiomicrospira crunogena*, a sulfur-oxidizing bacterium, suggesting that the lipids of *S. velum* are either derived directly from the endosymbionts or are synthesized using carbon derived from the endosymbionts.

The stable isotope and lipid composition profiles of *S. velum* were very similar to those of *Solemya borealis*, a newly discovered symbiosis, and *Inanidrilus leukodermatus*, a symbiotic annelid. This demonstrates the usefulness of stable isotopes and lipid composition studies in the analysis and characterization of these types of symbioses. In particular, both techniques are valuable as initial screening tools for the presence of symbiotic sulfur-oxidizing bacteria.

In order to further characterize differences between *S. velum* and typical marine bivalves, total and free amino acids were determined for *S. velum*, and *Mya arenaria*. Both the relative and absolute amounts of free amino acids differed significantly between the two species. In *S. velum*, the absolute concentrations of the sulfur amino acid taurine were greater than the total free amino acid concentrations typically found in bivalves. The possible roles for taurine as a sink for oxidized sulfur compounds in the *Solemya velum* symbiosis was examined. Preliminary experiments suggest that taurine levels increase in the presence of reduced sulfur compounds; this demonstrates a possible link between sulfur-oxidation and taurine synthesis in this symbiosis.

Thesis supervisor: Judith McDowell Capuzzo, Senior Scientist.

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List of Abbreviations used in the text

ACN (Acetonitrile)
 ADP (Adenosine diphosphate)
 Ala (Alanine)
 APS (Adenosine-5'-phosphosulfate)
 Asp (Aspartic acid)
 Asn (Asparagine)
 ATP (Adenosine triphosphate)
 CA (Carbonic Anhydrase)
 Cys (Cysteine/Cystine)
 DI (Diglycerides)
 DOM (Dissolved organic matter)
 EFA (Essential fatty acid)
 EtOH (Ethanol)
 FAMES (Fatty acid methyl esters)
 FFA (Free fatty acid)
 GC (Gas chromatography)
 GC-MS (Gas chromatography-mass spectrometry)
 GABA (Gamma amino butyric acid)
 Glu (Glutamic acid)
 Gln (Glutamine)
 Gly (Glycine)
 HCL (Hydrochloric acid)
 HH (Hadley's Harbor)
 His (Histidine)
 HPLC (High pressure liquid chromatography)
 Ile (Isoleucine)
 LBB (Little Buttermilk Bay)
 Leu (Leucine)
 MeOH (Methanol)
 Met (Methionine)
 M (Monoglycerides)
 MUFA (Monounsaturated fatty acids)
 NAD(P)H (Nicotinamide-adenine dinucleotide [phosphate])
 Nor (Norleucine)

OH-P (Hydroxy-proline)
Orn (Ornithine)
Phe (Phenylalanine)
PITC (Phenylisothiocyanate)
PL (Phospholipids)
POM (Particulate organic matter)
Pro (Proline)
PUFA (Polyunsaturated fatty acid)
RuBPCase (Ribulose-5-biphosphate carboxylase)
SE (Steryl esters)
Ser (Serine)
ST (Sterols)
Tau (Taurine)
TCA (Trichloroacetic acid)
TEA (Triethylamine)
TRI (Triglycerides)
Trp (Tryptophan)
Tyr (Tyrosine)
Val (Valine)

CHAPTER 1

INTRODUCTION

1.1 The *Solemya velum* symbiosis

"It is surprising that any species of a group (Solemyidae) which evolved so early along such specialized lines have survived" - C.M. Yonge, 1939.

The protobranch bivalve genus *Solemya* is widely distributed throughout the world and ranges in habitat from the intertidal zone to depths of 2000 m (Abbott, 1974), predominantly occurring in sulfide-rich sediments. *Solemya* is capable of rapid swimming and burrowing, and species may occupy discrete burrows (as in the case of *Solemya velum* [Morse, 1913; Frey, 1968; Stanley, 1970; Jenner, 1977]) or leave no trace of a burrow (e.g. *S. togata* and *S. parkinsoni* [Yonge, 1939; Owen, 1961]). In *S. velum* the burrows are kept open, and the walls of the burrows are covered with a rust-colored substance which slightly cements the adjacent sediment Frey, 1968). During burrowing, the large foot of *S. velum* protrudes from the anterior opening of the body and points downward until contact is made with the sediment. The foot protrudes into the sediment and expands allowing the animal to get a grip or foothold. This permits the animal to erect itself and the foot is pushed further into the sediment until the animal is hidden. Burrows of *S. velum* typically extend 7-15 cm into the sediment. *Solemya velum* generally builds a Y-shaped burrow (Figure 1.1) which permits access to both oxygenated seawater and sulfide-rich sediment porewaters.

In contrast to the majority of molluscs, which have a prominent alimentary tract easily discovered by conventional dissection methods, species belonging to the genus *Solemya* (particularly northern hemisphere species) have very small digestive systems. Some species are entirely gutless (Reid, 1980). Consequently, the nutritional strategies utilized by these bivalves

have been the subject of controversy for half a century (Yonge, 1939; Owen, 1961; Reid, 1980; Reid and Bernard, 1980; Cavanaugh, 1985a; b). Most living protobranchs are indirect deposit feeders and digestion is both extracellular and intracellular (Barnes, 1980). Extracellular digestion occurs in the stomach. Enzymes are secreted by areas of the digestive diverticula. Intracellular digestion occurs within the cells of the sections of the diverticula. The ducts of the digestive diverticula are ciliated and divided into an incurrent and excurrent tract. Food is brought to the mouth by the feeding palps and enters the digestive diverticula via the incurrent tract. In *Solemya velum* (Figure 1.2), the feeding palps are extremely reduced and the digestive system consists of a narrow thread-like tube running the length of the body.

Figure 1.1 Burrows of *Solemya velum* showing typical 'chalice' shape (from Frey, 1968).

The nutritional requirements of the gutless and semi-gutless representatives of the *Solemya* genus would appear to be provided via a variety of trophic pathways (Yonge, 1939; Owen, 1961; Reid, 1980; S. Gallager, unpublished results). It is possible that in *Solemya* particulate organic matter (POM) may be carefully sorted by the gill cilia prior to stomach ingestion. In this way, only the smallest particles will be passed to the reduced gut (Yonge, 1939). In this context, Morse (1913) found that in *S. velum* particles are

highly sorted by the gills so that only the smallest particles are collected from the gills by the reduced labial palps and passed to the mouth. In addition, predigestion of POM may be facilitated through the release of digestive enzymes by the gill and mantle epithelia, these smaller particles may then enter the reduced gut, or be pinocytosed by the gills. This predigestion may also result in the presence of dissolved organic matter (DOM) within the mantle cavity, allowing epithelial uptake to take place across the gill and mantle surface. Active transport of dissolved amino acids is exhibited by the gills and mantle surface of *S. velum* (S. Gallager, unpublished results). Recently, however, the nutritional potential of unusual sulfur-oxidizing chemoautotrophic bacteria endosymbiotic¹ within certain cells (bacteriocytes) of the gill tissue of both *S. velum* and *S. reidi* (Cavanaugh, 1983a; b; Felbeck, 1983a; Figure 1.3), has received widespread attention (Cavanaugh, 1985a; b; Fisher and Childress, 1986; Powell and Somero, 1986a,b). In this dissertation, the role of these bacteria in the nutrition of *S. velum* was investigated using biochemical markers for endosymbiont activity.

Chemoautotrophs (alternatively referred to as chemolithotrophs or, more precisely, chemoautolithotrophs) are those procaryotes capable of deriving their cell carbon from CO₂ (autotrophy) and their energy and reducing power (ATP and NAD(P)H) from the respiration of inorganic substances (chemolithotrophy). Epifluorescent and transmission electron microscopy, coupled with lipopolysaccharide analysis, reveal the endosymbionts of *S. velum* to be gram-negative rods of between 2-10 µm in length and 1 µm in diameter (Cavanaugh, 1983a,b). The bacteria occur intracellularly within bacteriocytes located in the hypertrophied gills of this species (Figure 1.3). They are surrounded by intercalary cells which lack symbionts (Cavanaugh, 1983a, 1985a). The recent study of Cavanaugh (1988) has revealed that ribulose-1-5-biphosphate carboxylase (RuBPCase) is located only in the bacterial cells found in the gills of *S. velum*, providing direct

¹ The term symbiosis can be defined in many ways (Keeton, 1980). In this discussion the term will be defined in it's broadest sense as any instance in which two or more organisms live together, whether both (mutualism) or only one (commensalism) of the organisms benefit from the association.

evidence that it is the bacterial cells that are responsible for the autotrophic activity measured in the gills. In that study, antibodies elicited by the purified large RuBPCase from tobacco cross-reacted on immunoblots with a protein of similar molecular mass in extracts of gill, but not foot, tissue. Further immunoelectron microscopy studies demonstrated the presence of labelled antisera in the bacterial endosymbionts (Cavanaugh, 1988).

1.2 General biology of invertebrate-chemoautotroph symbioses.

Chemoautotrophic bacteria have also been localized both biochemically and ultrastructurally in specialized tissues of other phyla (Table 1.1). In fact, many species appear to harbor more than one morphological type of bacteria including *Inanidrillus* (= *Phallodrillus*) *leukodermatus* and *Phallodrillus planus* (Giere and Langheld, 1987), *Lucinella divaricata* and *Thyasira flexuosa* (Herry and Le Pennec, 1987; Wood and Kelly, 1989b), *Ridgeia piscesae* (DeBurg et al., 1989), deep-sea Thyasirids (Southward et al., 1986), and *Spisula subtruncata* (Soyer et al., 1989). Physiological and biochemical studies of invertebrate-chemoautotroph symbioses (hereafter called animal-bacteria symbioses) have shown that in most of the habitats studied, including rift vents, fault vents, mangrove swamps, sewage outfall areas, eelgrass beds, and reducing sediments, a key feature of these symbioses is simultaneous access to both reduced sulfur compounds and oxygen. Provided the endosymbionts possess the necessary enzymatic machinery, the energy present in the sulfides can be exploited via oxidative pathways. Animal-bacteria symbioses capable of growth on methane have also been recorded (Childress et al., 1986; Cavanaugh, et al., 1987); but these species will not be discussed in this thesis in any depth.

Host-symbiont interactions in animal-chemoautotrophic bacteria symbioses, like those of other host-symbiont relationships probably confer nutritional advantages to the host, and an understanding of host-symbiont relationships and nutritional

interactions in other types of symbioses is an important prerequisite for the study of animal-bacteria symbioses. For example, in molluscs such as *Tridacna maxima* that have photosynthetic algae (Muscatine, 1980a), or *Elysia viridis* which houses isolated chloroplasts (Trench *et al.*, 1973) the presence of the symbiont allows the host to directly utilize the products of a phototrophic metabolism. In other mollusc-symbiont interactions, the host utilizes foodstuffs otherwise unavailable to it (e.g., cellulose in the wood-boring mollusc symbioses). In coral-algae symbioses, the algae are capable of reducing nitrate from seawater (Muscatine, 1980b), and efficient element cycling takes place within the symbioses allowing the proliferation of corals in low nutrient waters. By analogy with these other types of symbioses, it is likely that in animal-bacteria symbioses, the host is provided with the products of chemoautotrophic metabolism, and ultimately is utilizing an energy source (reduced sulfur compounds) otherwise unavailable to it.

The best studied molluscan symbioses are those involving photosynthetic symbionts. There is a considerable literature concerning the nature of the photosynthetates translocated from symbiont to host, rates of symbiont turnover, species distribution patterns, and symbiont acquisition. In symbioses involving intact chloroplasts, acquisition usually just involves feeding on the appropriate algae, the chloroplasts then get transported and incorporated into the cytoplasm of the cells of the digestive gland (Hinde, 1978). The major products that appear to be translocated from chloroplast to host are triose phosphates, simple sugars and amino acids (Hinde, 1978). In molluscs with algal symbionts, such as *Tridacna maxima*, the algal symbionts are predominantly housed in the sinuses of exposed regions of the mantle. The main products translocated in this species include glycerol, glucose and alanine (Muscatine, 1967; Muscatine and Porter, 1977). Interestingly, in *T. maxima*, the clam appears to secrete a "host-factor" which affects the quantity and quality of the metabolites translocated by the symbionts (Muscatine, 1967).

Figure 1.2 a) *Solemya velum* external view b) *S. velum* with shell removed showing the large foot (F) and gills (Ct). The gonads (Gn) are contained within the foot. The highly reduced digestive system is hidden by the gills. In life, the gills are a rich purple/red color, often with white flecks at the edges.

Figure 1.3 a) Phase contrast micrograph of endosymbionts of *Solemya velum*; b) Transmission electron micrograph of transverse section of gill filament showing bacteriocyte of *S. velum*, rod-shaped cells are apparent along with intercalary cells lacking symbionts. b= bacteria; mv = microvilli; nb = nucleus of bacteriocyte; ni = nucleus of intercalary cell. Scale bar = 3 μ m. c) electron micrograph of endosymbiont revealing gram-negative cell ultrastructure and peribacterial membrane (arrows). Scale bar = 0.2 μ m. From Cavanaugh, 1983a, 1985a, reproduced with the permission of the author

Figure 1.2

Figure 1.3

Table 1.1 Representative marine organisms thought to harbor chemoautotrophic sulfur-oxidizing symbionts.

Organism	Tissue	Reference
Phylum Mollusca		
Class Bivalvia		
<i>Solemya velum</i>	Gills	Cavanaugh, 1983a
<i>Solemya reidi</i>	"	Felbeck, 1983
<i>Codakia obicularis</i>	"	Berg and Alatalo, 1984
<i>Lucinoma annulata</i>	"	Vetter, 1984
<i>Lucinoma borealis</i>	"	Southward, 1986
<i>Lucinoma floridana</i>	"	Fisher and Hand, 1984
<i>Lucinoma radians</i>	"	Giere, 1985
<i>Lucinoma costata</i>	"	"
<i>Lucinoma multilineata</i>	"	"
<i>Lucinoma aequizonata</i>	"	Distel and Felbeck, 1987
<i>Lucinella divaricata</i>	"	Herry and Le Pennec, 1987; Herry <i>et al.</i> , 1989
<i>Loripes lucinalis</i>	"	"
<i>Thyasira flexuosa</i>	"	Southward, 1986
<i>Thyasira sarsi</i>	"	"
<i>Thyasira gouldi</i>	"	"
<i>Thyasira equalis</i>	"	"
<i>Myrtea spinifera</i>	"	Dando <i>et al.</i> , 1985
<i>Calyptogena magnifica</i>	"	Cavanaugh, 1983a
<i>Calyptogena pacifica</i>	"	Cavanaugh, 1983a
<i>Calyptogena elongata</i>	"	Vetter, 1985
<i>Bathymodiolus thermophilus</i>	"	Fiala-Médioni, 1984
<i>Bathymodiolus Spp.</i>	"	Fiala-Médioni <i>et al.</i> , 1986
(Galapagos)		
<i>Teunia jenchchronana</i>	"	Distel and Felbeck, 1988a
<i>Spisula subtruncata</i>	"	Soyer <i>et al.</i> , 1987
Class Gastropoda	"	
Undescribed gastropod		Stein <i>et al.</i> , 1988
Phylum Annelida		
Class Oligochaeta		
<i>Inanidrilus (=Phallodrilus) leukodermatus</i>	Sub-cuticular spaces	Giere <i>et al.</i> , 1984
<i>Phallodrilus planus</i>	"	Felbeck <i>et al.</i> , 1983
Phylum Pogonophora		
<i>Siboglinum fiordicum</i>	Trophosome	Southward <i>et al.</i> , 1986
<i>Siboglinum atlanticum</i>	"	Southward <i>et al.</i> , 1986
<i>Siboglinum ekmani</i>	"	"
<i>Sclerolinum brattstromi</i>	"	"
<i>Oligobranchia gracilis</i>	"	"
Phylum Vestimentifera		
<i>Riftia pachyptilia</i>	"	Cavanaugh <i>et al.</i> , 1981
<i>Lamellibranchia barhami</i>	"	Felbeck <i>et al.</i> , 1981
<i>Escarpia spicata</i>	"	"
<i>Ridgeia piscesae</i>	"	DeBurgh <i>et al.</i> , 1989
<i>Ridgea spp.</i>	"	"

In the mutualistic, sulfur-oxidizing symbioses (i.e. both species are presumed to benefit from the association) listed in Table 1.1, the animal host is thought to provide the sulfur-oxidizing symbiont access to the inorganic compounds necessary for chemosynthesis (O_2 , CO_2 , reduced sulfur) and shelter (Figure 1.5). It is not yet known how intake of CO_2 from a pool of seawater bicarbonate is ensured. The uncatalyzed conversion of HCO_3^- to CO_2 is a relatively slow process (Miller and Colman, 1980; Cook *et al.*, 1986). In addition, intake of inorganic carbon compounds may be limited by unstirred boundary layers and passage across the several membrane layers separating bacteria from host. Some algae and cyanobacteria possess a CO_2 -concentrating mechanism that involves the enzyme carbonic anhydrase (CA; Lucas and Berry, 1985). CA activity has also been reported in both the algal and animal fractions of cnidarian symbioses (Graham and Smillie, 1976; Isa and Yamazato, 1984). Recently, Weis *et al.* (1989) provided evidence to show that CA in the animal host may function as a CO_2 supply mechanism in 22 species of tropical cnidarian symbioses. It is conceivable that a similar mechanism may operate in animal-bacteria symbioses.

Exactly how sulfide and other reduced sulfur compounds, lethal to invertebrates at the pmg/liter level, are transported to the bacteria in *Solemya velum* is unknown. The site of sulfide toxicity in most animals is mitochondrial cytochrome C-oxidase, the terminal member of the mitochondrial electron transport chain. In many species, external sulfide concentrations of $>1\mu M$ will inhibit cytochrome C-oxidase activity by 50% (Nicholls, 1975; Hand and Somero, 1983). However, in the animal-bacteria symbioses that have been examined, at least three distinct strategies for avoiding sulfide poisoning appear to be exploited.

In the *Riftia pachyptilia* symbiosis, blood hemoglobin is capable of binding and transporting sulfide to the bacterial symbionts (Arp and Childress, 1983). Sulfide binding to the hemoglobin prevents inhibition of the cytochrome C-oxidase at sulfide concentrations that would otherwise be 100% inhibitory (Powell and Somero, 1983, 1986b). Interestingly, sulfide binding

has no effect on the oxygen binding capabilities of the hemoglobin. In the *Solemya reidi* symbiosis a different strategy for avoidance of sulfide poisoning seems to have evolved. The initial steps of sulfide oxidation appear to be achieved by the mitochondria of the animal host (Powell and Somero, 1986a). Thiosulfate, a non-poisonous form of reduced sulfur, is the predominant end-product of this oxidation. The high concentrations of thiosulfate found in the blood of *S. reidi* (up to 300 μ moles/l of blood; Anderson *et al.*, 1987) are probably the result of this preliminary oxidation. In *S. reidi*, levels of blood thiosulfate appear to be dependent on both the availability of O₂ and on the activity, viability and size of the symbiont population. In *Calymene magnifica*, *Lucinoma annulata* and *S. reidi*, special hematins in the blood catalyze the oxidation of sulfides and may also serve a detoxification function (Powell and Arp, 1989). In *Solemya velum*, two different hemoglobins occur in the gills. In the absence of sulfide, the gill hemoglobin may be oxygenated and deoxygenated. In the presence of sulfide, about half of the gill hemoglobin is reversibly converted to a ferric form which can bind to sulfide to form a ferric hemoglobin sulfide, the remaining hemoglobin can bind oxygen (Doeller, 1984; 1988). These gill hemoglobins may thus mediate oxygen and sulfide delivery to the endosymbionts.

The biochemical pathways involved in the oxidation of sulfur by these symbioses remain to be elucidated. In many species, including *S. velum* (Chen *et al.*, 1987), enzymes involved in the oxidation of reduced sulfur compounds, such as adenosine-5'-phosphosulfate reductase (APS reductase) have been localized in symbiont-containing tissues (Table 1.2). Yet, the putative bacteria of only a few species of symbioses are thought to have been isolated and cultured (Wood and Kelly, 1989a, b). It is not yet known whether these isolates are in fact the symbionts of the species in question or just bacterial contaminants. Consequently, we know almost nothing regarding the biochemical pathways of sulfur oxidation in the symbionts. In fact, the pathways of sulfur oxidation are not well understood even in the major groups of free-living colorless sulfur-oxidizing bacteria. This is because the oxidation of

sulfides also occurs spontaneously in aerated seawater, without any bacterial involvement, and many of the intermediate products are unstable and may undergo other reactions, producing 'intermediates' which are not actually involved in the oxidation pathways at all.

The pathways involved in sulfide oxidation are best known in bacteria of the thiobacilli genus (Figure 1.4). Sulfide oxidation generally follows the following stoichiometry and pathway in the thiobacilli with two moles of oxygen being consumed for every mole of sulfide or thiosulfate oxidized and 1.5 moles of oxygen consumed per mole of elemental sulfur utilized. The thiobacilli may be among the most important sulfide oxidizers in sediments and soils, and they can be isolated in large numbers from marine and freshwater sediments. In general the sulfur cycle of a typical marine sediment can be represented diagrammatically, as in Figure 1.6. The thiobacilli and other colorless sulfur bacteria are restricted to the narrow zone where the H_2S and O_2 gradients meet and the two compounds exist in equilibrium.

Figure 1.4 General pathways of sulfur oxidation in the thiobacilli. Intermediates are not shown. Enzymes involved: (1) Enzyme unknown; (2) Sulfur oxygenase enzyme; (3) Sulfite oxidase; Adenosine-5'-phosphosulfate reductase; ADP sulfurylase; ATP sulfurylase; (4) Rhodanese. Rhodanese is present in a number of sulfur-oxidizing bacteria and other prokaryotes and eukaryotes, and has been purified from *Thiobacillus denitrificans*, *Ferrobacillus ferrooxidans*, and *Chromatium* (Bowen et al., 1965; Tabita et al., 1969; Smith and Lascelles, 1966). Its role is not fully understood, but it is probably the "thiosulfate cleaving enzyme". It is found in both the gill and foot tissue of *S. velum* (Cavanaugh, 1985b). Sulfite oxidase is found in both eucaryotes and procaryotes. APS-reductase appears to occur only in procaryotes (Cavanaugh, 1985b) and plays a role in the oxidation of sulfite. ADP-and ATP sulfurylase is found in both eucaryotes and procaryotes and is involved in the conversion of sulfite to sulfate via an APS intermediate.

Figure 1.5 Diagrammatic representation of the metabolic organization found in the gills of the a) *Solemya velum* and b) *S. reidi* symbioses. Modified from Felbeck *et al.*, 1983; Cavanaugh, 1985b; and Anderson *et al.*, 1986.

Figure 1.6 General pathways of sulfur cycling in marine sediments.

The sulfur-oxidizing bacteria in the marine symbioses discussed here probably oxidize sulfur using similar pathways to those utilized by the thiobacilli. The occurrence of sulfur-oxidizing bacteria in marine invertebrates may be an important component of the sulfur and carbon cycles in marine sediments hitherto omitted from sediment sulfur cycling process rates. The animal host effectively provides the symbionts with suitable microhabitats outside their typical zones of occurrence. In this way, sulfides that may otherwise be oxidized chemically in the oxidized upper sediment layers may be biologically oxidized, allowing the energy produced to be held within the ecosystem and used for carbon

fixation. It can probably be assumed that the bond energy released during sulfide oxidation in animal-bacteria symbioses is ultimately trapped in biologically useful molecules such as ATP and NAD(P)H, which can then be used to drive the reductive steps of CO₂ fixation (via the Calvin-Benson cycle) and the synthesis of organic carbon molecules which may subsequently be translocated to the bivalve tissue (Figure 1.5).

Although few of the details of sulfur-oxidation are known in animal-bacteria symbioses there does appear to be a direct relationship between sulfide oxidation and CO₂ fixation rates in *Solemya reidi* (Anderson *et al.*, 1987). In addition, external thiosulfate concentrations affect respiratory gas flux in *Solemya reidi*, with O₂ and CO₂ uptake rates increasing as the thiosulfate concentration is increased to 100 μ M, and then decreasing again with further thiosulfate increases (Anderson *et al.*, 1987). Thus, it seems in *S. reidi*, that there is an optimum S₂O₃ concentration for maximum CO₂ fixation and respiration rates (Anderson *et al.*, 1987). In *S. velum*, CO₂ fixation rates are found to increase with increases in external thiosulfate and hydrogen sulfide concentrations (Cavanaugh, 1983a,b,1985b). However, Cavanaugh (1985b) found no effect of thiosulfate on O₂ consumption rates in this species, an unusual finding, considering the oxygen required for sulfur-oxidation processes. Doeller (1984), found a two-fold increase in the metabolic rate of *S. velum* in the presence of a reduced sulfur source. It is interesting to note here, that in some species, the form of reduced sulfur compound present in the external medium appears to affect the CO₂ fixation rate. In *Inanidrilus* (= *Phallodrilus*) *leukodermatus*, carbon dioxide uptake and fixation is stimulated by S₂O₃ but suppressed by S⁻² (Giere *et al.*, 1988). In both *S. reidi* and *S. velum*, both forms of reduced sulfur compound stimulate CO₂ fixation rates in the symbionts (Anderson *et al.*, 1987, Cavanaugh, Pers Comm.).

The presence of symbiotic relationships is not an unusual phenomenon in the marine environment; in fact organisms without symbiotic associations are unlikely to exist naturally. There are many examples of symbioses involving animals and plants such as

reef-building corals, and the giant clams (tridacnidae) which harbor symbiotic dinoflagellates. In many instances, the presence of symbionts is vital for the survival of the host. For example, the feeding and communication behaviors of fish with luminescent bacterial symbionts are in many cases dependent on the bacteria of the light organs (Dyer, 1989 and references therein). Termites without their bacterial and protist symbionts will eat wood but starve to death (Grossovsky and Margulis, 1982). In reef-building corals the photosynthetic activity of the algae is essential for the rapid precipitation of CaCO_3 which is required for reef maintenance (Ahmadjian and Paracer, 1986 and references therein).

The acquisition of chemoautotrophic symbionts appears to have been a major evolutionary driving force in many of the invertebrates involved (Reid and Brand, 1986). The presence of chemoautotrophic symbionts is generally correlated with host-gut reduction, as just described. Interestingly, the giant clams *Tridacna maxima* and *T. crocea*, which house algal symbionts appear to have guts that are proportionately smaller than the guts of the symbiont-free clam *T. gigas*. Thus, the loss of normal digestive systems in association with the presence of symbionts is not confined to animal-bacteria symbioses. Although, it is only in animal-bacteria symbioses that there are examples of complete gut loss. In addition, the evolution of increased gill size and the expansion of subfilamental tissues in the gills of some bivalves, and the reduction of feeding palps in many bivalves containing symbiotic bacteria, demonstrate the morphological changes which have accompanied the evolution of these symbioses (Cavanaugh, 1985a; Reid, 1980; Reid and Brand, 1986).

The development of these symbioses required that at one time the presence of bacterial contaminants failed to elicit the digestive or immune responses of the host. Intracellular establishment of the bacteria, as occurs in many of the bivalves outlined in Table 1.1, could have occurred during ctenidial establishment of gill epiflora which has been observed to occur in some vent molluscs (De Burgh and Singla, 1984). The processes leading to the eventual establishment of the stable symbiosis must have occurred slowly,

particularly in the intracellular bacterial symbioses which represent the coexistence of at least two genomes of divergent evolutionary origins occupying the same cytoplasmic environment. The two components of the symbioses must develop the means to utilize the metabolic products of each other and develop harmonic proliferation in order to ensure neither component of the symbiosis overgrows the other. The *Solemyidae* are a very early group of bivalves appearing in the Devonian (Yonge, 1939). It is interesting to note that a symbiosis between *Amoeba proteus* and bacteria has been observed to develop in laboratory cultures in as little as 20 years (Jeon, 1983; Reisser *et al.*, 1985).

Dyer (1989) suggests that there is a strong selection pressure in symbiotic associations for genes to be exchanged between the members of the symbiosis. Dyer and Obar (1984) propose the following scheme for development of more efficient symbioses. (1) Two organisms exchange nutrients in a mutually advantageous manner which results in an increase in fitness for both species. (2) In order to ensure harmonic proliferation mechanisms develop to ensure neither species overgrows the other, these could include coordination of cell division and respiratory rates. (3) Non-functional or redundant genes may be lost and/or important genes traded between partners. In the most advanced symbioses, partners are probably coordinated at the genetic level. Perhaps the apparent ability of the mitochondria of the *Solemya reidi* symbiosis to preform the initial steps in the sulfur oxidation pathway (Powell and Somero, 1986a) is the result of gene transfer between symbiont and mitochondrial genomes. There are a variety of mechanisms in which genetic transfer may take place between different genomes for example, genes have been exchanged between plastids and mitochondria in corn, possibly via transposons or viruses (Stern and Palmer, 1984). In addition, Sikorski *et al* (1990) recently reported two examples of horizontal transfer of DNA from prokaryotes to eukaryotes.

The mechanisms by which the bacterial endosymbionts are transmitted from one generation to the next are not well understood. Three possible mechanisms exist:

- 1) Maternal transmission during egg development, egg deposition or egg fertilization.
- 2) Lateral transmission from host to host.
- 3) Intake of free-living bacteria from the external sea water.

In the annelid *Phallodrilus leukodermatus*, the females coat the eggs with symbionts during egg deposition (Giere and Langheld, 1987). Direct transmission has not been observed in any bivalve host, to date. It is unlikely that symbiont transmission is left to chance. In many of the species studied, the symbionts appear to be quite host specific (Distell *et al.*, 1988); in addition, many symbiont-free bivalves inhabit the same sediments as bivalves with symbionts, yet they do not harbor any symbionts. In the *Solemya reidi* symbiosis, recognizable bacteria appear in the pericalymma larval cells soon after fertilization (Gustafson and Reid, 1988). The pericalymma breaks up and autolyses at metamorphosis, and the cells and bacteria are carried into the rudimentary oesophagus. The bacteria remain here and multiply until the rudimentary oesophagus disintegrates and they are carried to the developing gills (Gustafson and Reid, 1988). The bacteria are not observed, however, in the undivided fertilized egg.

Table 1.2 Enzyme activities of some species of animal-bacteria symbioses from sulfide-rich habitats.

Organism	Calvin-Benson Pathway enzymes		Sulfur-oxidizing enzymes				
	RuBPCase	Ru-5-P Kinase	ATP-Sulfurylase	APS-Reductase	Rhodanase	Adenyl Sulfate Reductase	Sulfate Adenyl Transferase
Phylum Vestimentifera							
<i>Riftia pachyptilia</i> (1)	0.22	19.01	74	7.6	7.6		
<i>Riftia</i> spp. (1)	1.13		133	30.1	5.2		
<i>Lamellibranchia barhami</i>	0.4		30				
+ unknown vent sp.(1)							
Phylum pogonophora							
<i>Siboglinium fiordicum</i> (5)	0.082			0.0185		0.008 - 0.016	0.11 - 0.15
(post-annular region)							0.062
Phylum Annelida							
<i>Phallosdrilus leukodermatus</i> (6)	0.48 - 0.62						
Phylum Mollusca							
<i>Solemya velum</i> (3a)	0.03 - 0.15						
(3b)	0.81						
<i>Calyplogena magnifica</i> (1)	0.4						
<i>Calyplogena pacifica</i> (1)	0.6		25				
<i>Solemya reidi</i> +	2.4	4.4	77	4.1	0.7		
<i>Solemya panamensis</i> (1)							
<i>Parvilucina tenuis culpta</i> (1)	0.01		3.8				
<i>Codakia obicularis</i> (2)	0.2		43		1.4		
<i>Lucina pennsylvanica</i> (2)	+						
<i>Myrtea spinifera</i> (4)	1.028 - 0.047						
<i>Thyassira flexuosa</i> (7)	0.085 - 0.147					0.016 - 0.042	0.203 - 0.293
<i>Thyassira sarsi</i> (7)	0.144					0.001 - 0.017	0.220 - 0.394
<i>Lucinoma annulata</i> (1)	0.1	1.25	0.6			0.6 - 1.8*	0.089 - 0.15

* Positive result, not quantified.

(1) Felbeck et al., 1981, and references therein.

(2) Berg and Alatalo, 1984, intact gill tissue.

(3a) Cavanaugh, 1983b, intact gill tissue

(3b) Cavanaugh, 1983a, cell-free gill tissue extract.

(4) Dando et al., 1985, tissue homogenate

(5) Southward et al., 1986, tissue homogenate.

(6) Giere et al., 1988, whole animal.

(7) Dando and Southward, 1986, tissue homogenates.

* measured at 8°C

Note blanks indicate that the enzyme was not detected or not measured.

1.3 Nutrition in animal-bacteria symbioses: Introduction to thesis research.

In this thesis the utilization of endosymbiont nutrients by the host species was investigated using *S. velum* as a general model of animal-bacteria symbioses. Animal-bacteria symbioses are often difficult to collect and return to the laboratory intact; in addition, they are extremely difficult to maintain under laboratory conditions. Consequently, performing detailed physiological experiments of nutrition in these animals is fraught with difficulties. In this study, biochemical markers were used to study the transfer of metabolites from endosymbiont to host. In particular, biochemical parameters which are unaffected by damage to the animal during collection or preservation techniques were sought. *S. velum* is a suitable candidate for such research as relatively large numbers may be collected and examined for potential "bacterial markers". Once such markers are identified, it is possible to apply similar techniques to the study of nutrition in rarer or less accessible symbioses. In other words, the appropriate techniques may be "calibrated" using the *S. velum* symbiosis, and applied to other symbioses when the opportunities arise.

The nutritional requirements of an animal can be categorized as specific or non-specific. While a variety of substances can satisfy the non-specific needs of an animal (e.g. caloric or carbon needs), specific nutritional requirements can be satisfied by only one, or a limited suite of compounds (Phillips, 1984). Because bacteria are unlikely to provide all the specific requirements of animal cells (Phillips, 1984), it is highly unlikely, that in these types of symbioses, translocation of bacterial material is the sole feeding strategy employed by the host. Yet we know very little regarding the nutritional state of animal-bacteria symbioses.

1.3.1 Non-essential nutrients

From an energetic standpoint the chemoautotrophic bacterial contribution to host nutrition has not been adequately documented. Cavanaugh (1985a) has measured bacterial CO₂ fixation rates of 40

$\mu\text{g}^{-1}\text{h}^{-1}$ in *S. velum*, which could provide between 25% (assuming 40% translocation) and 65% (assuming 95% translocation) of the energetic requirements of this species. In *S. reidi* the percentage translocated is not known exactly, but it is probably in excess of 25% (Fisher and Childress, 1985). The qualitative composition of the products translocated from symbiont to host have been explored in the *Solemya reidi* and *Bathymodiolus* spp. symbioses (Felbeck, 1983a, Fisher and Childress, 1986, Fiala-Médioni *et al.*, 1986), and differ from the products translocated in photosynthetic symbioses such as glucose and maltose. In *S. reidi*, aspartate is the major radiolabelled product found in the host tissue after the intact symbiosis has been incubated in radiolabelled CO_2 . No radiolabelled carbohydrates were found. Because aspartate can be used in a number of metabolic pathways (e.g., the citric acid cycle, after conversion to oxaloacetate, gluconeogenesis, after conversion to oxaloacetate and phosphoenolpyruvate; and protein synthesis; Lehninger, 1975) it may be particularly useful for the transfer of reduced carbon from endosymbiont to host. In *Bathymodiolus* spp. incubated with radiolabelled bicarbonate *in situ*, 41-57% of the radioactivity found in the gills of the animal were located in small molecules (amino acids and sugars), 43-59% of the radioactivity was found in macromolecules, and 7-15% occurred in the lipids of the animal (Fiala Médioni *et al.*, 1986a).

Further evidence to suggest the nutritional importance of the symbionts is provided by the stable isotope ratios of animal-bacteria symbioses. The analysis of stable isotope ratios is an important method of identifying and quantifying trophic processes in both field and laboratory situations (Fry and Sherr, 1984). Stable isotope ratios are calculated as parts per thousands differences from standard reference materials. Samples with negative isotope ratios are depleted in the lighter isotope relative to the standard, whereas those with positive isotope ratios are enriched in the heavier isotope. Changes in stable isotope ratios generally occur in the early stages of food web processes when supplies of the element in question are limiting (Fry and Sherr, 1984; Hoefs, 1987). Because isotope signatures are passed along trophic pathways with only

small variations, stable isotope ratios can provide an estimate of the origin of the element in question (Fry and Sherr, 1984). Considerable evidence exists to suggest that the isotopic signatures resulting from photosynthetic and chemosynthetic carbon fixation differ markedly (Ruby *et al.*, 1987). Consequently, the isotope ratio of an organism deriving its reduced carbon compounds from chemosynthetic endosymbionts should differ from the isotope ratios of organisms deriving their carbon from photosynthetic organisms. The stable isotope ratios of a number of species with symbionts have been investigated, and most show unusual ratios of carbon stable isotope ratios, which differ from the isotope ratios expected if the animals were utilizing phytoplankton-derived carbon (Rau, 1981a,b; Spiro *et al.*, 1986). This suggests the utilization of symbiont-derived carbon by the host species. Chapter 3 of this thesis concerns the results of a detailed stable isotope analysis of *S. velum* and an enriched endosymbiont fraction undertaken to determine the contribution of the endosymbiotic bacteria to the carbon and nitrogen budgets of this species. In addition, stable isotope ratios are used to support additional biochemical data throughout the dissertation.

1.3.2 Essential Nutrients

All animals must obtain specific essential nutrients in order to survive, grow, and reproduce. Many nutritional requirements of marine invertebrates have only recently been elucidated by aquaculture research.

Lipids

a) Fatty Acids.

Fatty acid synthesis in marine invertebrates involves the successive linking of acetyl-CoA moieties to a malonyl-CoA molecule to form the unsaturated fatty acid, palmitic acid (16:0) (Lehninger, 1975; Malins and Sargent, 1976). Fatty acid synthetase, a multi-enzyme system located in the cytosol is responsible for *de novo* fatty acid synthesis. Further elongation of the palmitate produced

usually takes place in the membrane of the endoplasmic reticulum. This microsomal reaction requires acyl-CoA derivatives of at least 10 carbons. A quantitatively less important elongation pathway occurs in the mitochondria and is basically the reverse of the β -oxidation pathway used in fatty acid catabolism. Monounsaturations of fatty acids is achieved with a δ -9 desaturase². This has a maximum affinity for stearic acid (18:0) which is desaturated to oleic acid (18:1 ω 9), and to a lesser extent palmitic acid (16:0) into palmitoleic acid (16:1 ω 7). Although most metazoans can synthesize unbranched, even-length fatty acid chains from acetate and desaturate at the 9th carbon from the methyl end, further desaturations towards the methyl end of the carbon chain (ω 3, ω 6) are either impossible, or proceed too slowly to be of use³. Therefore, *de novo* animal synthesis of polyunsaturated fatty acids (PUFAs) is unlikely to result in an ω -number less than 7 (Lehninger, 1975).

Yet, animals require ω 3 and ω 6 fatty acids. EFAs are vital components of membranes, where they are important regulators of membrane fluidity and play a role in membrane transport processes. Additionally, they are precursors for prostaglandin synthesis and act as energy sources in a manner similar to saturated and monounsaturated fatty acids (Guarnieri and Johnson, 1970 and references therein). These essential fatty acids (EFAs) are abundant in both algae, higher plants, and animals, although they are only synthesized *de novo* by plants. Once they have been ingested by metazoans, the EFAs can be elongated and desaturated (Figure 1.7).

PUFAs serve several important functions in metazoans (Guarnieri and Johnson, 1970). They are used in energy storage and function as metabolic regulators and hormones. In most marine animals the typical fatty acid pattern is one in which the EFAs 20:5 ω 3 and 22:6 ω 3 predominate (Sargent, 1976; Joseph, 1982). In those animals studied to date (terrestrial species for the most part)

²The delta classification refers to the number of carbon atoms from the carboxylic carbon of the fatty acid. An alternative classification scheme is the omega classification. In the omega classification, the carbon number indicates the position of the double bond closest to the methyl end of the carbon skeleton.

³Nematodes are apparently unique among metazoans in their ability to synthesize long-chain PUFAs from acetate (Nichols, 1975).

EFA deficiencies can result in decreased EFA levels, impaired membrane functioning, reduced fertility and malfunctioning of membrane-bound enzyme systems. In bivalves EFA deficiencies result in poor growth (Langton and Waldock, 1981).

PUFAs, including the EFAs are absent in most bacteria (Phillips, 1984), although a few species of deep-sea bacteria are capable of EFA synthesis (DeLong and Yayanos, 1986). In most sulfur oxidizing bacteria, the fatty acid desaturation pathway proceeds via a pathway different from that found in eucaryotes (Goldfine, 1972; Fulco, 1983). This pathway can proceed in the absence of oxygen and is called the anaerobic pathway. Generally, the anaerobic pathway leads to the formation of *cis*-vaccenic acid (18:1 ω 7) as the major end-product, with 16:1 ω 7 produced as a secondary product. Desaturation of fatty acids using this pathway will not lead to the methylene interrupted double bond system (1,4-arrangement) typical of the PUFAs of higher organisms. Instead, sequential action of the β - γ -dehydrase could lead only to a 1,3-double bond system (not found in bacteria) or alternate cycles of β - γ -dehydration and α - β -dehydration could lead to a 1,5-double bond system (Goldfine, 1972; Fulco, 1983). This has occasionally been found in bacteria. There have been no reports of PUFAs in any species of sulfur-oxidizing chemoautotroph.

ω9 series **ω6 series** **ω3 series**

Figure 1.7 Elongation and desaturation pathways of the three major PUFA families in marine and terrestrial organisms (fatty acids in bold are the most common PUFAs in marine bivalves).

b) Sterols-

Sterols are synthesized from acetyl Co-A, and synthesis takes place in several stages. First, mevalonate, a six-carbon compound is synthesized from acetyl-CoA. In the next major stage, isoprenoid

units are formed from mevalonate by the loss of CO₂. Isoprenoid units are the building blocks of sterols. Condensation of six isoprenoid units yields squalane, which then gives rise to lanosterol. Cholesterol is formed from lanosterol after several further steps including the loss of three methyl groups (Lehninger, 1975)

In eucaryotic cells, sterols account for approximately 1% of tissue dry weight, and some marine invertebrates (including all crustaceans and many bivalves) have obligate sterol requirements. Reviews of the sterol composition of molluscs (e.g. Voogt, 1972; Goad, 1976) demonstrate that marine molluscs contain complex mixtures of C₂₆, C₂₇, C₂₈, C₂₉ and C₃₀ sterols, with cholesterol (C₂₇) generally occurring in the highest concentrations. Many of the larger sterols found in marine molluscs are commonly synthesized by algae and are presumed to be derived from the diet (See Volkman, 1986). Data on the ability of bivalves to synthesize sterols are controversial. Although it is generally stated that bivalves are incapable of synthesizing sterols, Fagerland and Idler (1960) reported the incorporation of radiolabelled acetate in the sterols of *Mytilus californianus* and *Saxidomus giganteus*, and recently, Teshima and Patterson (1981) demonstrated the synthesis of cholesterol, demosterol, isofucosterol, and 24-methylenecholesterol from acetate in *Crassostrea virginica*. Teshima and Kanazawa (1974) reported the synthesis of cholesterol, 22-dehydrocholesterol, demosterol and 24-methylenecholesterol from mevalonate in *Mytilus edulis*. Voogt (1975), however, did not recover any radioactivity in the sterols of *M. edulis* after the addition of labelled acetate, and the majority of bivalves studied appear to be capable of only limited *de novo* sterol synthesis (See review of Voogt, 1983). Bacteria, for the most part, do not contain sterols (Lee *et al.*, 1980) although small amounts are found in some species (McCaffrey *et al.*, 1989, and references therein).

In the marine environment, where photosynthetic phytoplankton generally form the base of the food chain, the inability of animal to synthesize EFAs and certain sterols is no great problem. These compounds will be derived from the diet. However,

in the case of animal-bacteria symbioses with small or no digestive systems, it is unlikely that the symbionts can provide the animal host with all the essential dietary nutrients. Perhaps additional trophic pathways are utilized, the animal or bacterial components of the symbiosis can indeed synthesize these nutrients, or the animal host has evolved strategies for avoiding the detrimental effects of essential nutrient deficiencies.

In chapter 4 of this thesis, the lipid composition of the *S. velum* symbiosis is analyzed and compared with the lipid composition of symbiont-free bivalves. Potential endosymbiont and plant 'biomarkers' are identified, and the role of endosymbionts and plants in the lipid nutrition of this symbiosis discussed. In chapter 7 the biochemical composition of the *S. velum* symbiosis is compared with two other putative animal-bacterial symbioses, *S. borealis* and *Inanidrilus* (= *Phallodrilus*) *leukodermatus* in order to determine whether the bacterial biomarkers found in *S. velum* are useful in the study of other symbioses.

Amino acids

Although amino acids are of central importance in the metabolism of all organisms, principally because they are precursors of proteins, different organisms vary in their ability to synthesize amino acids, and in the forms of nitrogen used for this purpose. Most metazoans, including many of the molluscan species studied have a requirement for the 10 dietarily essential amino acids common to most metazoans (arginine, histidine, lysine, threonine, phenylalanine, tryptophan, methionine, valine, leucine and isoleucine) (Bishop *et al.*, 1983). Additionally, some molluscan species may also require serine, glycine, cysteine and proline (Bishop *et al.*, 1983). Furthermore, many bivalves utilize taurine (N- or 2-methyl amino sulfonic acid) as an intracellular osmotic effector, and have high intracellular concentrations of this amino acid. Yet the rate of taurine biosynthesis in marine molluscs seems too low to account for the high levels found in bivalve tissues (Bishop *et al.*, 1983). Consequently, it is probable that this amino acid is also derived from the diet, and in many cases may be an essential

dietary nutrient. Marine invertebrates exhibit a fairly consistent distribution of essential amino acids (expressed as a percentage of total amino acids) in their tissues. In order to achieve this amino acid composition, essential amino acids must be obtained in the diet in roughly the same proportion as they occur in the animal tissue (Bishop *et al.*, 1983).

Microorganisms vary greatly in their ability to synthesize amino acids. Some, such as *Leuconostoc mesenteroides*, require a wide suite of amino acids in the diet, while many autotrophic species can manufacture all the required amino acids from NH_4^+ . Biosynthesis of these nutritionally essential amino acids by bacteria generally involves the utilization of glutamate, aspartate or other amphibolic intermediates (Lehninger, 1975). There is little information in the literature concerning the amino acid synthesis capabilities of sulfur-oxidizing bacteria. There is evidence for the synthesis of essential amino acids by the bacteria in certain types of animal-bacteria symbioses. The wood-boring mollusc *Bankia setacea* is incapable of synthesizing the essential amino acids (Trytek and Allen), and wood, which is very low in nitrogen and amino acids, provides the bulk of the diet for this species. This bivalve contains large concentrations of symbiotic bacteria which are capable of both cellulose degradation and nitrogen fixation, and the bacteria are responsible for digesting the dietary wood in addition to supplementing this nitrogen-poor diet. The symbionts are also capable of synthesizing all the dietarily essential amino acids (Trytek and Allen, 1980). It is possible that in *S. velum* and other similar symbioses the symbionts also provide the host with essential amino acids.

In chapter 5 of the thesis I have examined the amino acid composition of the *S. velum* symbiosis and compared it with the amino acid composition of the symbiont-free bivalve, *Mya arenaria*. The amino acid compositions of two additional symbioses and a species of sulfur-oxidizing bacterium are also reported in chapter 7. The large amounts of taurine found in the free amino acid pool of *S. velum* led to further studies on the possible sources and functions of this amino acid, these experiments are described in chapter 6.

Table 1.3 Amino acids of physiological importance (asterisks denote the essential amino acids; Lehninger, 1976).

1) Amino acids with non-polar R groups

Alanine	(Ala)
Valine*	Val
Leucine*	Leu
Isoleucine*	Ile
Proline	Pro
Phenylalanine*	Phe
Tryptophan*	Try
Methionine*	Met

2) Amino acids with uncharged polar R groups

Glycine	Gly
Serine	Ser
Threonine*	Thr
Cysteine	Cys
Tyrosine	Tyr
Asparagine	Asn
Glutamine	Gln

3) Amino acids with negatively charged R groups at pH 6.0)

Aspartic acid	Asp
Glutamic acid	Glu

4) Amino acids with positively charged R groups at pH 6.0

Lysine*	Lys
Arginine*	Arg+
Histidine*	His

Taurine	Tau
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CHAPTER 2

GENERAL METHODS AND BACKGROUND DATA

2.1 Site Characteristics

2.1.1 Methods

The animals used for most of the studies described here were collected by sieving from sub-tidal, reducing sediments at Little Buttermilk Bay, a small inlet in Cape Cod Massachusetts (Figure 2.1). Additional specimens of *Solemya velum* and *Tellina tenuis* were obtained from Hadley's Harbor, Cape Cod, Massachusetts. A specimen of *Solemya borealis* was collected from Buzzards Bay (Appendix 1), *Inanidrilus leukodermatus* was collected from calcareous sediments in Bermuda (Appendix II).

Sediment total organic carbon content (TOC), total H, N, S content, water content, and sediment grain size were determined for the Little Buttermilk site by collecting sediment cores of 7 cm in diameter and 25 cm in length. Cores were extruded in 2 cm sections. Total water content was determined by drying known weights of sediment to constant weight at 60°C and reweighing. For TOC analyses, inorganic carbon was removed by treatment with 3N HCL. TOC, H, N, analyses were carried out on selected sediment core sections by Bonnie Woodward; Dr. B. Howes carried out the total S measurements.

For determination of sediment grain size each sediment section was dried and sub-divided into $<63 \mu\text{m}$ (silt and clay) and $>63 \mu\text{m}$ (sand and gravel) components. The $>63 \mu\text{m}$ component was sieved through sieves of progressively smaller mesh size (ϕ scale) on a mechanical shaker and the weight of sediment retained on each sieve determined. This allowed the sediment to be sub-divided into silt, very fine sand, fine sand, medium sand, coarse sand, very coarse sand, and gravel fractions. The organic matter in the sediment sections was not destroyed with peroxide prior to analyses; thus the sediment includes organic debris and fecal pellets.

2.1.2 Results

Water content decreases from about 50% of the total wet weight between 0-2 cm to about 30% between 10-12 cm (Figure 2.2), the percentage of sand increase with depth, while the percentage of silt shows a slight decline with depth. TOC, H, N and S concentrations decline with depth as would be expected with sediment remineralization (Figure 2.3). Upon closer examination it can be seen that the sediments at Little Buttermilk Bay consist predominantly of fine, medium and coarse sands on a dry weight basis (Figure 2.4), with the percentage of coarse sands increasing with sediment depth. Silt, as a percentage of the total dry weight, decreases rapidly in the upper 2 cm of the sediment cores, and then remains constant (Figure 2.4)

Figure 2.1 a) Location of Little Buttermilk Bay in Cape Cod, Massachusetts; b) Mean annual water temperature.

Figure 2.2 Sediment characteristics at Little Buttermilk Bay (% sediment wet weight).

Figure 2.3 TOC, H, N, and S of Little Buttermilk Bay sediments (% sediment dry weight).

Figure 2.4 Sediment grain size at Little Buttermilk Bay (% dry weight).

Figure 2.4 continued.

2.2 Animal Morphometrics

Various morphometric parameters (length, width [at longest and widest points of the shells), wet weight, dry weight, gill, foot, and visceral mass wet and dry weights) were measured on each specimen of *S. velum* and *M. arenaria* collected from Little Buttermilk Bay. These are outlined in figures 2.5-2.17. In this way, realistic wet to dry weight regression values for each animal could be used in subsequent calculations. Mean length and width measurements of the animals used in this thesis were 1.52 cm and 0.59 cm respectively for *S. velum* and 5.16 cm and 3.0 cm respectively for *M. arenaria*. Abbreviations used in the figures are: W = width; L = length; WW = wet weight; DW = dry weight.

Figure 2.5 Length versus width regression for *Solemya velum* (squared polynomial regression).

Figure 2.6 Length versus soft tissue wet weight regression for *Solemya velum* (squared polynomial regression).

Figure 2.7 Width versus soft tissue wet weight regression for *Solemya velum* (squared polynomial regression).

Figure 2.8 Gill wet weight/dry weight regression for *Solemya velum* (least squares regression).

Figure 2.9 Foot wet weight/dry weight regression for *Solemya velum* (least squares regression).

Figure 2.10 Visceral mass wet weight/dry weight regression for *Solemya velum* (least squares regression).

Figure 2.11 Length versus width regression for *Mya arenaria* (squared polynomial regression).

Figure 2.12 Length versus soft tissue wet weight regression for *Mya arenaria* (squared polynomial regression).

Figure 2.13 Width versus soft tissue wet weight regression for *Mya arenaria* (squared polynomial regression)...

Figure 2.14 Gill wet weight/dry weight regression for *Mya arenaria* (least squares regression).

Figure 2.15 Foot wet weight/dry weight regression for *Mya arenaria* (least squares regression).

Figure 2.16 Visceral mass wet weight/dry weight regression for *Mya arenaria* (least squares regression).

Figure 2.17 Total soft tissue wet weight/dry weight regression for *Mya arenaria* (least squares regression).

2.3 Stable Isotope Analyses

Specimens of *Solemya velum*, *Tellina agilis*, and *Mya arenaria* were collected from the top 10 cm of the sediments at Little Buttermilk Bay and Hadley's Harbor, Cape Cod, Massachusetts during June and July 1987. Epifluorescence and phase contrast microscopy indicated that the latter two species contained no endosymbionts; consequently these species served as non-symbiotic controls. Sediment cores (15.7 cm³) were collected from both field sites; sediments were dried at 60°C, pulverized, acidified with 2M HCL and re-dried without rinsing, prior to nitrogen and carbon stable isotope

analysis. For animal tissue analysis, gills and foot tissue (the latter with gonads removed) were dissected immediately upon return to the laboratory, washed in sterile artificial sea water (ASW; autoclaved, 0.22 μ filtered) and dried at 60°C for approximately 24 h. Ten gill and foot samples from *S. velum* were pooled for each $\delta^{34}\text{S}$ measurement.

Differential and density gradient centrifugation were employed to remove animal cells and resulted in gill material highly enriched in bacterial tissue with respect to the original gill (Figure 2.18). For each bacterial enrichment, gills were removed from 9-10 *S. velum*, washed 3 times in sterile ASW, and gently homogenized over ice in a Wheaton glass homogenizer. The crude homogenate was centrifuged at 1,000xg in a Sorvall SS34 rotor at 4°C for 3 min to remove the larger tissue fragments. The supernatant was removed, and the pellet was resuspended in sterile ASW and centrifuged at 1,000xg for a further 3 min. The resulting pellet was discarded while both supernatants were combined and centrifuged at 3,000xg for 5 min at 4°C. The supernatant resulting from this centrifugation was removed and again the pellet was resuspended and recentrifuged at 3,000xg. After discarding this pellet supernatants were combined and centrifuged in a refrigerated Eppendorf microfuge at 15,000xg for 20 min. Microscopic examination of these bacterial pellets indicated a large reduction in the presence of animal cell nuclei and other organelles. Three "bacterial" samples were prepared in this way. A fourth "bacterial" sample was resuspended in 90% Percoll (density = 1.030g ml⁻¹) and 10% 1.0 M NaCl and centrifuged at 5,000xg for 30 min. The resulting pellet was washed 3 times by centrifugation at 15,000xg for 45 s. to remove Percoll. The 4 bacterial pellets (39 gill pairs) were dried at 60°C for approximately 24 h and analyzed for C and N stable isotope ratios. There was no significant difference in the stable isotope measurements of bacterial pellets prepared in these alternate ways.

Samples of *Solemya borealis* and *Inanidrilus* (= *Phallodrilus*) *leukodermatus* were dissected and dried to constant weight at 60°C. *Thiomicrospira crunogena*, a chemoautotrophic sulfur-oxidizing

bacterium, was isolated from 2,600 m at 21°N from the hydrothermal vent area near the East Pacific Rise (Jannasch *et al.*, 1985) and viable cultures were maintained at -70°C. This species is a gram negative vibrioid bacterium 0.4-0.5 mm in width and 0.5-3.0 mm in length. Eight liters of culture were grown in an artificial seawater medium containing 430 mM NaCl, 7.5 mM (NH₄)₂SO₄, 6.1 mM MgSO₄·7H₂O, 3.1 mM KH₂PO₄, 2.4 mM NaHCO₃, 2.0 mM CaCl₂·2H₂O, 20 mM Tris HCL and 8 ml of trace element solution SL-8 (Biebl and Pfennig, 1978) in double-distilled water; 10 mM final concentration of Na₂S₂O₃ was added as a sulfur source. Media was filter sterilized and added to autoclaved culture flasks. The pH of the media was adjusted to pH 7.5 with NaOH. Cells were harvested by centrifugation of 200 ml aliquots at 10,000xg for 20 min. and dried at 60°C.

Samples for carbon and nitrogen determinations were combusted in sealed Vycor tubes at 900°C for 1 h, followed by cryogenic combustion of CO₂ and N₂ gases. Sulfur samples were combusted to sulfate at 590°C for 12 h using a recently developed sealed tube combustion method with NaNO₃:KNO₃:NaCl (1:1:1) as the oxidizing agent (Fry, Marine Biological Laboratory, in preparation). The sulfate was precipitated with barium and subsequently decomposed to yield pure SO₂ for isotopic analysis (Yanigusawa and Sakai, 1983). Values thus obtained for δ³⁴S represent the isotopic composition of both organic sulfur constituents and stored elemental sulfur. All isotope measurements were determined using a Finnigan 251 ratio mass spectrometer. Standards were high-purity gases from commercial cylinders previously calibrated against National Bureau of Standards reference materials (δ¹³C, δ¹⁵N) or Canyon Diablo troilite (CDT, δ³⁴S). δ¹³C, δ¹⁵N and δ³⁴S values are reported relative to Pee Dee Belemnite (PDB), atmospheric N₂ and CDT respectively using the standard delta notation.

$$\delta X = [(R_{\text{sample}}/R_{\text{std}}) - 1] \times 10^3$$

where X = ¹³C, ¹⁵N, or ³⁴S

and R = ¹³C:¹²C, ¹⁵N:¹⁴N, or ³⁴S:³²S.

Figure 2.18 Endosymbiont "enrichment" procedure

2.4 Total protein and carbohydrate determination

Solemya velum and *Mya arenaria* were collected from Little Buttermilk Bay during October and November 1988, divided into gills, foot and visceral mass (defined here as remaining soft parts excluding adductors) and dried to constant weight at 60°C. Five to ten mg of tissue were ground to a fine powder and homogenized with 2 mls of HPLC-grade distilled water; 500µl aliquots of the homogenate were used for each total protein and carbohydrate determination. Total protein was estimated using the biuret assay, with bovine serum albumin as the standard, carbohydrate was determined using the H₂SO₄/phenol technique, with d-glucose as the standard (Raymont, 1964). Five specimens of each species were used in the analyses.

2.5 Phenylisothiocyanate Derivatization of Amino Acids.

2.5.1 Method Development

The formation of a phenylcarbamyl derivative of amino acids, through the use of phenylisothiocyanate (PITC, the Edman reagent) was first demonstrated by Koop *et al.* (1982) for the analysis of amino acids liberated by carboxypeptidase digestion of peptides from cytochrome P-450. Modifications of the technique for the derivatization of free amino acids released by the acid hydrolysis of pure proteins, and subsequent high pressure liquid chromatography (HPLC) analysis are outlined in Bidlinger *et al.* (1984); Henrickson and Meredith, (1984), and Yang and Sepulveda (1985). As retention times, reproducibility and resolution differ markedly between different HPLC systems, the published methodologies had to be significantly modified for use in this study, as none of the published methods produced adequate resolution of all the common amino acids. Moreover, prior to this study PITC derivatization had not been used in the analysis of complex biological tissues. Consequently, once the technique itself had been optimized for the determination of free amino acids it was necessary to determine the appropriate methods for the hydrolysis and derivatization of whole tissue samples.

Advantages of this technique over traditional ion-exchange and O-phthalaldehyde derivitization methods include the formation of stable reaction products with all primary and secondary amino acids, and the presence of a linear relationship between the concentration of the amino acid derivative and the electrical signal of the detector. Detection limits are in the picomolar range.

Optimal derivatization techniques

Optimal amino acid derivatization, peak separation and detection were achieved using the following protocols. These methods differ from the published PITC derivitization methodologies in that it was necessary to change the pH and strength of the HPLC eluents, modify the gradient elution program significantly and to increase the length of each HPLC run.

Amino acid standards, PITC, triethylamine (TEA) and glacial acetic acid were purchased from Sigma; HPLC grade solvents and water were obtained from Burdick and Jackson. All glassware was washed in a strong dichromic acid solution, rinsed 10 times in water and sonically cleaned with distilled water, methanol and acetone. For derivatization of amino acids, 20 μ l of free amino acid standard plus internal standards (norleucine and gamma-amino butyric acid [GABA]) was placed in an Alltech microvial and dried under vacuum. The amino acid mixture was coupled with 40 μ l of PITC solution (ethanol:water:TEA:PITC = 7:1:2:1, made fresh daily to prevent the formation of PITC degradation products; PITC was stored at -20°C under nitrogen) for 15 minutes and dried again under vacuum. PITC-amino acids derivitized in this way can be stored frozen for at least three months without affecting the results.

Amino acids were separated on a Beckman high-resolution reverse-phase C₁₈ column (25cm length, 4.6 mm diameter), maintained at 48°C and connected to an Isco 2360 gradient programmer, Varian 2010 HPLC pump and Spectroflow 757 UV/visible absorbance detector. The Beckman high-resolution column was found to produce better separation than the Spherisorb ODS column used in most of the published methodologies. PITC-amino acids were detected at 254 nm. Chromatographic data were

collected and analyzed using a Nelson Analytical 444/446 chromatography data system.

The following gradient program and eluent system was found to produce optimal separation of the PITC-amino acids.

Eluent A - 0.03% sodium acetate in 6% acetonitrile in water and 0.005% triethylamine (TEA). The pH of this eluent significantly affects the retention time of many peaks. Optimum pH was found to be in the range 6.15-6.25, adjusted with glacial acetic acid.

Eluent B - 50% acetonitrile in water

The gradient programmer was programmed to deliver the eluents to the Varian 2010 pump in the following program (Table 2.1) at a rate of 0.8 mls min⁻¹.

Table 2.1 Gradient elution program for the detection of amino acids.

Eluent A	Eluent B	Time
100%	0%	Start
65%	35%	In 20 minutes
20%	80%	In 10 minutes
0%	100%	In 1 minute
0%	100%	Hold for 10 minutes
100%	0%	In 5 minutes
100%	0%	Hold for 20 minutes (to re-equilibrate column)

Blanks of the entire procedure were run (Figure 2.19) and demonstrate that amino acid contamination was not a problem.

INTF:700
F S.=10

Figure 2.19. Blank derivitization at highest detector sensitivity. Demonstrates contamination levels of less than 10 pm.

Two hundred to one thousand μl of eluent A was added to the derivitized sample prior to derivitization, and 20 μl -100 μl injected. Response factors and retention times were determined for each amino acid relative to both internal standards. Once the retention times of each amino acid was known, a free amino acid standard mixture containing between 50 and 350 $\mu\text{M}\cdot\text{L}^{-1}$ of the following amino acids was prepared : aspartic acid (asp), glutamic acid (glu), hydroxyproline (OH-P), asparagine (asn), glutamine (gln), serine (ser), glycine (gly), histidine (his), arginine (arg), taurine (tau), alanine (ala), proline (pro), NH_4^+ , tyrosine (tyr), valine (val), methionine (met), cysteine (cys), isoleucine (ile), leucine (leu), phenylalanine (phe), tryptophan (trp), ornithine (orn) and lysine

(lys). The non-physiological amino acids norleucine and gamma-amino butyric acid (GABA) were used as internal standards. A typical chromatogram is presented in Figure 2.20. Different concentrations of this amino acid mixture were prepared and derivitized in order to determine retention time reproducibility, response factor reproducibility, derivitization reproducibility, and detector response linearity. Generally, the reproducibility of all aspects of the analysis were within 5% (Table 2.2), and detector response was determined to be linear for all the amino acids studied. (Figure 2.21)

Figure 2.20 Typical chromatogram using conditions described in the text.

Table 2.2 Errors associated with PITC derivitization of amino acids.

Amino Acid	% S.D. Relative Retention Time with respect to GABA (N=4)	% S.D. Retention Time (Absolute Values, N=9)	% S.D. Relative Response Factor with respect to GABA (N=4)	% S.D. Injection Area Normalized to GABA (N=4)
Aspartic Acid	0.8	1.0	1.9	0.1
Glutamic Acid	0.5	1.7	4.4	0.4
Hydroxy-Proline	0.9	1.5	3.6	1.8
Asparagine	2.0	1.6	1.0	1.3
Serine	1.8	1.5	2.5	0.8
Glutamine	2.5	1.6	4.0	0.7
Glycine	1.8	1.4	3.2	1.5
Histidine	1.6	1.5	3.9	8.1
Arginine	3.0	0.9	1.9	5.1
GABA*	0.0	1.2	0.0	0.5
Threonine	5.5	1.2	3.8	1.9
Alanine	2.3	1.2	2.3	3.2
NH ₄ ⁺	0.5	1.2	2.3	2.1
Proline	1.8	1.4	2.6	1.6
Tyrosine	1.1	1.2	3.6	4.6
Valine	0.8	0.8	5.9	3.5
Methionine	0.6	0.9	0.5	2.3
Cysteine/Cystine	1.2	0.8	3.9	3.3
Isoleucine	0.6	0.9	2.7	1.7
Leucine	0.4	0.9	3.0	2.6
Norleucine*	0.4	1.0	0.2	0.0
Phenylalanine	0.4	0.8	4.0	3.7
Tryptophan	0.4	0.7	0.6	4.6
Lysine	0.5	0.7	6.7	4.2

Figure 2.21 Linearity of PITC derivitization

Figure 2.21 Continued

Figure 2.21 Continued

Figure 2.21 Continued

2.5.2 Amino Acid Analyses of animals and bacteria.

Specimens of *Solemya velum*, *Mya arenaria*, *Solemya borealis*, and *Inanidrilus leukodermatus* were collected and dissected as described previously (pages 69 and 83), small aliquots of the *Thiomicrospira crunogena* bacterial pellets were also analyzed.

Total hydrolyzable amino acids (THAA)

For THAA analysis each tissue fraction was placed in a tared 10 ml ampule and wet weight determined. Two mls of 6N HCL was added to each ampoule and the sample was spiked with known amounts of the internal standards norleucine and GABA. The known ratios of these amino acids also served as a check on the sample preparation techniques. Each ampoule was flushed successively with nitrogen and evacuated under vacuum to remove oxygen, Ampoules were sealed under vacuum and kept at 110°C for 36 h and subsequently frozen at -20°C prior to PITC derivitization. Samples were filtered through 0.45µm PFTE filters prior to derivitization. Conversion of asparagine and glutamine to aspartic and glutamic acids occur during hydrolysis, and yields of serine, tryptophan and threonine are reduced during acid hydrolysis.

Free amino acids

Each tissue fraction was placed in a tared 5 ml homogenization tube and 2 ml of 5%trichloroacetic acid (TCA) in ethanol:water (50:50) were added to each. The samples were homogenized after the addition of internal standards and sealed under nitrogen. The free amino acids were extracted for 36 h, each sample was then filtered through 0.45 µm PFTE filters and stored at -20°C until derivitization.

PITC derivitization

THAA and free amino acid samples were transferred to 5 ml derivitization vials and the HCL and TCA removed under vacuum. 100 µl of ethanolic solution (ethanol:water:TEA = 1:1:1) were added to each vial and removed under vacuum, to ensure complete removal of HCL and other reagents. Derivitization to PITC-amino acids was achieved by adding 330-550 µl of PITC solution

(depending on sample size) to the sample. After 15 minutes the PITC solution was removed under vacuum, the sample was diluted with 1-2 ml of eluent A, filtered through a 0.22 μm PFTE syringe filter and analyzed for PITC-amino acids.

Chromatography was as described previously, PITC-amino acids were separated on a Beckman high resolution C_{18} reverse-phase column at 48°C and analyzed at 254 nm using a flow rate of 0.8 ml min^{-1} and the gradient program already described. Amino acids were identified and quantified by comparison with the retention times and response factors determined for amino acid standards and the internal standards. Blanks were run for each derivitization and any amino acid concentrations in the blanks were subtracted from the sample concentrations (contamination levels were always less than 1%).

For $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements, the filtered, TCA-extracted and HCL hydrolyzed samples, were evaporated under nitrogen and combusted at 900°C , followed by cryogenic combustion of CO_2 and N_2 as described in section 2.3. For $\delta^{34}\text{S}$ determination of the free amino acids, TCA extracts of five specimens of *S. velum* were combined, passed through an activated copper column to remove any elemental sulfur, and evaporated under nitrogen. Samples were combusted to sulfate at 590°C for 12 h with $\text{NaNO}_3:\text{KNO}_3:\text{NaCl}$ (1:1:1) as the oxidizing agent. The sulfate was precipitated with barium and subsequently decomposed to yield pure SO_2 for isotopic analysis (See section 2.3). Isotope measurements were determined using a Finnigan 251 ratio mass spectrometer. Standards were high purity gases from commercial cylinders calibrated against National Bureau of Standards reference materials. The stable isotope ratios are reported relative to Pee Dee Belemnite (PDB), N_2 in air and CDT Triolite using the standard delta notation:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$$

Where $X = {}^{13}\text{C}$, ${}^{15}\text{N}$, or ${}^{34}\text{S}$

and $R = {}^{13}\text{C}/{}^{12}\text{C}$, ${}^{15}\text{N}/{}^{14}\text{N}$, or ${}^{32}\text{S}/{}^{34}\text{S}$.

2.6 Lipid analyses

Specimens of *Solemya velum*, *Mya arenaria*, *Solemya borealis*, and *Inanidrilus leukodermatus* were collected and dissected as described previously (pages 69 and 83), small aliquots of the *Thiomicrospira crunogena* bacterial pellets were also analyzed.

For lipid analysis, frozen tissues were thawed and dried to constant weight at 60°C and were ground to a fine powder. Comparasion of the lipid composition of *Mya arenaria* with values previously reported for this species and other typical marine bivalves (Joseph, 1982) demonstrate that double bond oxidation is unlikely to have occurred to any large extent. The dry weights of *T. crunogena* extracted could not be determined because of the large amounts of sulfur produced externally by these bacteria. Removal of the sulfur prior to extraction was not attempted because of the low culture yield. The lipids of 5-50 mg of animal tissue or bacterial culture were extracted by successive sonication with 40 ml isopropyl alcohol, 40 ml chloroform:methanol (1:1), and 40 ml chloroform:methanol (3:1) after the addition of two internal recovery standards (a C₁₉ fatty acid and a C₂₁ fatty acid methyl ester). Forty ml isopropyl alcohol was added to the sample, along with the internal standards and the sample was sonicated for 8-10 min. using a Tekmar sonic insertion probe disruptor. After centrifugation, the isopropanol was decanted into a separatory funnel containing 60 ml 1/6 saturated NaCl (made with KMnO₄-distilled water). The sample was then re-extracted with 40 ml chloroform:methanol (1:1), and with 40 ml chloroform:methanol (3:1). After each extraction, the tubes were centrifuged, and the supernates were combined in the separatory funnel. Lipids were partitioned into the isopropanol/chloroform phase and drained into a round-bottom flask containing ~30 ml granular Na₂SO₄ which had been Soxhlet extracted. The aqueous phase in the separatory funnel was re-extracted twice with 25 ml chloroform and added to the round bottom flask. An additional 25 ml of chloroform was then added to the round-bottom flask. The flask was vigorously swirled and allowed to stand for at least 2 h to allow the Na₂SO₄ to dry the solution. The solution was then decanted into a glass

funnel containing a glass wool plug and 25 ml Na_2SO_4 . The funnel was rinsed with two aliquots of 25 ml chloroform, and the lipid extract was rotary evaporated to a small volume.

Half the lipid extract was used for total fatty acid, sterol, hydrocarbon and hopanoid analysis and was transferred in chloroform:methanol (3:1) to air tight centrifuge tubes. The volume of sample was reduced under nitrogen and two ml toluene was added to each centrifuge tube. Two ml 0.5 N KOH in methanol was added to each tube, and the samples were sealed under nitrogen. Fatty acids were then saponified by placing the centrifuge tubes in boiling water for 20 min. Fatty acids were converted to fatty acid methyl esters (FAMES) by reaction of the saponified sample with 2-3 ml 10% BCl_3 -methanol (Alltech). The samples were again sealed under nitrogen and were boiled for 5 min.

Lipid groups were separated from each other by fractionation on a silica gel column (7 g soxhlet extracted Bio-Sil A, 100-200 mesh, 5% deactivated with water in 0.9 x 35 cm columns) and packed over copper previously activated with 2N HCL using the following elution scheme (Table 2.3); the copper served to remove elemental sulfur). Recovery of the internal standards usually exceeded 95%.

Table 2.3 Column chromatography elution system.

<i>Fraction</i>	<i>Eluent</i>	<i>Compounds eluted</i>
1	20 ml hexane	alkanes, some monoenes
2	20 ml toluene/hexane (25:75 v/v)	polyunsaturated alkenes, aromatics
3	20 ml toluene/hexane (50:50 v/v)	polyunsaturated alkenes, aromatic compounds
4	20 ml ethyl acetate/hexane (5:95 v/v)	fatty acid methyl esters, wax esters, steryl esters
5	20 ml ethyl acetate/hexane (10:90 v/v)	fatty acid methyl esters, ketones
6	20 ml ethyl acetate/hexane (15:85 v/v)	n-alcohols, hopanols, 4-methyl sterols
7	20 ml ethyl acetate/hexane (20:80 v/v)	4-desmethyl sterols
8	20 ml ethyl acetate	polar material
9	20 ml methanol	polar material

The remaining lipid extract was separated by thin layer chromatography (TLC) into (1) polar lipids, (2) monoacylglycerides, (3) free sterols, (4) combined diacylglycerides, triacylglycerides and free fatty acids, and (5) steryl and methyl esters on Whatman LK5D Linear K Silica Gel TLC plates (Figure 2.22). The solvent system consisted of dichloroethane:chloroform:acetic acid:acetone:isopropyl alcohol (92:8:0.1:0.03:0.03). Plates were pre-run in chloroform:methanol (3:1) to remove any contaminants. Lipid bands were determined by running authentic lipid class standards on each plate followed by visualization with 2',7'-dichlorofluoresceine. Lipid classes were scraped off each plate and eluted from the silica gel with methanol, toluene and hexane in

mini-columns made of pasteur pipettes with a small plug of soxhlet extracted glass wool. The fatty acids of each lipid class were saponified and free fatty acids converted to FAMES as described above. Recovery of internal standards usually exceeded 80%.

Figure 2.22 Diagrammatic representation of TLC lipid class bands obtained using the conditions described in the text. PL = phospholipids; M = monoglycerides; FFA = free fatty acids; ST = sterols; DI = diglycerides; TRI = triglycerides; SE = steryl esters.

All FAME samples were analyzed by gas chromatography by coinjection on a J&W Scientific Durabond DB-5 30 m fused silica column (0.32 mm i.d., 0.25 mm film thickness) and a J&W Scientific Durabond DB-Wax 30 m fused silica column (0.25 mm i.d., 0.25 mm film thickness). Both columns were mounted in Carlo Erba 4160 high resolution gas chromatographs equipped with flame ionization detectors. Identification of individual FAMES was based on a comparison of the retention times of authentic standards on both

columns. If a substance coeluted with a standard on both columns it was assumed to be the same as the standard. The results for individual fatty acids from the column yielding the lowest concentration for that fatty acid were used in data interpretation to ensure that coelution of unknown compounds were not affecting the results. FAME identifications were confirmed by electron-impact gas chromatography mass spectrometry (GC-MS) using a J&W Scientific DB-5 fused silica column installed in a Carlo Erba high resolution gas chromatograph interfaced to a Finnigan 4500 quadropole mass spectrometer and controlled by an INCOS data system. The *cis* configuration of the double bond in 18:1 ω 11 was verified by coinjection with *cis*- and *trans*-vaccenic acid standards; these compounds are resolved on the DB-5 column. Alkane and hopanol fractions were analyzed on the DB-5 fused silica column. Sterol composition was determined by GC-MS. Prior to analysis the sterols were acylated by reaction with 40 μ l pyridine and 40 μ l acetic anhydride overnight under nitrogen.

All glassware used in lipid analyses was cleaned successively with soap and water, distilled water, methanol, acetone, toluene, hexane and methylene chloride. Analytical blanks of the entire lipid extraction procedure were periodically run to check analytical techniques. Recoveries of the internal standards generally exceeded 95%.

For $\delta^{13}\text{C}$ measurements, the fatty acids of *T. crunogena* and fatty acid and sterol samples pooled from two animals of each bivalve species were evaporated to dryness under nitrogen and sealed under vacuum while frozen. Samples were combusted at 900°C, followed by cryogenic combustion of CO_2 as described previously. Isotope measurements were determined using a Finnigan 251 ratio mass spectrometer. Standards were high purity gases from commercial cylinders calibrated against National Bureau of Standards reference materials. The $\delta^{13}\text{C}$ ratios are reported relative to Pee Dee Belemnite (PDB) using the standard delta notation:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$$

Where $X = {}^{13}\text{C}$

and $R = {}^{13}\text{C}/{}^{12}\text{C}$.

The effects of derivitization on the $\delta^{13}\text{C}$ ratios of the fatty acids were determined by analyzing the $\delta^{13}\text{C}$ ratios of a 19:0 fatty acid standard before and after saponification and derivitization. In addition, the $\delta^{13}\text{C}$ ratios were adjusted for the effects of solvent residues by running blanks and calculating the blank corrected $\delta^{13}\text{C}$ values using the following equation.

$$\delta_o V_o = \delta_s V_s + \delta_b V_b$$

Where : $\delta = \delta^{13}\text{C}$

V = Voltage from mass 44 ion beam $\sim$ ($\approx$ sample size)

o = observed

s = sample

b = blank

$$\text{and } \delta_s = \frac{\delta_o V_o - \delta_b V_b}{V_s}$$

Since $V_o = V_s + V_b$, $V_s = V_o - V_b$

$$\Rightarrow \delta_s = \frac{\delta_o V_o - \delta_b V_b}{V_o - V_b}$$

This correction did not affect the isotope ratios by more than 5%.

2.7 References

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CHAPTER 3

THE ROLE OF ENDOSYMBIOTIC BACTERIA IN THE NUTRITION
OF *SOLEMYA VELUM*: EVIDENCE FROM STABLE ISOTOPE
ANALYSES OF ENDOSYMBIONTS AND HOST.

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3.1 Abstract

Stable isotope compositions were investigated in the protobranch bivalve *Solemya velum*, a species known to harbor endosymbiotic chemoautotrophic bacteria, in order to determine the dominant nutritional sources for this species. C, N, and S stable isotope ratios were determined in gill and foot tissue of *S. velum*; C and N isotope values were also examined in an enriched bacterial fraction, separated from intact gills by differential centrifugation.

The bacterial fraction and host tissues had similar $\delta^{13}\text{C}$ values (-30.9 ‰ to -33.9 ‰), suggesting that endosymbiotic bacteria provide as much as 98% of the carbon requirements of *Solemya velum*. The $\delta^{15}\text{N}$ values of both *S. velum* and the endosymbionts were comparable (4.4‰ to -9.8‰) and considerably lower than those of bivalve controls that do not harbor endosymbionts (*Mya arenaria* and *Tellina agilis*, $\delta^{15}\text{N}$ = 6.3‰ to 8.5‰). Uptake of pore-water ammonium by the endosymbionts and subsequent translocation of nitrogenous compounds to the host may account for the low $\delta^{15}\text{N}$ of *S. velum*. In this context, the symbionts may provide 100% of the host nitrogen. The $\delta^{34}\text{S}$ values of *S. velum* (-26.7‰ to -31.15‰) suggest the use of biogenically produced H_2S as a sulfur source. Both the sulfur and nitrogen stable isotope ratios are among the

lowest reported for animal tissues. This study represents the first attempt to estimate the bacterial contribution to host nutrition by examining the stable isotope ratios of both bacteria and host tissue and demonstrates the large contribution of bacterial endosymbionts to the diet of *S. velum*.

3.2 Introduction

Symbiotic associations between marine invertebrates and chemolithotrophic bacteria occur in at least 4 invertebrate phyla (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981; Giere *et al.*, 1984; Southward *et al.*, 1981). The majority of these animals are deep-sea dwellers and, in many species, the gut is either greatly reduced or totally absent. This lack or reduction of the digestive apparatus, coupled with the presence of large bacterial populations within specific animal tissues, strongly indicates a trophic function for the bacteria. Quantifying the extent of the bacterial contribution to nutrition using standard energetic, respiration and enzyme kinetic studies, however, is difficult. In many cases, the physiological condition of the animal is sub-optimal. Moreover, the results of such studies, generally carried out over short periods, may not be representative of longer time scales.

An alternative approach to studying the nutritional importance of the bacterial symbionts is the use of stable isotope ratios. The analysis of stable isotope values has become an important method of identifying and quantifying trophic pathways and processes in both field and laboratory situations. In many situations, stable isotope studies may be preferable to more traditional methods of food web analysis. Stable isotope ratios reflect only those materials actually assimilated by the organism (Rounick and Winterbourn, 1983). Furthermore, the stable isotope value of an organism can provide a temporally integrated history of an organism's diet, avoiding the need for long-term laboratory and field studies and reducing the sample sizes required in some cases. Thus, stable isotope studies hold particular promise for the study of trophic interactions in delicate, ephemeral, or relatively inaccessible ecosystems, such as hydrothermal vents.

Stable isotope ratios are calculated as parts per thousand differences from standard reference materials. Samples with negative isotope ratios are depleted relative to the standard, whereas those with positive isotope values are enriched in the

heavy isotope. The stable isotope ratios of certain elements of biological importance occur in constant ratios globally (Table 3.1).

Table 3.1 Average isotopic ratios of light elements on earth.

Isotope	Ratio
H/D	64.1×10^2
$^{12}\text{C}/^{13}\text{C}$	89.1
$^{14}\text{N}/^{15}\text{N}$	27.7×10
$^{16}\text{O}/^{18}\text{O}$	50.1×10
$^{32}\text{S}/^{34}\text{S}$	22.6

The stable isotope ratios of a given biological sample will usually differ from these global values, however, because chemical reactions occurring with a non-limiting supply of reactants will preferentially utilize the lighter of the two isotopes. The power of this technique lies in the fact that these stable isotope ratios are altered by both biological and non-biological processes in a highly predictable manner (Peterson and Fry, 1987). A few well characterized reactions are responsible for controlling the isotopic compositions of most organic compounds. Because the isotopic signatures resulting from these key, or root, reactions are generally passed along trophic pathways with small, predictable variations, stable isotope values can provide estimates of the origins of the elements in question (Rau 1981a; Rau *et al.*, 1983; Fry and Sherr, 1984; Rounick and Winterbourn, 1986; Spiro *et al.*, 1986). Furthermore, if the diet of an organism consists of two isotopically distinct food sources, the isotopic composition of the organism should reflect the relative utilization of each of the potential food items (Fry and Sherr, 1984). In this context, a simple two-source mixing model can be used to determine the contributions of two food sources (Figure 3.1).

Figure 3.1. Simplified schematic representation of the application of two-source mixing models for the determination of relative dietary contributions from two food sources (A and B) to the stable isotope ratios of an organism (C).

The extent of isotope fractionation by chemosynthetic processes is not well characterized. Studies conducted thus far suggest that the reduction of inorganic carbon during chemoautotrophy generally results in a greater depletion of the heavier isotope (i.e., more negative $\delta^{13}\text{C}$) than that resulting from photosynthetic processes (Degens 1969; Ruby *et al.*, 1987). If so, given the same inorganic carbon source, chemoautotrophic bacteria should have a $\delta^{13}\text{C}$ value several per mil lower than phototrophically-based biomass. The stable isotope ratios of a number of species containing symbiotic bacteria have been investigated; all show unusual $^{13}\text{C}:^{12}\text{C}$ ratios (Rau 1981a; 1985; Southward *et al.*, 1981; Felbeck 1983; Spiro *et al.*, 1986), although pogonophoran and vestimentiferan worms exhibit unexpectedly heavy isotope values. Consequently it has been inferred that much of the host carbon is supplied by the bacteria and derived from chemosynthetically-fixed CO_2 (Rau and Hedges 1979; Rau 1981a; Felbeck 1983). Furthermore, unusually low $^{15}\text{N}:^{14}\text{N}$ ratios in

hydrothermal vent animals known to contain symbionts are also suggestive of a non-photosynthetically derived food source (Rau 1981b).

These investigations provide only qualitative information, however, as they do not involve any separate determination of the stable isotope ratios of the endosymbionts. A knowledge of both the bacterial and host isotope signatures, and the isotopic composition of the more typical phytoplankton-based food source is a prerequisite for quantifying the extent of nutritional coupling between host and symbiont. Consequently, in the present study, ratios of the stable isotopes C, N and S were examined in tissues of *Solemya velum* (Say), a bivalve species known to harbor endosymbiotic chemoautotrophic bacteria. C and N isotope ratios were also investigated in "bacterial tissue" separated from intact gills by differential and density-gradient centrifugation. In this way the contribution of the endosymbionts to nutrition of the animal host could be assessed quantitatively through the use of a simple, two-source mixing model.

$$\delta S. \text{ velum} = (F)\delta_{\text{bacteria}} + (1-F)\delta_{\text{control bivalve}}$$

where F is the fractional contribution from the bacteria

$$F = (\delta S. \text{ velum} - \delta_{\text{control bivalve}}) / (\delta_{\text{bacterial pellet}} - \delta_{\text{control bivalve}})$$

and δ is $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$.

Isotopic ratios of the bacterial pellet were assumed to represent the endosymbiont values, while isotopic values of the control bivalves were taken to represent a phytoplankton-based food source. In order to exclude isotopic variability that may arise as a result of biochemical differences between gill and foot tissues, only foot tissue was used in the model.

3.3 Results and Discussion

This report represents the first attempt to assess the nutritional importance of bacterial endosymbionts through determination of the stable isotope ratios of host tissue and bacteria. The results suggest that *Solemya velum* does not rely on the typical marine phytoplankton-based food chain as a nutritional source. For clarity each element is reported and discussed separately.

3.3.1 $\delta^{13}\text{C}$ Ratios

A dramatic difference was apparent between the $\delta^{13}\text{C}$ values of *Solemya velum* (-30.9‰ to -33.9‰) and the control bivalves *Mya arenaria* and *Tellina agilis* (14.2‰ to -17.8‰; Table 3.2, Fig. 3.2a). Animal tissues usually show an enrichment in $\delta^{13}\text{C}$ of 1-2‰ in comparison with dietary carbon (DeNiro and Epstein, 1978). This enrichment of the heavier isotope may be due to the preferential loss of ^{12}C in respired CO_2 , selection of ^{13}C during assimilatory processes, or discrimination between isotopes during enzyme-mediated biochemical processes. The $\delta^{13}\text{C}$ values of the control animals would appear to reflect a dietary carbon source ultimately derived from marine phytoplankton ($\delta^{13}\text{C}$ generally -18‰ to -24‰, references in Gearing *et al.*, 1984), and subsequently modified slightly by metabolic reactions and trophic interactions. The large difference between the $\delta^{13}\text{C}$ values of *S. velum* and the non-symbiont controls (Fig. 3.2a) indicates that straightforward heterotrophy is unlikely to be an important trophic mechanism in *Solemya velum*. Assuming a 1‰ increase in $\delta^{13}\text{C}$ relative to dietary sources the $\delta^{13}\text{C}$ ratios of the food of *S. velum* is probably in the -32.9 to -34.9‰ range, values very different from those expected for photosynthetically-derived food sources.

The observed $\delta^{13}\text{C}$ enrichment of approximately 1-2‰ between the bacterial pellet and foot tissues of *Solemya* at both sites (Table 3.2) is typical of the small metabolic fractionations that occur during producer-consumer interactions. This finding suggests a tight trophic coupling between endosymbiont and host. The reason for a further $\delta^{13}\text{C}$ depletion of 1‰ in gill tissue is not as clear. It

may be due to the chemical composition of the gills. The lipid fraction of biological tissues generally exhibits lower $\delta^{13}\text{C}$ values (Figure 3.4, Degens *et al.*, 1968 and references within; Prahl and Meulhausen, 1989); consequently it is conceivable that the membrane lipids associated with the extensive epithelial gill surfaces of *S. velum* may produce heavy isotope depletion. This appears to be the case in *S. velum* as the lipids of this species have much lower $\delta^{13}\text{C}$ ratios than whole animal $\delta^{13}\text{C}$ values (See Chapter 4). Similar differences between foot and gill tissue carbon isotope ratios are also found in the gills of *T. agilis* (Table 3.2) and *Dosinia lupinis* (Spiro *et al.*, 1986), both non-symbiont bivalves. For this reason, only bivalve foot tissue was used in the mixing model.

The $\delta^{13}\text{C}$ ratios of *S. velum* showed a consistent difference of approximately 1‰ between the two sites examined. This could be due to variability in (1) the concentration or the isotopic composition of the dissolved inorganic carbon pool at each site, (2) the enzymatic fractionation of carbon isotopes occurring during carbon fixation, or (3) differences in the intracellular concentration of CO_2 or HCO_3^- .

The carbon stable isotope values determined for *S. velum* fall within the range reported for other symbiont-containing bivalves (Rau, 1981a; Spiro *et al.*, 1986; Berg and Alatalo, 1984, Table 3.2). Because marine chemoautotrophic bacteria appear to fractionate carbon isotopes to a greater extent than do photosynthetic organisms (Degens 1969; Ruby *et al.*, 1987), the simplest explanation for the ^{13}C depletion in these organisms is that much of their organic carbon is derived from the chemoautotrophic fixation of seawater-derived CO_2 , or respired CO_2 . The hypothesis that most, or all, of the host cell carbon is ultimately derived from bacterial chemoautotrophy is supported by the close similarity between the $\delta^{13}\text{C}$ values of the foot (animal tissue), the gills (animal + bacterial tissue) and the bacterial pellet (predominantly bacterial tissue). If the carbon isotope ratios of the control bivalves *Mya arenaria* and *Tellina agilis* are representative of a plant-based food source, and the bacterial pellet is representative of the endosymbiont $\delta^{13}\text{C}$ value, then the contribution of each of these two food sources to the carbon budget of *S. velum* can be estimated using the two-source mixing model (Fig. 3.3).

Table 3.2 C, N, and S stable isotope ratios in *Solemya velum*, *Mya arenaria*, *Tellina agilis*, and sediments from Little Buttermilk Bay and Hadley's Harbor, Cape Cod, Massachusetts.

Species Little Buttermilk Bay				Species Hadley's Harbor			
	$\delta^{13}\text{C}_{\text{‰}}$	$\delta^{15}\text{N}_{\text{‰}}$	$\delta^{34}\text{S}_{\text{‰}}$		$\delta^{13}\text{C}_{\text{‰}}$	$\delta^{15}\text{N}_{\text{‰}}$	$\delta^{34}\text{S}_{\text{‰}}$
<i>Solemya velum</i>				<i>Solemya velum</i>			
Bacterial Pellet*	-33.6	-6.9		Bacterial Pellet*	-31.7	-7.9	
	-32.5	-8.6					
	-32.3	-8.0					
Gill Tissue	-33.9	-9.3		Gill Tissue	-32.8	+0.4	
	-33.6	-8.7	-28.2 [#]		-32.7	-0.3	-26.7 [#]
	-33.2	-9.8			-32.4	-4.6	
Foot Tissue	-32.1	-8.2		Foot Tissue	-31.0	+1.3	
	-31.7	-7.7	-31.1 [§]		-31.3	+4.4	-29.2 ^{§§}
	-31.5	-8.6			-30.9	-4.6	
<i>Mya arenaria</i>				<i>Tellina agilis</i>			
Gill Tissue	-17.2	+8.5		Gill Tissue	-15.4	+6.3	
	-17.5				-15.5	+6.5	
					-15.6		
Foot Tissue	-17.7	+8.3		Foot Tissue	-14.2	+6.8	
	-17.8				-14.6	+8.2	
					-15.2		
Body [¢]	-17.1	+8.2					
Sediment*	-17.4	+4.7		Sediment*	-18.2	+4, +4.9	
					-19.3		

* Data for 9-10 gill pairs, [#] $\delta^{34}\text{S}$ ratios for 10 pooled gill pairs, [§] $\delta^{34}\text{S}$ ratios for 10 pooled foot tissues, [¢] McDowell Capuzzo, Leavitt and Fry, unpublished results, • integrated value for the top ten cm.

Figure 3.2 Stable isotope ratios for *Solemya velum* in comparison with non-symbiont containing bivalves and potential nutritional sources at Little Buttermilk Bay (LBB) and Hadley's Harbor (HH). a) $\delta^{13}\text{C}$; b) $\delta^{15}\text{N}$; c) $\delta^{34}\text{S}$.

(¹) Wada *et al.*, 1975; (²) Sweeney and Kaplan, 1980; Hoefs, 1987; (³) Hoefs, 1987; (⁴) Haines and Montague, 1979; Incze *et al.*, 1982; (⁵) Gearing *et al.*, 1984; (⁶) Hoefs, 1987; (⁷) Fry and Krouse, in prep.; (⁸) Determined using the isotope fractionation values reported for thiobacilli in Fry *et al.*, 1986 and references therein.

Figure 3.3. Bacterial contribution to carbon and nitrogen nutrition in *Solemya velum* at Little Buttermilk Bay (LBB) and Hadley's Harbor (HH), calculated from the two-source mixing model described in the text. $\Delta\delta$ values denote isotopic differences between the foot tissue of control bivalves (■, bacterial contribution = 0%), the bacterial pellet (*, bacterial contribution = 100%), and *Solemya velum* foot tissue (o). Horizontal lines represent the ranges of isotopic values recorded.

Table 3.3 Stable isotope values for representative symbiont-containing bivalves and symbiont-free bivalves.

Species	Tissue	Location	Environment Type	Delta 13C (Per mille)	Delta 15N (Per mille)	Delta 34S (Per mille)
<i>Mya arenaria</i> *	Gill	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-17.2, -17.5 (1)	8.5 (1)	
<i>Mya arenaria</i> *	Foot	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-17.7, -17.8 (1)	8.3 (1)	
<i>Tellina agilis</i> *	Gill	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-15.4 (1)	6.3, 6.5 (1)	
<i>Tellina agilis</i> *	Foot	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-14.2, -15.2 (1)	6.8, 8.2 (1)	
<i>Solemya velum</i>	Gill	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-33.9 to -32.4 (1)	-9.8 to 0.4 (1)	-28.2 to -26.7 (1)
<i>Solemya velum</i>	Foot	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-32.1 to -30.9 (1)	-8.6 to 4.4 (1)	-31.1 to -29.2 (1)
<i>Solemya velum</i>	"bacteria"	Cape Cod (Massachusetts, U.S.)	Reducing Sediments	-33.6 to -31.7 (1)	-8.6 to -6.9 (1)	
<i>Calyptogena magnifica</i>	Mantle (foot, d34S)	21 Degrees North (E. Pacific Rise)	Hydrothermal Vent	-32.7, -32.6 (2)	3.2, 4.9 (3)	0.4, -1.7 (4)
<i>Calyptogena magnifica</i>	Mantle	Rose Garden (Galapagos Islands)	Hydrothermal vent	-32.1, -32.0 (2)	2.1, 2.8 (3)	
<i>Lucinoma borealis</i>	Gill	Salcomb (Devon, England)	Non-reducing Sediments	-29.0, -28.1 (5)		
<i>Lucinoma borealis</i>	Remaining Soft Parts	Salcomb (Devon, England)	Non-reducing Sediments	-25.9, -25.3 (5)		
<i>Thyasira sarsi</i>	Gill	Knappen (Norway)	Non-reducing Sediments	-31 (5)		
<i>Thyasira sarsi</i>	Remaining Soft Parts	Knappen (Norway)	Non-reducing Sediments	-28.2 (5)		
<i>Soelmya reidi</i>	Not Specified	Santa Monica (California, U.S.)	Reducing Sediments	-30 (6)		
<i>Bathymodiolus thermophilus</i>	Foot, gill Mantle	Galapagos islands Marianas Vents	Hydrothermal vent	-32.9 to -32.7 (7), (8)	-3.0, -0.5 (8)	

(1) This study; ((2) Rau, 1981a; (3) Rau, 1981b; (4) Fry et al., 1983; (5) Spiro et al., 1986

(6) Felbeck, 1983; (7) Rau and Hedges, 1979; (8) VanDover and Fry, 1989.

* Symbiont-free

From the $\delta^{13}\text{C}$ values of *S. velum*, the bacterial pellet, and the controls it would appear that the endosymbionts may provide as much as 98% of the host carbon (Fig. 3.3), clearly demonstrating the importance of the endosymbiotic bacteria in the carbon budget of *S. velum*. These estimates are supported by the results of preliminary experiments on the ability of *S. velum* to ingest phytoplankton cells, Appendix III). The results of previous qualitative studies (Felbeck 1983; Cavanaugh 1985; Spiro *et al.*, 1986; Anderson *et al.* 1987) also indicate chemoautotrophic endosymbionts are of nutritional importance to the host species. This study, however, represents the first quantitative evidence regarding the bacterial contribution to the host's dietary needs. The similarity between $\delta^{13}\text{C}$ values of *S. velum* and other bivalve-bacteria symbioses (Table 3.3) would suggest a close nutritional coupling between host and symbiont in the other species also.

Figure 3.4 Summary of the major isotope fractionation processes occurring in the marine environment.

3.3.2 $\delta^{15}\text{N}$ Ratios

Nitrogen stable isotope fractionation in *S. velum* also suggests unusual trophic pathways in this species (Fig. 3.2b). Nitrogen in animal tissues is predominantly derived from the diet. Fractionation of the isotopes of this dietary nitrogen by animals results in a +1.3‰ to +5.3‰ enrichment of the heavier isotope (average 3.4‰, Minawaga and Wada, 1984). Consequently, isotopic ratios found in the control species (+6.3 to +8.5 ‰; Table 3.2) represent a dietary nitrogen source with a $\delta^{15}\text{N}$ of +1‰ to +7‰. These values fall within the typical range for photosynthetic organisms, indicating a phytoplankton-based nitrogen source for these species (Fig. 3.1b). The nitrogen isotopic ratios in tissues of *S. velum*, however, are among the most $\delta^{15}\text{N}$ depleted values reported to date (-9.8‰ to +4.4‰), differing greatly from those of the control species (Fig. 3.2b).

Biological utilization of NO_3^{2-} or NH_4^+ can result in low animal tissue $\delta^{15}\text{N}$ ratios if these sources are unusually depleted in ^{15}N . Assuming a metabolic $\delta^{15}\text{N}$ enrichment of 3‰, the low nitrogen isotope ratios would represent a nitrogen source with $\delta^{15}\text{N}$ values of +1‰ to -12‰. Although we did not directly analyze porewater NO_3^{2-} or NH_4^+ , the $\delta^{15}\text{N}$ values of the bulk sediment (4.0‰ to 4.9‰) at both sites does not suggest that a $\delta^{15}\text{N}$ -depleted nitrogen source is present in these sediments. All the specimens of *Solemya velum* used in this study were collected from the top 10 cm of the sediment, where mineralization of sedimentary organic matter may produce an abundant supply of NH_4^+ . Such a supply can permit significant biological discrimination against the heavier isotope (Wada and Hattori 1978; Kohl and Shearer 1980; Macko *et al.*, 1987). Consequently, the low $\delta^{15}\text{N}$ values of *S. velum* and the endosymbionts suggest the utilization of a non-limiting nitrogen source, such as pore water NH_4^+ . Thiobacilli are capable of utilizing a wide range of nitrogen sources including ammonia, nitrate, and urea (Keunen and Beudeker, 1982). Thus, by analogy with thiobacilli, chemoautotrophic endosymbionts may provide nitrogen for the host by NH_4^{2+} and NO_3^{2-} assimilation. In addition, nitrogen reduction enzymes have been reported to occur in *Solemya reidi*,

although there appears to be little evidence for this in *S. velum* (Cavanaugh, pers. comm.).

The nitrogen stable isotope fractionation values of *S. velum* also suggest that bacteria play a significant role in host nutrition (Table 3.2, Fig. 3.2b), but application of the mixing model to the nitrogen stable isotope ratios yields ambiguous results (Fig. 3.3). Although at Little Buttermilk Bay bacteria may provide 100% of the host nitrogen, quantifying the bacterial contribution to host nitrogen at Hadley's Harbor is difficult because of the large variability in $\delta^{15}\text{N}$ ratios of *S. velum* at this site. This large variability in *S. velum* from Hadley's Harbor was unusual. Similar large variances in $\delta^{15}\text{N}$ ratios (-12.9‰ to 3.0‰) have recently been reported for a symbiont-containing mussel collected from the Louisiana slope seep area (Brooks *et al.*, 1987) and animals from the Florida escarpment (Paull *et al.*, 1985). In these bivalves $\delta^{15}\text{N}$ variability could be due to both differences in the dominant form of nitrogenous compound present (a variable known to affect the $\delta^{15}\text{N}$ values of biological tissues; Wada and Hattori 1978) and isotopic fractionations resulting from metabolic processes.

3.3.3 $\delta^{34}\text{S}$ Ratios

The $\delta^{34}\text{S}$ values found in *S. velum* are extremely low (-26.7 to -31.1‰, Table 3.2), similar to those reported for pore water H_2S (Goldhaber and Kaplan 1975, Fry *et al.*, 1986, Tables 3.4, 3.5), and much lower than $\delta^{34}\text{S}$ values of bivalves that utilize a phytoplankton-based trophic web (Fig. 3.2c). Isotope ratios for animals from marine food webs based on phytoplankton have sulfur isotope ratios similar to those of seawater sulfate (+20‰). The extremely negative $\delta^{34}\text{S}$ values found in *S. velum* at Little Buttermilk Bay and Hadley's Harbor suggest that biogenic, reduced sulfur acts as the sulfur source for this species. Dissimilatory sulfur reduction in marine sediments occurs with a large isotope fractionation effect (-30 to -70‰, Goldhaber and Kaplan, 1975; Fry *et al.*, 1986; Tables 3.4, 3.5) and results in sediment sulfides characterized by very negative sulfur isotope ratios. Consequently, the $\delta^{34}\text{S}$ ratios in the reducing sediments characteristic of *S. velum*

habitats could be in the -30‰ range. In organic-rich sediments, 50-90% of the decomposition of organic matter may occur via sulfate reduction (Howarth and Teal, 1980; Jorgenson, 1982). This sulfur may then be oxidized by the symbionts of chemoautotrophic symbioses, or even by the animal tissue itself, as found in the west coast species *Solemya reidi* (Powell and Somero 1986), providing the energy source for chemoautotrophy. Alternatively, the pore-water sulfides may be incorporated into organic biomolecules or stored as elemental sulfur.

The utilization of reduced sulfur by this and other invertebrate-chemoautotroph symbioses allows energy that would normally be lost from the ecosystem to be harvested. Reduced sulfur compounds are reoxidized at the interface between the oxidized and reduced layers of the sediment either biologically or chemically. If the reoxidation occurs chemically the energy produced is lost to the ecosystem. Animal-bacteria symbioses, such as *S. velum*, extend the habitat available for the oxidation of reduced sulfur species to well below the oxic-anoxic boundary (Spiro *et al.*, 1983).

Table 3.4 Distribution and isotopic abundance of sulfur in the top 16 cm of sediments of the western Baltic Sea (from Hartmann and Nielsen, 1969).

Depth in Sediment	Dissolved SO_4^{2-} $\delta^{34}\text{S}$	Dissolved Sulfide $\delta^{34}\text{S}$
0-1.5	+20‰	-
1.5-3	+28‰	-
3-5	+29.3‰	-24.6‰
5-7	+33.3‰	-21.9‰
8-10	+39.7‰	-18.7‰
10-13	+41.2‰	-16.3‰
13-16	+47.6‰	-13.1‰

Table 3.5 Distribution of $\delta^{34}\text{S}$ in sulfides from representative Californian sediments (from Goldhaber and Kaplan, 1975).

Location	$\delta^{34}\text{S}$ Range	$\delta^{34}\text{S}$ Mean
East Cortez Basin	-33 to -41‰	-37‰
Santa Barbara Basin	-18 to -34‰	-26‰
Santa Catalina Basin	-30 to -40‰	-36‰
Santa Monica Basin	-18 to -23‰	-20‰
San Diego Trough	-36 to -42‰	-39‰
Long Basin	-46 to -51‰	-48‰
Marina del Rey Harbor (Los Angeles)	-7 to -19‰	-13‰

Qualitative statements regarding the contribution of endosymbiotic bacteria to the nutrition of animal-bacteria symbioses have been reported elsewhere. These studies utilizing the incorporation of $\text{NaH}^{14}\text{CO}_3$ (Felbeck, 1983; Cavanaugh, 1985; Fisher and Childress 1986) and estimates of $\text{CO}_2:\text{O}_2:\text{H}_2\text{S}$ ratios (Anderson *et al.*, 1987) all suggest that the symbionts are important in the nutrition of the host. Quantification of the nutritional role of endosymbionts has been difficult, if not impossible with these methods, especially in the case of deep-sea species, that are frequently damaged during collection. Examining the stable isotope ratios of both components of the symbiosis as described in this study allows the use of a two source mixing model to determine the bacterial isotope contribution to the animal tissue. The contribution of stable isotopes directly corresponds to the contribution of the element in question. These methods are equally applicable to all the animal-bacteria symbioses known and show particular promise for the study of hydrothermal vent species, as damage to the organism during collection will not significantly alter the isotopic composition of the animal and bacterial components.

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CHAPTER 4

INCORPORATION AND UTILIZATION OF BACTERIAL LIPIDS BY
THE *SOLEMYA VELUM* SYMBIOSIS.

Portions of this chapter are in press for publication in Marine Biology with Judith McDowell Capuzzo as co-author, and have been published in "Trophic relationships in the marine environment". Proc. 24th Europ. Mar. Biol. Symp (Barnes, M. and R.N. Gibson, Eds). Aberdeen University Press, pp. 553-564 with Judith McDowell Capuzzo as coauthor.

4.1 Abstract

A detailed analysis of the lipid composition of *Solemya velum*, a bivalve containing endosymbiotic chemoautotrophic bacteria, was undertaken in order to determine the presence of lipid biomarkers of endosymbiont activity. The symbiont-free clam *Mya arenaria* and the sulfur-oxidizing bacterium, *Thiomicrospira crunogena* were analyzed for comparative purposes. The $\delta^{13}\text{C}$ ratios of each species were also measured to elucidate potential carbon sources for the lipids of each bivalve species.

Both fatty acid and sterol composition differed markedly between the two bivalves. The lipids of *S. velum* were characterized by large amounts of 18:1 ω 7 (*cis*-vaccenic acid), 16:0, and 16:1 ω 7 fatty acids and low concentrations of the highly unsaturated fatty acids characteristic of most marine bivalves. Cholesterol accounted for greater than 95% of the sterols in *S. velum*; small amounts of 24-ethylcholest-5-en-3 β -ol were found in some specimens. In contrast, *Mya arenaria* had fatty acid and sterol compositions similar to typical marine bivalves characterized

by large amounts of the highly unsaturated fatty acids 20:5 ω 3 and 22:6 ω 3 and a variety of plant-derived sterols. The fatty acids of *T. crunogena* were similar to those of *S. velum* and were dominated by 18:1 ω 7, 16:0 and 16:1 ω 7 fatty acids.

The *cis*-vaccenic acid found in *S. velum* is almost certainly symbiont-derived and serves as a potential biomarker for symbiont-lipid incorporation by the animal host. The high concentrations of *cis*-vaccenic acid (up to 35% of the total fatty acid content) in both symbiont-containing and symbiont-free tissues of *S. velum* demonstrate the importance of the endosymbionts in the lipid metabolism of this bivalve. Furthermore, the presence of *cis*-vaccenic acid in all the major lipid classes of *S. velum* demonstrates both incorporation and utilization of this compound. The small amounts of polyunsaturated fatty acids found in *S. velum* and the absence of sterols of plant origin further suggests that this symbiosis relies on endosymbiont chemoautotrophy for the majority of its nutritional requirements.

The $\delta^{13}\text{C}$ ratios of the fatty acids and sterols of *S. velum* were significantly lighter (-38.4‰ to -45.3‰) than those of *M. arenaria* (-23.8‰ -24.2‰) and were similar to the values found for the fatty acids of *T. crunogena* (-45‰); this suggests that the lipids of *S. velum* are either derived directly from the endosymbionts or are synthesized using endosymbiont-derived carbon.

5.2 Introduction

"Adaptations of organisms to specialized features of the marine environment are likely to be reflected, to at least some extent, in their lipid composition " - J.R. Sargent

Symbiotic chemoautotrophic bacteria have been localized both biochemically and ultrastructurally in at least four marine invertebrate phyla (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981; Southward *et al.*, 1981; Giere *et al.*, 1984). Many of these species are deep-sea dwellers and accessibility for experimental study is limited. Consequently, most of the biochemical and physiological studies of these symbioses have been confined to shallow-water species, such as the protobranch bivalve *Solemya velum* (Cavanaugh, 1985; Cavanaugh *et al.*, 1988). This small bivalve (adults are usually 1-2 cm in length) inhabits Y-shaped burrows in sulfide-rich sediments and is characterized by extreme gut-reduction and the presence of high concentrations of symbiotic, gram-negative, sulfur-oxidizing bacteria within specific gill bacteriocyte cells (Cavanaugh, 1983, 1985,).

In marine invertebrate-chemoautotrophic bacteria symbioses (hereafter called animal-bacteria symbioses), the host species contains large concentrations of gram-negative bacteria which are usually intracellular but which may occur in extracellular spaces. Generally, the bacteria are contained in membrane-bound vacuoles within host cells (Southward *et al.*, 1981; Felbeck, 1983, Cavanaugh, 1983; Bosch and Grassé 1984a; Fiala-Médioni, 1984; Fiala-Médioni *et al.*, 1986a;b; deBurgh *et al.*, 1989). The bacteria appear to be chemoautotrophic, deriving their energy and reducing power from the respiration of inorganic substances, and their cell carbon from the fixation of CO₂. Most of the symbioses discovered to date are thought to involve sulfur-oxidizing bacteria; and high activities of the enzymes involved in sulfur metabolism and CO₂ fixation have been reported in the symbiont-containing tissues of some species (Felbeck, 1983; Felbeck *et al.*, 1981, Cavanaugh, 1983). In addition, Cavanaugh *et al.* (1988) has confirmed that in

the *S. velum* symbiosis ribulose-biphosphate carboxylase, the enzyme involved in carbon fixation, is localized only in the bacterial cells.

In many animal-bacteria symbioses, the digestive system of the host species is very small or absent entirely, indicating a trophic function for the bacteria. In this context, stable isotope studies (Chapter 3) suggest that *S. velum* may obtain up to 100% of its carbon and nitrogen requirements from bacterial symbionts. Similarly, the detailed study of Anderson *et al.* (1987) suggests net autotrophy in the west coast species *S. reidi*. The distinct membrane separating the bacteria from the plasma membrane may play a role in regulating nutrient or metabolite transfer between host and symbiont, or in protecting the bacteria from host enzymes (deBurgh *et al.*, 1989).

Understanding the details of the trophic relationships between marine invertebrates and chemoautotrophic bacterial symbionts is difficult using the standard physiological techniques of respirometry and enzyme kinetics. These methods require optimal animal and bacterial physiological condition, yet deep-sea and hydrothermal vent species are frequently damaged during collection. Moreover, optimal laboratory conditions for the maintenance of most species are not known, and bacterial productivity in animal-bacteria symbioses appears to decline as the length of laboratory maintenance increases (Pers. Obs.; Anderson *et al.*, 1987). Comparative biochemical and physiological studies may be useful in identifying chemical markers of endosymbiont activity (Distel *et al.*, 1988; Chapter 3). Symbiont-derived chemical markers could serve as valuable screening tools for the presence of symbionts in untested species. In addition, the identification of chemical markers, which are unaffected by physical damage to the organism during collection and short-term preservation, could be extremely useful in the study of nutrition in deep-sea symbioses.

Lipids have been used in the past as biological markers for algae and bacteria in marine sediments (Volkman and Johns, 1977, Perry *et al.*, 1979; Volkman *et al.*, 1980), to determine the biomass of bacterial symbionts in ascidians (Gillen *et al.*, 1988), and as

possible organic tracers for establishing estuarine feeding relationships and benthic microbial community structure (Jeffries, 1972; Bobbie and White, 1980). The lipid content of bivalve molluscs is generally dependent on dietary lipid intake (Pollero *et al.*, 1979; Moreno *et al.*, 1980; Piretti *et al.*, 1987). Consequently, the lipids of symbiont-containing bivalves will reflect a symbiont-based diet if the symbionts are being utilized for nutritional purposes. In addition, it may be possible to identify utilization of specific bacterial lipids by the host as certain lipids (e.g. hopanoids, *cis*-vaccenic acid, branched fatty acids) are generally associated with bacteria (Goldfine, 1972; Ourisson *et al.*, 1979; Fulco, 1983).

Similarly, stable isotope ratios are important biochemical markers which have been used to estimate the contribution of bacterial symbionts to host nutrition (Rau, 1985; Chapter 3). The reduction of inorganic carbon during chemoautotrophy generally results in a greater depletion of the ^{12}C isotope relative to the ^{13}C isotope (i.e., more negative $\delta^{13}\text{C}$ value) than occurs during photosynthetic carbon fixation (Degens, 1969; Ruby *et al.*, 1987). Consequently, chemoautotrophic bacteria may have more negative $\delta^{13}\text{C}$ values than photosynthetic organisms. These differences in $\delta^{13}\text{C}$ ratios are often transferred through the food-chain allowing determination of the origin of the carbon in an organism. The results of stable isotope studies suggest that, in some species, bacterial endosymbionts may be vital in the provision of carbon and nitrogen to the host species (Chapter 3). To date little information exists concerning the nature of the compounds being utilized.

In this chapter, a detailed comparative study of the biochemical composition of the *S. velum* symbiosis, the symbiont-free bivalve *Mya arenaria*, and *Thiomicrospira crunogena*, a sulfur-oxidizing bacteria was undertaken. In particular, I have attempted to identify potential endosymbiont lipid biomarkers. The results of a study of the fatty acid, sterol, hydrocarbon, hopanol and lipid class composition of these species are reported. In addition the lipid carbon stable isotope ratios were analyzed to allow the lipid carbon source for each species to be estimated.

The methods used in the analyses discussed here are outlined in Chapter 2, Sections 2.3 and 2.6.

4.3 Results

4.3.1 Alkanes, Hopanols and Sterols

Concentrations of n-alkanes and hopanols greater than those observed in blanks were not observed in either species of bivalve or the bacterium *T. crunogena*. Additionally, no sterols were present in the bacterial samples. Although both species of bivalve contained sterols (Table 4.1, Figure 4.1), significant interspecific differences in sterol composition were apparent. In intact *S. velum* samples cholest-5-en-3 β -ol (cholesterol; 5420 ng mg⁻¹ dry wt.) accounted for almost 97% of the total sterol content (Table 4.1a). In contrast, whole *Mya arenaria* specimens contained at least 13 different sterols with cholest-5-en-3 β -ol (45.5%), 24-methylcholesta-5,24-dien-3- β -ol (12.5%), 24-ethylcholest-5-en-3 β -ol (possibly sitosterol, 8.1%), an unknown cholestadienol (7.1%), and 24-methylcholesta-5,22-dien-3 β -ol (probably brassicasterol; 6.6%) occurring in the largest amounts (Table 4.1b). The gills of *S. velum* contained the highest concentrations of sterols and the largest relative amounts of 24-ethylcholest-5-en-3 β -ol (12.3% of the total), whereas the foot tissue had the least amounts. In *M. arenaria*, the gills also had the highest concentrations of sterols, the visceral mass had the lowest levels. The sterol content of *S. velum* (~0.6% dry wt) was slightly larger than that of *M. arenaria* tissues (0.4% dry wt.), probably due to the relatively larger gills of *S. velum*.

Table 4.1. a) *Solemya velum* and b) *Mya arenaria*. Sterol composition of whole bivalves and individual tissues^(a).a) *Solemya velum*

Sterol	Gill (N=3)		Foot (N=3)		Visceral Mass (N=3)		Whole Animal (N=8)	
	ng mg ⁻¹ dry wt.		ng mg ⁻¹ dry wt.		ng mg ⁻¹ dry wt.		ng mg ⁻¹ dry wt.	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Cholest-5-en-3 β -ol(b)	6120	1030	5130	790	5540	360	5420	990
24-Ethylcholest-5-en-3 β -ol(c)	860	370	130	130	180	50	190	140
Total	6980	1400	5260	920	5730	410	5610	1130

b) *Mya arenaria*

Sterol	Gill (N=3)		Foot (N=3)		Visceral Mass (N=3)		Whole Animal (N=8)	
	ng mg ⁻¹ dry wt.		ng mg ⁻¹ dry wt.		ng mg ⁻¹ dry wt.		ng mg ⁻¹ dry wt.	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
26,27-Dinorergosta-5,22-dien-3 β -ol(d)	100	4	120	11	68	3	72	8
Cholesta-5,17(20)-dien-3 β -ol	30	5	33	7	7	13	18	11
Cholest-5-en-3 β -ol	3130	230	2560	320	1850	180	2030	370
5- α -Cholestan-3 β -ol(e)	300	16	410	320	0(i)	0	12	23
Unknown Cholestadienol	580	2	490	110	260	64	320	117
Cholesta-5,24-dien-3 β -ol(f)	180	46	290	15	270	155	190	37
24-Methylcholesta-5,24-dien-3 β -ol	750	150	810	39	490	67	560	95
24-Methylcholesta-5,22-dien-3 β -ol(g)	510	19	490	28	330	84	270	110
24-Ethylcholesta-5,22-dien-3 β -ol(h)	240	10	100	25	111	83	88	23
Unknown 24-Ethylcholestadienol	160	100	200	17	80	29	120	26
24-Ethylcholest-5-en-3 β -ol(c)	280	43	300	60	440	450	240	170
24-Ethylcholesta-5,24(28)-dien-3 β -ol(i)	140	9	110	1	190	200	77	43
Unknown Sterols	320	20	320	10	200	7	200	29
Total	6700	660	6200	960	4290	1330	4190	1070

(a) The trivial names of most of the sterols identified are listed below. We were unable to distinguish between the possible epimers of some of the sterols, in these cases the most likely epimer is listed.

(b) Cholesterol; (c) Possibly sitosterol; (d) Identification based on similarity of sterol mass spectra with standard; (e) Cholestanol; (f) Possibly desmosterol;

(g) Possibly brassicasterol; (g) Possibly stigmasterol; (h) Fucosterol or isofucosterol; (i) Below detection limits of 1 ng mg⁻¹ dry wt.

Figure 4.1 Sterol composition of whole *Solemya velum* and *Mya arenaria* specimens (Means $\pm$ s.d.). For clarity, only whole specimen values are plotted.

4.3.2 Fatty acids

The fatty acids of *T. crunogena* (Table 4.2, Figure 4.2) were dominated by the three fatty acids 16:0, 16:1 ω 7 and 18:1 ω 7 (*cis*-vaccenic acid). These three lipids accounted for 87.3% of the total fatty acids¹ found and are the most commonly reported fatty acids in Gram-negative bacteria (Perry *et al.*, 1979; Parkes and Taylor, 1983; Gillan and Johns, 1986); 64.4% of the fatty acids of this species had one unsaturation. No fatty acids of greater than C₁₈ to C₃₄ (the upper limit of the method) in length were found and the *iso* and *anteiso* branched fatty acids characteristic of some species of gram-negative bacteria (e.g., *Legionella*) were not observed in *T. crunogena*.

Table 4.2 Fatty acid composition of *Thiomicrospira crunogena* (expressed as % of total $\geq 0.1\%$).

Fatty Acid	Sample 1 (a)	Sample 2 (a)	Average
14:0	1.0	1.0	1.0
15:0	1.2	1.3	1.3
16:0	24.8	23.9	24.3
16:1 ω 7	43.6	42.7	43.2
17:0	1.9	2.0	2.0
18:0	6.7	7.5	7.1
18:1 ω 9	1.4	1.6	1.5
18:1 ω 7	19.6	19.9	19.7
Unsaturation Index (b)			64.4

(a) Each sample consisted of approximately 1/4 of the culture yield. Absolute amounts of lipids cannot be reported as the large amounts of sulfur in each sample prevented weight determination.

(b) Unsaturation index is calculated as the number of double bonds present in a fatty acid times the total percent of that fatty acid present.

¹ The ω classification of fatty acids refers to the position of the double bond closest to the methyl end of the fatty acid chain. For example, a C₁₈ fatty acid with two double bonds, the closest of which is 3 carbon units from the methyl end of the lipid is described as 18:2 ω 3. In the Δ -classification, the position of the double bonds is reported in relation to the carboxyl end of the fatty acid chain, thus the fatty acid just described would be 18:2 Δ 12,15.

Figure 4.2. Fatty acids of the sulfur-oxidizing bacterium *Thiomicrospira crunogena* (percent of total fatty acids greater than 1 percent).

The fatty acids of *S. velum* and *M. arenaria* differed significantly in terms of absolute and relative amounts. Differences within the tissues of each species were also apparent.

Differences between bivalves - The absolute fatty acid concentrations of whole *S. velum* samples (mean values = 32.1 $\mu\text{g mg}^{-1}$ dry wt., Table 4.3, Figure 4.3a) were greater than those of *M. arenaria* (16.1 $\mu\text{g.mg}^{-1}$ dry.wt., Table 3). In *S. velum*, the high lipid content is partly due to the presence of extremely large quantities of the bacterial lipid *cis*-vaccenic acid (18:1 ω 7). This lipid occurs in average concentrations of 8.1 $\mu\text{g mg}^{-1}$ dry wt. in *S. velum*. In whole

S. velum samples, the only three fatty acids occurring in concentrations exceeding 10% of the total are the three fatty acids 18:1 ω 7, 16:1 ω 7 and 16:0, which account for >50% of the entire fatty acid pool. (Figure 4.3a). The fatty acid 18:2 ω 6 occurred in relatively high concentrations in *S. velum*. The only polyunsaturated fatty acid (PUFA) found in high concentrations in *S. velum* was arachidonic acid (20:4 ω 6) which occurred in average concentrations of 3.2 $\mu\text{g mg}^{-1}$ dry wt. (Table 3). In contrast, the major fatty acid present in *M. arenaria* was the PUFA 22:6 ω 3 (Table 4.4, Figure 4.3b) which occurred in concentrations of 3.1 $\mu\text{g mg}^{-1}$ dry wt. Palmitic acid (16:0) and eicosapentaenoic acid (20:5 ω 3) were the next most abundant fatty acids in *M. arenaria*, occurring in concentrations of 2.7 and 2.1 $\mu\text{g mg}^{-1}$ dry wt. respectively. These three fatty acids accounted for 48% of the total fatty acids in *M. arenaria*, although an unknown 20:1 fatty acid also occurred in relatively high concentrations (Figure 4.4b). The ratio of 16:1 to 16:0 differed between the two bivalves. In both *S. velum* and the bacterial samples, there were larger amounts of 16:1 ω 7 than 16:0, whereas the opposite was true for *M. arenaria*.

It is interesting to note the differences in the degree of unsaturation between the fatty acids of the two species (Tables 4.3, 4.4). In both species, 70-80% of the fatty acids have at least one unsaturation, a typical pattern in marine bivalves (See Gardner and Riley, 1972). However, greater than 40% of the fatty acids in *M. arenaria*, but only about 17% of the fatty acids in *S. velum*, have at least 3 unsaturations. This accounts for the large differences in desaturation index² between the two species. Yet, the absolute concentrations of PUFAs present in both bivalve species are similar because of the higher overall concentrations of fatty acids in *S. velum*. The branched *iso* and *anteiso* fatty acids occurred in only small amounts in both species.

² A measurement of the proportion of unsaturated fatty acids present, calculated as the sum of the number of unsaturations present per fatty acid times the percentage of the total fatty acids represented by that fatty acid

Table 4.3 Fatty acid composition of *Solemya velum* (mean $\pm$ s.d.)

Fatty Acid	Whole Animal		Gill		Foot		Visceral Mass	
	Mean (N=8)	s.d.	Mean (N=3)	s.d.	Mean (N=3)	s.d.	Mean (N=3)	s.d.
	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.
14:0	1450	1120	840	330	580	220	1190	770
14:1 ω 5	22	15	35	20	36	10	9	11
a-15:0	27	17	41	20	31	7	8	9
15:0	64	56	73	23	260	280	24	21
i-16:0	2	3	6	10	0	0	0	0
16:0	3630	1890	4720	1300	2360	500	2350	570
16:1 ω 7	4690	2220	7020	2290	2360	750	2900	630
a-17:0	37	15	36	13	43	16	34	17
17:0	58	22	80	23	64	26	43	15
18:0	1530	320	2180	900	1250	400	1190	140
i-18:0	29	37	130	110	19	34	15	26
18:1 ω 9	950	520	860	410	700	440	1110	940
18:1 ω 7	8070	3410	10800	4730	5000	1540	6200	2570
18:2 ω 6	1430	510	1540	360	1310	420	1460	660
18:3 ω 3	52	61	120	210	230	400	0	0
18:3 ω 6	12	14	12	17	11	11	11	19
18:4 ω 3	32	29	58	70	47	19	49	47
20:0	27	21	38	40	61	57	16	15
20:1* (a)	0 (b)	0	0	0	0	0	0	0
20:1 ω 9	860	320	670	100	910	330	800	310
20:1 ω 7	430	230	940	850	200	140	300	150
20:2*	2110	790	2950	1660	1770	820	1690	620
20:2 ω 6	222	150	202	290	62	110	180	160
20:3*	1360	440	1630	950	1510	62	1240	520
20:3 ω 3	38	78	86	68	0	0	130	220
20:4 ω 6	3240	1030	3620	1270	1770	670	2770	1150
20:5 ω 3	497	600	22	31	270	250	580	360
22:0	6	9	18	13	26	23	9	8
22:1 ω 9	24	45	47	81	41	39	47	82
22:2 ω 6	4	10	12	20	30	53	0	0
22:2*	1050	480	1190	470	1120	550	1160	590
22:3 ω 3	50	48	46	64	86	100	32	60
22:3*	0	0	0	0	0	0	0	0
22:4 ω 6	2	6	100	92	67	120	0	0
22:6 ω 3	68	73	19	17	200	350	91	63
24:0	6	8	4	4	21	36	6	11
24:1 ω 9	3	4	7	12	23	24	0	0
26:0	8	10	0	0	74	43	4	0
28:0	1	2	0	0	0	5	0	0
30:0	0	0	0	0	0	0	0	0
TOTAL	32100	14600	40100	16900	22500	8850	25600	10700
% $\geq$ 1 unsaturation		78.8		78.6		79.0		80.4
% $\geq$ 2 unsaturations		32.5		28.3		38.3		36.6
% $\geq$ 3 unsaturations		17.2		13.9		18.9		19.1
Unsaturation index		142.9		130.3		149.7		153.8

(a) Location of double bonds unknown

(b) Below detection limit of 1 ng mg⁻¹ dry wt.

Table 4.4 Fatty acid composition of *Mya arenaria* (mean $\pm$ s.d.)

Fatty Acid	Whole Animal		Gill		Foot		Visceral Mass	
	Mean (N=8)	s.d.	Mean (N=3)	s.d.	Mean (N=3)	s.d.	Mean (N=3)	s.d.
	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.	ng mg ⁻¹ dry wt.
14:0	240	53	190	24	370	35	210	33
14:1 ω 5	10	6	24	10	40	24	8	3
15:0	16	6	18	2	42	2	15	7
15:1	100	21	130	2	200	10	90	15
16:0	42	26	67	35	110	100	30	12
16:1 ω 7	2730	360	3540	460	4200	240	2490	160
17:0	790	200	700	120	1270	15	780	83
17:1	110	37	200	28	200	59	110	55
18:0	220	37	250	220	360	19	200	15
18:1	800	240	1000	120	1180	110	690	71
18:2	70	45	130	130	120	41	84	10
18:3	1010	180	840	190	1820	160	900	49
18:4	390	100	370	81	420	17	360	27
19:0	160	130	150	37	200	24	130	6
20:0	51	33	88	5	63	24	58	13
20:1	9	8	20	21	8	15	8	9
20:2	210	62	250	120	230	35	160	90
20:3	32	35	12	22	55	20	18	15
20:4	1320	320	3960	590	2100	280	1140	72
20:5	530	120	1030	78	1100	310	490	100
21:0	420	200	820	250	850	270	480	260
22:0	170	35	390	340	170	160	150	22
22:1	230	34	250	220	320	50	200	7
22:2	120	46	660	420	100	100	64	60
22:3	9	12	27	23	0	0	0	0
22:4	780	170	1700	540	1070	450	610	180
22:5	2060	450	3520	1140	2350	800	1830	710
23:0	7	9	7	11	0	0	6	10
23:1	14	10	82	62	13	23	15	13
23:2	5	14	26	44	0	0	12	21
23:3	4	11	0	0	0	0	12	20
23:4	28	17	150	190	94	84	10	17
23:5	300	120	450	390	340	260	180	160
24:0	60	60	180	152	220	140	37	64
24:1	3100	740	4630	880	3010	1190	2440	1060
25:0	3	6	7	12	0	0	0	0
25:1	1	2	27	41	5	8	0	0
26:0	3	5	2	4	7	12	5	9
27:0	1	2	5	8	0	0	0	0
28:0	0(b)	0	0	0	0	0	0	0
TOTAL	16100	3940	25900	6990	22600	5080	14000	3460
% $\geq$ 1 unsaturation		72.8		78.4		69.5		71.5
% $\geq$ 2 unsaturations		42.2		47.8		35.8		41.1
% $\geq$ 3 unsaturations		41.3		41.8		32.7		37.5
Unsaturation index		247.8		259.4		204.6		232.1

(a) Location of double bonds unknown

(b) Below detection limit of 1 ng mg⁻¹ dry wt.

Figure 4.3. Fatty acids of whole a) *Solemya velum* and b) *Mya arenaria* expressed as a percent of the total fatty acids found (mean $\pm$ s.d.). Asterisks denote fatty acids where the position of the double bond is unknown.

a) <i>Solemya velum</i>		b) <i>Mya arenaria</i>	
Fatty acid	Percent	Fatty acid	Percent
16:0	1.2	16:0	1.2
18:0	0.5	18:0	0.5
20:0	0.2	20:0	0.2
22:0	0.1	22:0	0.1
24:0	0.1	24:0	0.1
16:1	0.5	16:1	0.5
18:1	1.5	18:1	1.5
20:1	0.5	20:1	0.5
22:1	0.2	22:1	0.2
24:1	0.1	24:1	0.1
16:2	0.1	16:2	0.1
18:2	0.5	18:2	0.5
20:2	0.2	20:2	0.2
22:2	0.1	22:2	0.1
24:2	0.1	24:2	0.1
16:3	0.1	16:3	0.1
18:3	0.2	18:3	0.2
20:3	0.1	20:3	0.1
22:3	0.1	22:3	0.1
24:3	0.1	24:3	0.1
16:4	0.1	16:4	0.1
18:4	0.1	18:4	0.1
20:4	0.1	20:4	0.1
22:4	0.1	22:4	0.1
24:4	0.1	24:4	0.1
16:5	0.1	16:5	0.1
18:5	0.1	18:5	0.1
20:5	0.1	20:5	0.1
22:5	0.1	22:5	0.1
24:5	0.1	24:5	0.1
16:6	0.1	16:6	0.1
18:6	0.1	18:6	0.1
20:6	0.1	20:6	0.1
22:6	0.1	22:6	0.1
24:6	0.1	24:6	0.1
16:7	0.1	16:7	0.1
18:7	0.1	18:7	0.1
20:7	0.1	20:7	0.1
22:7	0.1	22:7	0.1
24:7	0.1	24:7	0.1
16:8	0.1	16:8	0.1
18:8	0.1	18:8	0.1
20:8	0.1	20:8	0.1
22:8	0.1	22:8	0.1
24:8	0.1	24:8	0.1
16:9	0.1	16:9	0.1
18:9	0.1	18:9	0.1
20:9	0.1	20:9	0.1
22:9	0.1	22:9	0.1
24:9	0.1	24:9	0.1
16:10	0.1	16:10	0.1
18:10	0.1	18:10	0.1
20:10	0.1	20:10	0.1
22:10	0.1	22:10	0.1
24:10	0.1	24:10	0.1
16:11	0.1	16:11	0.1
18:11	0.1	18:11	0.1
20:11	0.1	20:11	0.1
22:11	0.1	22:11	0.1
24:11	0.1	24:11	0.1
16:12	0.1	16:12	0.1
18:12	0.1	18:12	0.1
20:12	0.1	20:12	0.1
22:12	0.1	22:12	0.1
24:12	0.1	24:12	0.1
16:13	0.1	16:13	0.1
18:13	0.1	18:13	0.1
20:13	0.1	20:13	0.1
22:13	0.1	22:13	0.1
24:13	0.1	24:13	0.1
16:14	0.1	16:14	0.1
18:14	0.1	18:14	0.1
20:14	0.1	20:14	0.1
22:14	0.1	22:14	0.1
24:14	0.1	24:14	0.1
16:15	0.1	16:15	0.1
18:15	0.1	18:15	0.1
20:15	0.1	20:15	0.1
22:15	0.1	22:15	0.1
24:15	0.1	24:15	0.1
16:16	0.1	16:16	0.1
18:16	0.1	18:16	0.1
20:16	0.1	20:16	0.1
22:16	0.1	22:16	0.1
24:16	0.1	24:16	0.1
16:17	0.1	16:17	0.1
18:17	0.1	18:17	0.1
20:17	0.1	20:17	0.1
22:17	0.1	22:17	0.1
24:17	0.1	24:17	0.1
16:18	0.1	16:18	0.1
18:18	0.1	18:18	0.1
20:18	0.1	20:18	0.1
22:18	0.1	22:18	0.1
24:18	0.1	24:18	0.1
16:19	0.1	16:19	0.1
18:19	0.1	18:19	0.1
20:19	0.1	20:19	0.1
22:19	0.1	22:19	0.1
24:19	0.1	24:19	0.1
16:20	0.1	16:20	0.1
18:20	0.1	18:20	0.1
20:20	0.1	20:20	0.1
22:20	0.1	22:20	0.1
24:20	0.1	24:20	0.1
16:21	0.1	16:21	0.1
18:21	0.1	18:21	0.1
20:21	0.1	20:21	0.1
22:21	0.1	22:21	0.1
24:21	0.1	24:21	0.1
16:22	0.1	16:22	0.1
18:22	0.1	18:22	0.1
20:22	0.1	20:22	0.1
22:22	0.1	22:22	0.1
24:22	0.1	24:22	0.1
16:23	0.1	16:23	0.1
18:23	0.1	18:23	0.1
20:23	0.1	20:23	0.1
22:23	0.1	22:23	0.1
24:23	0.1	24:23	0.1
16:24	0.1	16:24	0.1
18:24	0.1	18:24	0.1
20:24	0.1	20:24	0.1
22:24	0.1	22:24	0.1
24:24	0.1	24:24	0.1

a) *Solemya velum* b) *Mya arenaria*

Figure 4.4. Fatty acids in the different tissue fractions of a) *Solemya velum* and b) *Mya arenaria* expressed as a percent of the total fatty acids found (mean $\pm$ s.d.). Asterisks denote fatty acids where the position of the double bond is unknown.

a) *Solenya velum* b) *Mya arenaria*

Differences between tissues - In both species the gills contained the highest fatty acid concentrations (Table 4.3, 4.4) with the mean values for *S. velum* and *M. arenaria* gills being $40.1 \mu\text{g mg}^{-1}$ dry wt. and $25.9 \mu\text{g mg}^{-1}$ dry wt. respectively. This is due to the high membrane content of bivalve gills. The high total fatty acid concentrations of *S. velum* are partly due to the large contribution of the hypertrophied gills to the soft tissue weight of this species (between 25% and 40% of the tissue weight) and partly due to the high concentrations of endosymbionts in the gill bacteriocytes. In the gills of *S. velum*, the concentrations of 18:1 ω 7 and 16:1 ω 7 were particularly high (Means = 10.8 and $7.0 \mu\text{g mg}^{-1}$ dry wt., respectively, Table 4.3; Figure 4.4a); in fact, in some specimens almost 1.5% of the gills dry weight was *cis*-vaccenic acid. The foot tissue of *S. velum* contained the lowest values, an expected finding considering the muscular nature of this tissue. In *M. arenaria*, the visceral mass and not the foot had the lowest absolute fatty acid concentrations; this is probably due to the presence of noticeable grains of sediment in the gut, which would contribute to the weight of the animal, but not to the fatty acid concentration. The gills of *M. arenaria* contained slightly greater amounts of 20:4 ω 6, 20:5 ω 3, 22:6 ω 3 and the unknown 20:1 and 20:3 fatty acids (Table 4.4, Figure 4.4b). The gills of *S. velum* had the lowest tissue desaturation index of the three *S. velum* tissue fractions, whereas in *M. arenaria* the desaturation index of the gills was the highest of the tissue measurements (Tables 4.3, 4.4).

4.3.3 Fatty acid composition of the major lipid classes

There were no significant differences between the distribution of fatty acids among the lipid classes of both bivalve species. In both species of bivalve approximately 40% of the fatty acids were found in the phospholipid pool, 30-40% in the combined triglyceride, diglyceride and free fatty acid fraction, 12-15% in the steryl and methyl ester pool, 6-10% in the triglyceride pool, and about 6% in the monoglycerides (Tables 4.5, 4.6, Figure 4.5). There were generally lower amounts of fatty acids in the tri- and diglyceride fraction of *S. velum* samples than in *M. arenaria*. A

proportion of the fatty acids in the steryl and methyl ester fraction are probably due to the formation of FAMES by reaction with methanol during the lipid extraction process, an artifact that cannot be avoided if chloroform:methanol extraction solvents are used. These FAMES then coelute with steryl esters using the TLC methodologies described previously. In *S. velum* the phospholipid fraction generally had relatively larger amounts of the fatty acids 20:4 ω 6, 16:1 ω 7 and 18:1 ω 7 and less 14:0, 16:0 and 18:1 ω 9 than the other lipid classes (Figure 4.5a). The fatty acid composition of the other lipid classes of *S. velum* were very similar (Figure 4.5a). In *M. arenaria*, the phospholipids had relatively larger amounts of the unknown 20:1, whereas the tri-, diglyceride and free fatty acid fraction had a large percentage of the fatty acids 20:5 ω 3 and 22:6 ω 3 (Figure 4.5b). In *M. arenaria* PUFAs are found distributed among all the lipid classes, whereas in *S. velum*, the predominant PUFA 20:4 ω 6, is more localized in the phospholipid pool. Sterol esters accounted for approximately 8% of the sterols of *S. velum* and 5% of the sterols of *M. arenaria* (Table 4.7).

Table 4.5 *Solemya velum*. Mean fatty acid and sterol concentrations of the major lipid classes analyzed^(a).

	Phospholipids		Monoglycerides		Triglycerides, diglycerides and free fatty acids		Steryl esters and methyl esters ^(b)	
	N=4 ng mg ⁻¹ dry wt.		N=4 ng mg ⁻¹ dry wt.		N=4 ng mg ⁻¹ dry wt.		N=4 ng mg ⁻¹ dry wt.	
Fatty acid	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
14:0	840	550	150	97	410	310	250	200
16:0	1970	200	440	170	1520	1140	730	530
16:1 ω 7	2180	350	230	300	660	550	310	410
18:0	720	120	220	74	700	510	400	150
18:1 ω 9	480	300	120	58	560	420	300	160
18:1 ω 7	4320	1300	490	654	1380	1370	670	590
18:2 ω 3	720	440	84	93	180	140	80	77
18:4 ω 3	18	11	19	42	46	50	17	30
20:1 ω 9	330	100	61	55	230	130	130	150
20:1 ω 7	98	56	45	42	120	95	78	95
20:2 ^{*(3)}	550	430	56	120	130	170	11	24
20:2 ω 6	160	240	50	71	36	66	27	31
20:4 ω 6	1320	870	160	160	250	210	220	160
20:5 ω 3	220	290	50	110	42	83	57	100
22:2 ω 6	140	240	13	29	56	120		
22:2 [*]	530	470			44	98	23	51
Total	14600	5980	2190	2060	6340	5460	3320	2770
% of total animal fatty acids	39.4 $\pm$ 4.5		6.5 $\pm$ 3.2		23.2 $\pm$ 4.7		12.2 $\pm$ 11.3	

(a) Only fatty acids exceeding 1% of the total are reported.

(b) Some fatty acids are methylated during the lipid extraction procedure, these methyl esters coelute with the steryl esters during TLC.

(c) Location of double bonds unknown.

Table 4.6 *Mya arenaria*. Mean fatty acid and sterol concentrations of the major lipid classes analyzed^(a).

	Phospholipids		Monoglycerides		Triglycerides, diglycerides and free fatty acids		Steryl esters and methyl esters ^(b)	
	N=4 ng mg ⁻¹ dry wt.		N=4 ng mg ⁻¹ dry wt.		N=4 ng mg ⁻¹ dry wt.		N=4 ng mg ⁻¹ dry wt.	
Fatty acid	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
14:0	120	32	43	15	200	63	130	150
16:0	1840	31	300	52	1520	430	730	560
16:1 ω 7	280	2	72	40	250	91	200	290
18:0	420	85	130	25	460	84	270	140
18:1 ω 9	610	36	130	49	440	120	360	360
18:1 ω 7	180	37	19	17	200	53	64	54
18:2 ω 3	71	49	39	24	91	35	51	40
20:1 ^{*(3)}	650	420	13	23	84	120	61	120
20:1 ω 9	130	31	40	23	140	55	57	45
20:1 ω 7	230	72	20	2	120	46	130	150
20:2 ω 6	62	38	39	67	12	16	9	6
20:4 ω 6	230	170	6	11	180	150	27	26
20:5 ω 3	390	160	39	12	680	260	96	97
22:6 ω 3	680	300	86	7	630	230	164	140
Total	5877	1450	975	367	4990	1750	2350	2180
% of total animal fatty acids	42.5 $\pm$ 0.5		6.6 $\pm$ 1.7		32.9 $\pm$ 14.6		15.3 $\pm$ 11.3	

(a) Only fatty acids exceeding 1% of the total are reported.

(b) Some fatty acids are methylated during the lipid extraction procedure, these methyl esters coelute with the steryl esters during TLC.

(c) Location of double bonds unknown.

Figure 4.5 Fatty acid composition of the major lipid classes of a) *Solemya velum* and b) *Mya arenaria*. Fatty acids expressed as a percent of the total fatty acids found in each lipid class (mean $\pm$ s.d.). Asterisks denote fatty acids where the position of the double bond is unknown.

Table 4.7 *Solemya velum* and *Mya arenaria*. Free and esterified sterol composition.*Solemya velum*

Cholesterol	ng/mg wet wt.		% of total sterols
	Mean (N=4)	s.d.	
Free	6004	520	92.0
Steryl esters	520	170	8.0

Mya arenaria

Cholesterol			
Free	2360	380	94.9
Steryl esters	103	75	5.1
Other Sterols			
Free	2480	640	96.1
Steryl esters	97	82	3.9
Total sterols			
Free	4840	920	95.6
Steryl esters	220	150	4.4

Table 4.8 a) *Solemya velum*, *Mya arenaria* and *Thiomicrospira crunogena* $\delta^{13}\text{C}$ ratios (parts per thousand relative to PDB); b) $\delta^{13}\text{C}$ ratios of the BCl_3 -methanol used in fatty acid methylation, and a commercial C_{19} fatty acid standard before and after methylation to a C_{19} FAME.

a)

	Intact tissue $\delta^{13}\text{C}$ (a)	Fatty acids δ^{13}	Sterols δ^{13}
<i>Solemya velum</i>	-31.5 to -33.9‰	-45.4‰	-38.5‰
<i>Mya arenaria</i>	-17.1 to -17.8‰	-23.8‰	-24.2‰
<i>Thiomicrospira crunogena</i>	-27.3‰	-45‰	

(a) Conway *et al.* 1989

b)

	$\delta^{13}\text{C}$
BCl_3 -methanol	-44.7‰
C_{19} Fatty acid	-26.8‰
C_{19} FAME after BCl_3 -methanol esterification	-27.5‰

4.3.4 Stable isotope values

Fatty acid and sterol $\delta^{13}\text{C}$ ratios for all the species examined are shown in Table 4.8, along with whole animal isotope values and $\delta^{13}\text{C}$ ratios of the methylation reagents and the C_{19} fatty acid and derivitized FAME. In all cases lipid $\delta^{13}\text{C}$ ratios were considerably more negative than corresponding whole tissue values, particularly in the *S. velum* and *T. crunogena* samples. Differences in the lipid isotope ratios between the two bivalves were extremely large (Table 4.8). The lipid isotope ratios in *S. velum* were between 14‰ and 22‰ more negative than corresponding values for *M. arenaria*. Furthermore, the extremely negative $\delta^{13}\text{C}$ ratios for *S. velum* fatty acids (-45.4‰) were very similar to those found in the sulfur-oxidizing bacteria *T. crunogena* (-45.0‰), suggesting a chemoautotrophic source for the lipid carbon of *S. velum*. Although the $\delta^{13}\text{C}$ ratios of the 10% BCl_3 -methanol derivitization reagents were extremely negative (-44 ‰), methylation of the animal and bacterial fatty acids is unlikely to have affected the $\delta^{13}\text{C}$ ratios of the resulting FAMES by more than -1‰ as evidenced by the small effect of methylation on the $\delta^{13}\text{C}$ ratio of the commercial C_{19} fatty acid standard (Table 4.8).

Sample	$\delta^{13}\text{C}$ (‰)
<i>S. velum</i> whole	-20.5
<i>S. velum</i> fatty acids	-45.4
<i>T. crunogena</i> whole	-21.0
<i>T. crunogena</i> fatty acids	-45.0
<i>M. arenaria</i> whole	-22.0
<i>M. arenaria</i> fatty acids	-38.0
Commercial C_{19} fatty acid	-37.0
Commercial C_{19} FAME	-37.5
10% BCl_3 -methanol reagent	-44.0

4.4 Discussion

The lipid content of many marine bivalves is related to dietary intake (Pollero *et al.*, 1979; Moreno *et al.*, 1980; Piretti *et al.*, 1987), particularly in the case of PUFAs and sterols, as many of these lipids are only synthesized *de novo* by plants and algae. Consequently, aspects of the diet of an animal can often be inferred from an examination of the lipid composition of the animal. The lipid composition of an organism may be particularly informative in cases where only a few animals are available for study, and laboratory feeding studies are not possible, as is the case with many animal-bacteria symbiosis.

The lipid composition of *S. velum* is unusual; the lipids of *S. velum* more closely resemble those of bacteria than those of typical marine bivalves with regard to both fatty acid composition and $\delta^{13}\text{C}$ ratios.

4.4.1 Sterols

Reviews of the sterol composition of molluscs (e.g. Voogt, 1972; Goad, 1976) demonstrate that marine molluscs contain complex mixtures of C_{26} - C_{30} sterols, with cholesterol (C_{27}) generally occurring in the highest concentrations (Table 4.9). Many of the larger sterols found in marine molluscs are commonly synthesized by plants and are presumed to be derived from the diet. Data on the ability of bivalves to synthesize sterols are equivocal. Although it is generally stated that bivalves are incapable of synthesizing sterols, Fagerland and Idler (1960) reported the incorporation of radiolabelled acetate in the sterols of *Mytilus californianus* and *Saxidomus giganteus*, and recently, Teshima and Patterson (1981) demonstrated the synthesis of cholesterol, demosterol, isofucosterol, and 24-methylenecholesterol from acetate in *Crassostrea virginica*. Teshima and Kanazawa (1974) reported the synthesis of cholesterol, 22-dehydrocholesterol, demosterol and 24-methylenecholesterol from mevalonate in *Mytilus edulis*. Voogt (1975), however, did not recover any radioactivity in the sterols of *M. edulis* after the addition of labelled acetate, and the majority of bivalves studied appear to be

capable of only limited *de novo* sterol synthesis (Voogt, 1983, and references therein). Thus, the majority of the sterols in *M. arenaria* probably reflect an algal-based diet, with perhaps some *in vivo* sterol interconversion.

The extremely limited range of sterols in *S. velum* is unusual and suggests a diet deficient in plant material. Little information exists in the literature concerning the sterol composition of protobranchs; cholesterol was the only sterol found in *S. velum* by Idler and Wiseman (1971), although *Nucula* sp. had eight different sterols. It is interesting to point out here that *S. velum* and *Nucula* sp. are the only protobranchs that have been looked at with regard to sterol composition, and the data on these species is generally cited to support the statement that cholesterol is the predominant sterol in protobranchs. This is probably not the case, however, as the data are being skewed by the large quantities of cholesterol found in *S. velum*. In fact, the sterol composition of *Nucula* sp. is typical for marine bivalves. The 24-ethylcholest-5-en-3 β -ol found in *S. velum* in our study may reflect the ingestion of small amounts of plant-derived particulate organic matter overall; however, the fact that 24-ethylcholest-5-en-3 β -ol was mainly associated with gill tissues suggest that the 24-ethylcholest-5-en-3 β -ol may be due to material adhering to the large gill surface area.

Overall, *S. velum* does not appear to be incorporating significant amounts of the sterols of exogenous particulate organic matter. This is further suggested by the extremely low (-38.5‰) $\delta^{13}\text{C}$ ratios found for the sterols of *S. velum* which would appear to have been synthesized using isotopically depleted carbon derived from bacterial chemosynthesis. The utilization of exogenous particulate matter would most probably result in carbon stable isotope ratios closer to those of *Mya arenaria* (-24.2‰) and other marine animals and algae (-11.2 to -22.8‰; Kokko *et al.* 1984). The origin of the high cholesterol concentrations in *S. velum* is uncertain. *S. velum* or the endosymbionts may be capable of cholesterol synthesis. If *in vivo* sterol biosynthesis occurs in the animal tissues of the *S. velum* symbiosis, then it is likely that the carbon is derived from compounds initially produced by the

bacterial endosymbionts. This would explain the unusually-low sterol $\delta^{13}\text{C}$ ratios found in the sterols of *S. velum*.

There have been several reports since 1967 of sterol-containing bacteria (McCaffrey ., 1989 and references therein). Sterols and hopanoids are both synthesized from squalene but steroid synthesis is considered to be evolutionarily more advanced and associated with eucaryotes. The low sterol carbon isotope ratios of *S. velum* may be the result of sterol synthesis by the endosymbionts, or synthesis of sterol precursors by the symbionts followed by conversion of these precursors to sterols by the animal host. *S. velum* and *M. arenaria* could also derive some of their sterol requirements by ingestion and/or direct epithelial uptake of dissolved sterols. Dissolved sterols (predominantly cholesterol and 24-ethyl cholesterol, mostly derived from plant material) have been reported in seawater samples (Gagosian, 1975; Goad, 1976 and references therein). Saliot and Barbier (1973) reported uptake of dissolved fucosteryl propionate by *Calliactis parasitica* and *Ostrea gryphea* and sterols appear to be absorbed from seawater by *Scapharca inaequalvis* (Piretti et al., 1989). This phenomenon is unlikely to occur to a large extent in *S. velum*, since the very negative $\delta^{13}\text{C}$ ratios for *S. velum* sterols are inconsistent with photosynthetically-derived carbon sources, although small amounts of sterols could be ingested in this way. It is important, however, to note that *S. velum* does appear to be capable of taking up dissolved amino acids from seawater (Gallagher, unpub. results, see Chapter 6).

Table 4.9 Percentage composition of sterols in bivalvia(1)

	A(2)	B	C	D	E	F(3)	G(4)	H	I(5)	J(6)	K(7)	L(8)	M
Order													
Protobranchia													
<i>Nucula sp.</i>	0.3	8.6	0.3	63.2		10.7	5.4	5.9		5.6			
<i>Solemya velum</i>				100.0									
<i>Solemya velum</i> (9)				97.0						3.2			
Order Filibranchia													
<i>Atrina pectinata</i>	1.0	16.0	1.0	26.0		23.0	1.0	21.0	2.0	5.0	1.0		
<i>Atrina fragilis</i>	3.6	11.2	7.8	31.1	0.9	23.2	1.8	7.3	2.7	5.1	1.0		5.4
<i>Limaria fragilis</i>		3.0		60.0		14.0	5.0	4.0	5.0	3.0	1.0		
<i>Arca nivea</i>	tr.(10)	8.0	1.0	36.0		24.0	10.0	12.0	3.0	6.0	1.0		
<i>Mytilus edulis</i>	5.9	8.6	0.9	58.7	8.1	9.6	0.6		6.4	1.2			
<i>Mytilus edulis</i>	2.0	25.0	4.0	30.0	4.0	23.0		12.0	2.0				
<i>Mytilus edulis</i>	5.0	9.1	1.0	34.0	10.5	21.5	3.0	9.0	3.0	3.5	0.3		0.3
<i>Ostrea edulis</i>	7.5	11.6	3.6	33.2		24.7	2.6	9.5	1.5	3.4	1.7		0.4
<i>Crassostrea virginica</i>	4.6	7.5	1.1	34.1		28.1	1.7	8.1	1.5	3.7	0.1	8.3	1.2
<i>Crassostrea virginica</i>	4.0	10.2		34.0	0.2	15.6	3.7	12.2	2.0	3.7	1.5	4.6	
Order Eulamellibranchia													
<i>Anodonta cygnea</i>	tr.	4.2	1.9	47.4		18.5	8.5		8.5	10.2			
<i>Anodonta cygnea</i>		2.0		42.0		24.0		25.0			7.0		
<i>Pseudoanodonta complanata</i>		4.0		48.0		12.0		26.0			10.0		
<i>Unio timidus</i>		2.0		52.0		16.0		27.0			3.0		
<i>Arctica islantica</i>	6.7	11.3	1.5	35.8		21.6	2.7	10.9	1.7	3.6		3.5	0.4
<i>Arctica islantica</i>	5.5	5.5	4.3	44.5	1.7	7.0	4.2	21.6		3.3		2.4	
<i>Cardium edule</i>	4.2	6.9	0.7	24.9	5.0	22.0	8.1	13.0	5.2	8.2		2.1	
<i>Tridacna maxima</i>	tr.	2.0	tr.	17.0		8.0	36.0	16.0		5.0	1.0	tr.	16.0
<i>Tridacna crocea</i>	1.0	4.0		22.0		17.0	44.0		4.0	3.0	tr.	4.0	1.0
<i>Tridacna noae</i>	tr.	1.0		12.0		5.0	65.0	13.0		tr.	tr.	tr.	4.0
<i>Tridacna squamosa</i>	tr.	3.0		24.0		11.0	39.0	10.0	1.0	3.0	tr.	tr.	7.0
<i>Hopponps hopponps</i>	tr.	3.0		29.0		14.0	34.0	13.0	tr.	4.0	tr.	tr.	3.0
<i>Tapes philippinarum</i>	4.0	6.0	1.0	35.0	9.0	14.0	tr.	17.0	4.0	8.0	3.0		
<i>Tapes japonica</i>		7.2		35.4		15.7		25.8				14.2	
<i>Spisula solidissima</i>	27.0	2.9	1.9	68.0		8.5	3.4	5.4		4.8		2.4	
<i>Spisula sachalinensis</i>	3.9	10.2		48.6		13.8		11.5		11.9			
<i>Mactra sulcataria</i>	3.0		6.7		39.6		14.1		19.4	2.4	10.5	4.3	
<i>Mya arenaria</i>	2.4	4.0	0.5	59.0		12.3	3.3	10.8	4.1	4.1		1.6	
<i>Mya arenaria</i>	3.4	9.5	0.6	38.1	4.0	13.2	7.7	16.6	2.0	3.9		1.0	
<i>Mya arenaria</i> (9)				48.4	4.6	6.5		13.3	2.1	5.7	1.8		17.1

(1) See references in review of Voogt, 1983.

(2) A= 22-trans-24-norcholesta-5,22-dienol; B= 22-trans-cholesta-5,22-dienol; C= 22-cis-cholesta-5,22-dienol;
D= cholesta-5-en-3- β -ol; E= Demosterol; F= 24-methylcholesta-5-22, dienol; G= 24-methylcholesta-5-en-3- β -ol;
H= 24-methylenecholesterol; I= 24-ethylcholesta-5,22-dienol; J= 24-ethylcholesterol; K= fucosterol;
L= 28-isofucosterol; M= other sterols

(3) Common in Cyanobacteria, Prymnesiophyceae, and Bacillariophyceae (Volkman, 1986).

(4) Common in Prasinophyceae, Dinophyceae, and Bacillariophyceae (Volkman, 1986).

(5) Common in Chlorophyceae, Prymnesiophyceae, and Dinophyceae (Volkman, 1986).

(6) Common in Cyanobacteria, Bacillariophyceae, and Prymnesiophyceae (Volkman, 1986).

(7) Common in most macroscopic brown algae (Volkman, 1986).

(8) Common in most macroscopic green algae (Volkman, 1986).

(9) Results from this laboratory.

(10) Trace amounts

4.4.2 Fatty acids

The fatty acid composition of *T. crunogena*, an obligate chemoautotroph (Jannasch *et al.*, 1985), was similar to those of the group III obligate thiobacilli subdivision of Katayama-Fujimura *et al.* (1982) and *Thioploca* sp. (McCaffrey *et al.*, 1989, Table 4.10). Bacteria can synthesize monounsaturated fatty acids (MUFAs) by using one of two biochemical pathways, which generally appear to be mutually exclusive (Goldfine, 1972; Fulco, 1983), although a few species are reported to utilize both pathways (Wada *et al.*, 1989). The anaerobic pathway, found only in bacteria, produces long chain MUFAs by the elongation of medium chain length *cis*-3-unsaturated intermediates (Figure 4.6). Generally, the anaerobic pathway leads to the formation of *cis*-vaccenic acid (18:1 ω 7) as the major end-product, with 16:1 ω 7 produced as a secondary product. Desaturation of fatty acids using this pathway will not lead to the methylene interrupted double bond system (1,4-arrangement) typical of the PUFAs of higher organisms. Instead, sequential action of the β - γ -dehydrase could lead only to a 1,3-double bond system (not found in bacteria) of alternate cycles of β - γ -dehydration and α - β -dehydration could lead to a 1,5-double bond system. This has occasionally been found in bacteria. In contrast to the anaerobic pathway, the second MUFA synthesis pathway results in a large variety of fatty acids. This aerobic, or O₂-dependent pathway, utilizes 16:0 or 18:0 as the preferred substrates, and double bond insertion occurs in the δ 9 position (in some species of bacteria, δ 5,8,9, and 10 isomers can also be found). In bacteria, this pathway involves an electron transport chain beginning with NAD(P)H and terminating with a non-heme, iron-containing desaturase (Fulco, 1983). Desaturation of fatty acid using the aerobic pathway can lead to the 1,4-arrangement of double bonds found in animals, plants and some bacteria. Because the two pathways appear to be mutually exclusive, we can infer from the high concentrations of *cis*-vaccenic acid in *T. crunogena* and other sulfur-oxidizers examined to date (e.g. *Thioploca* spp., Table 4.9; *Thiobacillus* sp., Katayama-Fujimura *et al.*, 1982) that the anaerobic MUFA synthesis pathway operates in these species.

The $\delta^{13}\text{C}$ ratios of the lipids of *T. crunogena* were particularly low in comparison with the ratios for intact bacteria (a depletion of -17.7‰ was evident). This depletion is much greater than the average plankton value of Prahl and Muehlhausen (1989), and may be the result of differences in the enzyme pathways involved in bacterial fatty acid synthesis.

Figure 4.6. The anaerobic MUFA pathway in bacteria (After Goldfine, 1972)

Three major differences are apparent when the fatty acids of both bivalve species are examined. (1) *Solemya velum*, like *Thiomicrospira crunogena*, contained extremely large amounts of the MUFAs 16:1 ω 7 and 18:1 ω 7 (the fatty acid predominantly produced by bacterial anaerobic MUFA synthesis), while *Mya arenaria* had only small amounts of these lipids. (2) The $\delta^{13}\text{C}$ ratios of the fatty acids of *S. velum* (-45.4‰) were similar to those of *Thiomicrospira crunogena* (-45‰) but differed markedly from the fatty acid $\delta^{13}\text{C}$ ratios of *M. arenaria* (-23.8‰). (3) *M. arenaria* contained high concentrations of PUFAs while the PUFA content of *S. velum* was low.

The *cis*-vaccenic acid found in all the *S. velum* samples analyzed in this study is almost certainly bacterial in origin; when the morphology of *S. velum* is considered (very small digestive system, large hypertrophied gills containing high concentrations of chemoautotrophic bacteria) it is highly likely that the *cis*-vaccenic acid is derived from the endosymbionts of this species. Similarly, the greater amounts of 16:1 ω 7 relative to 16:0 found in *S. velum* probably reflects the incorporation of endosymbiont lipids. Comparison of the fatty acids of *S. velum* with those of other bivalves and sulfur-oxidizing bacteria reveals the presence of these strong 'bacterial' signatures in *S. velum* (Table 4.10). The small amounts of *cis*-vaccenic acid found in *M. arenaria* taken concurrently from the same sediments indicates that the large amounts in *S. velum* are unlikely to be the result of external sediment contamination. *Cis*-vaccenic acid may be an important biomarker for animal-bacteria symbiosis and may be useful in screening other marine organisms for the presence of bacterial symbionts. In this context, two other putative animal-bacteria symbioses recently examined in this laboratory have been found to contain large amounts of *cis*-vaccenic acid, demonstrating the potential of this endosymbiont biomarker as a screening technique (Chapter 7).

The low $\delta^{13}\text{C}$ ratios found in the fatty acid pool of *S. velum* and the similarity between the lipid $\delta^{13}\text{C}$ ratios in *S. velum* and *Thiomicrospira crunogena* also demonstrate the utilization of

bacterial lipids by *S. velum*. Lipids are generally depleted in ^{13}C relative to the bulk organic carbon of most marine species (Prahl and Meuhlhhausen 1989). In marine plankton $\delta^{13}\text{C}$ ratios of total lipids differ from $\delta^{13}\text{C}$ ratios of plankton total organic carbon by $\sim -9.5\text{‰}$ (Prahl and Meuhlhhausen 1989), while in *Escherichia coli*, total lipid $\delta^{13}\text{C}$ ratios differ from total organic carbon values by about -7.1‰ (Monson and Hayes 1982). Thus, the very negative $\delta^{13}\text{C}$ ratios found in the fatty acids of *S. velum* suggest that the fatty acids of this species are synthesized using isotopically-light carbon (~ -30 to -40‰), such as that produced by chemoautotrophic bacteria (Degens 1969, Ruby *et al.* 1987, Conway *et al.* 1989). These lipids may either be synthesized by the chemoautotrophic symbionts of *S. velum* and transported to the host (as would appear to be the case for *cis*-vaccenic acid), or may be synthesized by *S. velum* using carbon derived from the bacterial symbionts.

Gillan *et al.* (1988)³ used the presence of bacterial fatty acids in sponges to determine the contribution of symbiotic bacteria to total sponge biomass. In our study, however, we found large concentrations of 18:1 ω 7 in the foot tissue of *S. velum* where endosymbionts are not found. This suggests that this bacterial lipid is also being utilized by non-bacterial cells and concentrations of bacterial lipids are not indicative of bacterial population size. In fact, if we use the techniques of Gillan *et al.* (1988) to calculate the contribution of *cis*-vaccenic acid to the biomass of *S. velum*, we estimate that the bacteria account for approximately 16-26% of the animal's biomass. This is a conservative estimate as it assumes the bacteria only contain one fatty acid, yet it is orders of magnitude greater than the actual biomass of bacteria in *S. velum* which can be estimated using the bacterial counts of Cavanaugh (1983). Thus, it is evident that the host tissue is incorporating lipids produced by

³ In the study of Gillan *et al.* (1988), the contribution of fatty acids attributable to symbiotic bacteria are estimated by subtracting the lowest observed abundances of bacterial fatty acids in sponges without symbionts from the fatty acid concentrations of bacteria-containing sponges. The fatty acid concentrations are converted to bacterial biomass by assuming a bacterial water content of 80%, and a fatty acid content of 3-5% on a dry weight basis. If we assume that the bacteria of *S. velum* contain about 3-5% fatty acids and the bacteria only provide *cis*-vaccenic acid, then 8070 ng/mg of *cis*-vaccenic acid corresponds to 160-260 μg of bacteria/mg dry weight bivalve, which is 16-26 % of the animals dry weight.

the bacterial endosymbionts. In addition, the presence of 18:1 ω 7 in all the major lipid classes examined, particularly the phospholipid and triglyceride pools (Table 4.5) suggests that this lipid plays a significant role in all aspects of the lipid metabolism of *S. velum*.

In an ideal mutualistic symbiosis, both members of the association must evolve the means to utilize the metabolic products of each other (Dyer, 1989). The incorporation of bacterial lipids into all the major lipid classes and all the animal tissues of the *S. velum* symbiosis suggests a tight metabolic coupling that exists between the two members of this symbiosis. It is suggested from this study that specific bacterial lipids are translocated from endosymbiont to the host and utilized intact. It is likely that in *S. velum*, *cis*-vaccenic acid assumes the physiological roles normally filled by oleic acid (18:1 ω 9), including energy storage and production and membrane structural functions.

Transfer of bacterial membrane material from endosymbiont to host may be a source for the bacterial lipids found in *S. velum*. DeBurgh *et al.* (1989) suggest that the vacuolar membrane surrounding the bacteria in animal-bacteria symbioses may play a role in regulating nutrient transfer. Portions of the bacterial membrane appear to be pinched off in *Lucinoma annulata* (Figure 4.7a,b; R. Vetter, Pers. Comm.). Furthermore, the accumulation of lipids under the bacterial zone of gill cells has been suggested by transmission electron microscopy (Fiala-Médioni *et al.*, 1984), abundant lipid inclusions have been seen in the gill cells of both *Bathymodiolus thermophilus* and *Calyptogena phaseoliformis* and Fiala-Médioni and LePennec (1987) suggest that these lipid inclusions might be related to the bacterial metabolism and represent some type of reserve for the host. In addition, Fiala-Médioni *et al.* (1986a) demonstrated the incorporation of 7-15% of the radiolabel in the lipidic fraction of *Bathymodiolus* after incubation in seawater containing radiolabelled bicarbonate. The recent *in vitro* studies of bacterial symbionts by Wood and Kelly (1989) suggest that the putative isolated symbionts of *Thyassira flexuosa* are highly leaky, resulting in the loss of bacterial metabolites to the surrounding medium. Our study provides

evidence to suggest the accumulation of bacterial lipids by the bivalve host and incorporation of these lipids into all the lipid class pools of this species. In addition, bacterial-lipids may be obtained by direct 'harvesting' or digestion of the bacterial endosymbionts. We can deduce from the lipid data for *S. velum* that the bacterial lipids do not appear to undergo desaturation and elongation to other fatty acids, as only low concentrations of other fatty acids of the $\omega 7$ -series were present⁴. In addition, the low $\delta^{13}\text{C}$ ratios of the fatty acids in *S. velum* strongly suggest that even fatty acids synthesized by the animal tissues are ultimately derived from bacterial carbon.

S. velum tissues contained smaller amounts of the PUFAs commonly found in marine bivalves (20:5 $\omega 3$ and 22:6 $\omega 3$, Tables 3,6), although the PUFA 20:4 $\omega 6$ occurred in extremely high concentrations. Monounsaturations of fatty acids in animal cells is achieved with a $\delta 9$ desaturase, which has a maximum affinity for stearic acid (18:0) and results in 18:1 $\delta 9$ (18:1 $\omega 9$). When PUFAs are formed, additional double bonds can only be introduced between the carboxyl group and the nearest existing double bond. Therefore, *de novo* animal PUFAs are unlikely to have an ω number less than 7. Yet animals require $\omega 3$ and $\omega 6$ PUFAs, and these essential fatty acids (EFAs) must be included in the diet. EFAs are vital components of membranes, where they are important regulators of membrane fluidity and play a role in membrane transport processes.

⁴ Animal fatty acid synthetases cannot insert a double bond into a fatty acid closer than the $\omega 7$ position, consequently, although *cis*-vaccenic acid could be elongated to 20:1 $\omega 7$, further desaturations would not occur towards the methyl end of the fatty acid chain, and the new fatty acid would still be a member of the $\omega 7$ series.

Table 4.10. *Solemya velum* and *Mya arenaria*. Total lipid fatty acid composition compared with sulfur-oxidizing bacteria and four other bivalves. (Fatty acids presented as % of total fatty acids > 1%).

Fatty acid ^(a)	<i>Thioploca</i> sp. ^(b)	<i>Thiomicrospira</i> <i>crunogena</i> ^(c)	<i>Solemya</i> <i>velum</i> ^(c)	<i>Mya</i> <i>arenaria</i> ^(c)	<i>Mya</i> <i>arenaria</i> ^(d)	<i>Mytilus</i> <i>edulis</i> ^(d)	<i>Solen</i> <i>strictus</i> ^(d)	<i>Mercenaria</i> <i>mercenaria</i> ^(d)	<i>Crassostrea</i> <i>virginica</i> ^(d)
14:1				1.5	2.2				
14:0		1.0	4.1	0.1	0.1	3.2	1.8	2.0	3.5
15:0	0.2	1.2	0.3	0.6					
16:1	41.4	43.6	14.4	4.9	4.5	7.5	1.2	4.8	4.2
16:0	17.7	24.8	11.0	17.0	12.5	15.9	16.3	12.3	28.9
17:0	0.1	1.9	0.2	1.4					
18:4			0.1	1.3	1.8	1.9	2.5	1.1	2.6
18:3			0.2	0.4	1.4	1.1	1.4	0.9	3.3
18:2			4.6	1.0	1.9	1.1	3.2	0.2	2.0
18:1 ω 9	0.4	1.4	2.9	6.2	6.2	11.1	10.6	8.4	3.9
18:1 ω 7	36.9	19.6	24.8	2.4					4.3
18:0	0.7	6.7	5.2	5.0	3.0	3.7	3.5	5.5	3.6
20:5			1.4	12.7	11.2	10.2	7.0	18.3	11.2
20:4			10.6	4.8	5.5	3.8	4.6	3.6	
20:3			4.5	0.8	0.8	0.2	0.3	0.1	
20:2			7.2	2.5	1.2	0.3	1.5	0.2	
20:1	0.1		4.0	14.3	1.1	7.7	4.4	8.6	7.1
22:6			0.2	19.0	13.4	13.7	14.0	15.0	9.7
22:5					0.5	0.2	2.1	2.0	
22:4				0.3	3.8		1.0	0.4	
22:3			0.2	2.0				0.1	
22:2			3.4	0.1			4.1		
22:0						1.4			

(a) Positional isomers are not reported for the majority of fatty acids as they were not determined in many of the studies cited.

(b) McCaffrey *et al.* 1989; (c) This study; (d) See references in Joseph 1982.

Additionally, they are precursors for prostaglandin synthesis and act as energy sources in a manner similar to saturated and monounsaturated fatty acids (Guarnieri and Johnson, 1970 and references therein).

EFAs are abundant in marine phytoplankton and higher plants, yet they are absent in the vast majority of bacteria studied to date (Goldfine, 1972; Phillips, 1984). These PUFAs have been recorded in a variety of deep-sea bacteria (DeLong and Yayanos, 1986); however, they can only be synthesized using the aerobic fatty acid synthesis system described previously. The high concentrations of *cis*-vaccenic acid in *S. velum* suggest that the bacterial endosymbionts utilize the anaerobic MUFA synthesis pathway and are thus incapable of ω 3 and ω 6 PUFA synthesis. Thus, while *Mya* and other typical bivalves will ingest adequate amounts of these fatty acids in the diet, species such as *S. velum*, with reduced digestive capabilities, would appear to be limited in their ability to obtain EFAs in the diet. In most marine bivalves, the PUFAs 20:5 ω 3 and 22:6 ω 3 predominate (Table 4.10) and the fatty acid profiles reported here for *M. arenaria* are in remarkably good agreement with those of other bivalve molluscs (Table 4.10). *S. velum* had only trace amounts of the ω 3 fatty acids, an observation consistent with the hypothesis that the endosymbionts serve as the primary nutritional source for the animal host.

The PUFA composition of *S. velum* raises two questions. How does *S. velum* function with a limited supply of EFAs, and what is the source of the EFAs found in their tissues? The PUFAs of most marine animals are characterized by large amounts of the ω 3 series of EFAs and fatty acids of the ω 3 series appear to be the main EFAs in fish [concentrations of ~1% of the dry weight of the diet are required for normal growth of marine trout (Sinnhuber *et al.*, 1972, Sargent, 1976)]. The high concentrations of ω 3 PUFAs in all the marine bivalves studied (e.g. Table 6.8) indicate that these PUFAs are very important in these organisms. The relatively large amounts of 20:4 ω 6 in *S. velum* suggest this fatty acid must fulfill some of the functions normally associated with 20:5 ω 3 and 22:6 ω 3 in most other bivalves, including maintenance of membrane

fluidity. Localization of 20:4 ω 6 in the phospholipid fraction of *S. velum* may be a PUFA conservation strategy to ensure proper membrane functioning, as the lipids of *S. velum* have a relatively smaller proportion of PUFAs than *M. arenaria* and other bivalves. The high concentrations of *cis*-vaccenic acid in the phospholipid pool of *S. velum* suggests that this lipid is also important in membrane structure.

In many bacteria *cis*-vaccenic acid is the major MUFA present and is an important component of bacterial membranes. Concentrations of *cis*-vaccenic acid may be increased in bacteria during acclimation to low temperatures, ensuring maintenance of membrane fluidity (Okuyama *et al.*, 1977). In animal systems two different strategies are used to ensure membrane fluidity during low temperature exposure (Hazel and Sellner, 1979 and references therein). In most cases, the ratios of PUFAs to saturated fatty acids are increased and the proportion of MUFAs remains fairly constant. In some instances, however, MUFA concentrations are increased while PUFA and saturated fatty acid concentrations remain stable. It would be interesting to see if the *cis*-vaccenic acid concentrations of *S. velum* increase during cold exposure. This lipid is almost certainly important in maintaining membrane integrity in *S. velum* tissues, as evidenced by the high concentrations of *cis*-vaccenic acid in the phospholipid pool of *S. velum*. Changes in the concentrations of bacterial lipids in the animal tissues during periods of cold stress would demonstrate the existence of mutual metabolic control mechanisms. This lipid may function with 20:4 ω 6 in lieu of the ω 3 PUFAs of typical marine bivalves. The animals examined in this study were collected in November; thus, the high concentrations of *cis*-vaccenic acid may be the result of cold exposure. If this is the case, one would expect levels to be even higher in December and January, when the annual temperatures at LBB reach a minimum (Chapter 2).

Figure 4.7 a) and b). Freeze fracture electron micrographs of the bacteria of *Lucinoma annulata* showing the bacterial membrane being pinched off. IM = inner bacterial membrane; OM = outer bacterial membrane; AM = animal membrane; Vs = vesicles between OM and IM in periplasm; Sg = sulfur globule. (Photographs provided by R. Vetter, Scripps Institution of Oceanography, tissues sectioned using freeze fracture techniques).

a)

b)

The source of the 20:4 ω 6 PUFA found in *S. velum* remains to be determined. Possibilities include *in vivo* synthesis by either the animal or bacterial components of the symbiosis or exogenous food sources. The presence of large amounts of fatty acids associated with the anaerobic bacterial MUFA synthesis pathway suggest that synthesis of PUFAs by the endosymbionts is unlikely. Additionally, animal fatty acid synthesis pathways are considered incapable of ω 6 fatty acid synthesis. Exogenous sources may provide the high concentrations of 20:4 ω 6 found in *S. velum*, yet large amounts of other lipids would be concomitantly taken in and the $\delta^{13}\text{C}$ ratios of *S. velum* fatty acids have a strong bacterial signature. Perhaps ingestion of small amounts of 20:4 ω 6 and other members of the ω 6 fatty acid series such as 18:2 ω 6 and 20:2 ω 6, and subsequent elongation by the animal may be important in contributing to the large 20:4 concentrations found in *S. velum*. Uptake of dissolved free fatty acids is another pathway by which lipids may be ingested in small amounts. This has been shown to occur in the bivalve *Crassostrea virginica* (Bunde and Fried, 1976) which can actively remove free fatty acids from seawater at ambient concentrations of 0.25 μM . Most of the fatty acids taken up by *C. virginica* were found to be concentrated in the phospholipid pool, and small amounts were also found in the sterols of the animal, indicating metabolism of the lipids taken up (Bunde and Fried, 1976). Low 20:4 turnover rates could also explain the high concentrations of this fatty acid.

To summarize, the fatty acid, sterol and lipid stable isotope composition of *Solemya velum* strongly suggests a chemoautotrophic diet. The major fatty acid was the monounsaturated fatty acid *cis*-vaccenic acid, a lipid normally found in large concentrations only in bacteria. This lipid may serve as a useful screening tool for the investigation of other animal-bacteria symbioses; preliminary analyses of other animal-bacteria symbioses (See Chapter 6) suggest that *cis*-vaccenic acid may be ubiquitous in animal-bacteria associations. *Cis*-vaccenic acid occurs in high concentrations in all the lipid classes of *S. velum*,

particularly the phospholipid and triglyceride pools, demonstrating its importance in both membrane structure and lipid storage processes. The sterols and $\omega 3$ and $\omega 6$ PUFAs derived from phytoplankton and normally found in high concentrations in marine bivalves were present in only trace amounts in *S. velum*, demonstrating a limited input of plant-derived material (This conclusion is supported by experiments examining the ingestion of algal cells, Appendix III). The $\omega 6$ PUFAs present in *S. velum* appear to be conserved for use in the phospholipid pool of this species; the source of this lipid remains to be determined. The $\delta^{13}\text{C}$ ratios of the lipids of *S. velum* strongly suggest an chemoautotrophic source for the lipid carbon.

The work described here clearly shows the usefulness of a biomarker approach for the study of animal-bacteria symbioses. Assumptions based on the lipid composition of the *S. velum* symbiosis support the results of earlier work with this species indicating that the bacterial endosymbionts form the major nutritional source for this species (Chapter 3). *S. velum* is highly suitable for general studies of nutrition of animal-bacteria symbioses, as large numbers may be collected and examined for endosymbiont biomarkers. In addition, the biomarker approach can be verified by direct physiological studies on intact living specimens. Consequently, new techniques can be calibrated on *S. velum*, and other relatively accessible symbioses, and applied to rarer specimens when the opportunities arise. The techniques described here may be very useful in the study of other symbioses.

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CHAPTER 5

A COMPARATIVE STUDY OF THE AMINO ACID COMPOSITION
OF *SOLEMYA VELUM* AND *MYA ARENARIA*.

5.1 Abstract

In order to determine comparative biochemical differences between bivalves with and without symbiotic bacteria, specimens of the protobranch bivalve *Solemya velum*, a bivalve species known to contain bacterial endosymbionts, and the soft-shelled clam *Mya arenaria* were collected from the same subtidal reducing sediments during October and November 1988. Total and free amino acids were determined for both species. Protein-bound amino acids were calculated as the difference between total and free amino acids. In addition the carbon and nitrogen isotope ratios of the total and free amino acids were measured in order to determine potential carbon sources for these molecules.

Both species had similar protein-bound amino acid compositions; approximately 50% of the protein-bound amino acids were essential amino acids. In *S. velum*, the large reduction of the digestive system suggests these amino acids are likely to be synthesized by the endosymbiotic bacteria and translocated to the animal tissue. These results are supported by the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios of the amino acids of *S. velum* which were very similar to the isotope ratios previously found in both the endosymbionts and whole tissues of *S. velum*. Both the relative and absolute amounts of free amino acids differed significantly between the two species. In *S. velum*, the absolute concentration of the sulfur amino acid taurine was greater than the total free amino acid concentrations typically found in bivalves. The possible roles for taurine in this animal-bacteria symbiosis are discussed.

5.2 Introduction

Chemoautotrophic bacteria occur as symbionts in at least four marine invertebrate phyla, including such diverse groups as bivalves, oligochaetes, pogonophoran, and vestimentiferan worms (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981; Giere *et al.*, 1984; Southward *et al.*, 1981). In most cases the bacteria appear to derive their cellular carbon from the fixation of CO_2 and their energy from the oxidation of reduced sulfur compounds such as H_2S , S , and $\text{S}_2\text{O}_3^{2-}$, although species capable of growth on methane have also been reported (Childress *et al.*, 1986, Cavanaugh *et al.*, 1987). Many of the host species are characterized by reduced digestive capabilities, suggesting a trophic function for the bacterial symbionts. In this context, recent studies (Anderson *et al.*, 1987, Chapter 3) suggest that intact animal-bacteria symbioses may obtain their entire carbon budgets through bacterial autotrophy, and much of their nitrogen requirements. The endosymbiotic bacteria, on the other hand, are provided with a sheltered environment, and the inorganic compounds necessary for chemoautotrophy (CO_2 , O_2 and reduced sulfur).

The invertebrate-chemoautotroph symbioses (hereafter called animal-bacteria symbioses) discovered to date generally occur in either inaccessible environments such as hydrothermal vents, or in relatively obscure species. Consequently, little information is available regarding the basic biology of the host species and the design and interpretation of experiments to elucidate the interactions between bacteria and host is difficult. In this dissertation, the trophic interactions occurring within animal-bacteria symbioses have been investigated using the shallow-water protobranch bivalve *Solemya velum* as a general model of animal-bacteria symbioses. In order to determine basic differences in physiology between the *S. velum* symbiosis and non-symbiont containing clams occupying similar habitats, and to provide background data regarding the biology of this species, a detailed examination of the biochemical composition and stable isotope ratios

of *S. velum* and the soft-shelled clam *Mya arenaria*, a bivalve lacking symbionts, has been conducted. Stable isotope ratios are important biochemical markers which have been used to estimate the contribution of bacterial symbionts to host nutrition (Rau, 1985; Chapter 3). The reduction of inorganic carbon during chemoautotrophy generally results in a greater depletion of the ^{12}C isotope relative to the ^{13}C isotope (i.e., more negative $\delta^{13}\text{C}$ value) than occurs during photosynthetic carbon fixation (Degens, 1969; Ruby *et al.*, 1987). Consequently, chemoautotrophic bacteria may have more negative $\delta^{13}\text{C}$ values than photosynthetic organisms. These differences in $\delta^{13}\text{C}$ ratios are often transferred through the food-chain allowing determination of the origin of the carbon in an organism. The carbon, nitrogen, and sulfur stable isotope ratios of *S. velum* are very negative, similar to the stable isotope ratios of an enriched bacterial endosymbiont preparation, and very different to those of organisms utilizing the typical phytoplankton-based food chain (Conway *et al.*, 1989). Determining the stable isotope ratios of the amino acids of *S. velum* should provide information on the carbon and nitrogen sources for these molecules.

In this chapter, the results of a comparative study of the gross biochemical composition and the amino acid composition of *S. velum* and *Mya arenaria* collected from the same subtidal reducing sediments are discussed. In addition, the carbon and nitrogen stable isotope ratios of the amino acid pools of both species are investigated. Methods used in this chapter are outlined in sections 2.4 and 2.5 of Chapter 2.

5.3 Results

5.3.1 Biochemical composition

Overall there were few discernable differences between the gross biochemical composition of the two species apart from the stable isotope ratios (Table 5.1). Although the foot and viscera of *M. arenaria* appear to have slightly greater carbohydrate levels than those of *S. velum*, these differences were not significant. The protein levels of the foot and visceral mass of *Mya* were greater

than those of the gills. This is not surprising considering the greater lipid levels found in the gills of this species. The high gill protein levels found in *S. velum* are in all probability artifacts caused by the interference of gill hemoglobin with the colorimetric Biuret assay. Using the total nitrogen values (Table 5.1) to estimate gill protein levels (assuming protein is 16% N and all the N is in the form of protein) yields a value of 60% for the protein content of the gills (dwt.), which is probably closer to the real value.

5.3.2 THAA and protein-bound amino acid composition

There were few obvious differences in the THAA content of the two species, apart from differences in taurine, alanine and glycine (Table 5.2, Figure 5.1a, b). The differences in these amino acids between the two species are due to the differential utilization of these amino acids in free amino acid pools of each species and are discussed below. The protein-bound amino acids of both species were estimated as the difference between THAA and FAA, and are presented in Figure 5.2 a,b, and Table 5.3). No obvious differences in the protein amino acids, apart from differences in glycine and alanine were apparent between the two species and both contained all the essential¹ amino acids. Amino acids present in amounts exceeding 5% of the total moles present were aspartic acid, glutamic acid, glycine, alanine, valine, isoleucine and lysine (see Figure 5.2a,b, and Table 5.3). The protein-amino acid pool of both species were similar to those reported for other bivalve species (e.g., *Crassostrea gigas*; Riley, 1980) and benthic marine organisms (Henrichs, 1980). Differences between tissues were apparent. In *S. velum*, gill tissue had significantly less glutamic acid and significantly greater amounts of alanine and glycine than either foot or visceral mass

¹ Essential amino acids are those amino acids that cannot be synthesized by most metazoans including molluscs and include arginine, histidine, valine, threonine, phenylalanine, tryptophan, methionine, valine, leucine, and isoleucine; Lehninger, 1975; Bishop et al., 1983). Although it is not known whether *Solemya velum* and *Mya arenaria* can synthesize these amino acids it is unlikely that these species will differ from most metazoans studied and we will assume that these amino acids are essential dietary requirements for both bivalves throughout the following discussion.

tissue ($P < 0.05$; Table 5.3). In *M. arenaria*, no significant differences were apparent between tissues.

Table 5.1 Comparative aspects of the biochemical composition of *Solemya velum* and *Mya arenaria*.

<i>Solemya velum</i>			
Parameter	Gill	Foot	Visceral Mass
% Water	78±4.7	75±6.9	72±6.9
% C	41.8±2.0	40.9±2.0	n.d
% N	9.6±0.5	11.5±0.9	n.d
% S	1.9	2.3	n.d
% Protein	85±3.0	76.0±11	73.0±8.0
% carbohydrate	9.0±3.0	8.1±1.2	8.1±2.2

<i>Mya arenaria</i>			
Parameter	Gill	Foot	Visceral Mass
% Water	82±2.5	78±2.6	79±0.4
% C		39.9	
% N		10.5	
% S		n.d.	
% Protein	53.3±4.0	65.3±0.2	70.0±2.8
% carbohydrate	6.1±0.7	11.8±0.2	13.3±2.0

5.3.3 Free amino acid composition

The free amino acid (FAA) pools of *M. arenaria* and *S. velum* differ dramatically in both relative and absolute amounts and composition (Table 5.4 Figure 5.3 a,b). Whereas the FAA pool of *S. velum* is totally dominated by taurine (63-73% of the total), the FAA pools of *M. arenaria* are characterized by large amounts of glycine (30-43%), alanine (23-38%) and taurine (16-24%). Such differences in relative composition are not unusual; different bivalves utilize different FAAs for osmoregulatory purposes, and even different tissues of the same species may have different relative and absolute amounts of FAAs (Table 5.5, Zurburg and DeZwinn, 1981). However, the absolute amounts of free amino acids in the *S. velum* symbiosis are noteworthy. On average, the whole body tissue of *S. velum*

contained $330 \mu\text{mol g}^{-1}$ FAA, of which $226 \mu\text{mol}$ was taurine (Table 5.5). Moreover, FAA concentrations were even higher in the foot tissue of this species (mean values were $393 \mu\text{mol g}^{-1}$ wet weight, with taurine accounting for $277 \mu\text{mol g}^{-1}$ wet weight, Table 5.5b). To our knowledge, taurine has not been found in such high concentrations in bivalve species studied to date. Although the gills of *S. velum* had the greatest relative amounts of taurine (73.7%, Table 5.4, Figure 5.3a), the foot tissue contained the highest absolute amounts of this amino acid ($277 \mu\text{mol g}^{-1}$ wet weight, Table 5.5b). The free amino acid concentrations found for *M. arenaria* were more typical of marine bivalves, with the average whole tissue value being $204 \mu\text{mol g}^{-1}$ wet weight (Tables 5.5, Figure 5.3b), although again, values for foot tissue were higher than those of other tissues ($312 \mu\text{mol g}^{-1}$ wet weight, Table 5.5b). Relative amounts of glycine, taurine and alanine varied between the tissues of *M. arenaria* (Table 5.4). In *S. velum*, the gills had more glutamic acid and less serine and alanine than either the foot or visceral mass tissue.

5.3.4 Stable isotope ratios

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotope ratios of the amino acid pools of the two bivalve species are outlined in Table 5.6, along with the whole animal values previously discussed in Chapter 3. Isotope ratios of the amino acids of both species are very similar to the whole tissue values for each species. In *S. velum*, very negative amino acid carbon and nitrogen isotope ratios are apparent (-27.6 to -30.4‰ and -8.0 to -9.2‰ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ respectively, Table 6). These values are similar to those found in the endosymbiont fraction and whole tissues of *S. velum* (Chapter 3) and very different from the carbon and nitrogen isotope ratios of the amino acids and whole tissues of the non-symbiont controls (-16.6 to -18.8‰ and 7.8 to 9.7‰ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ respectively), which are part of the typical photosynthesis-based trophic web.

Table 5.6 a) $\delta^{13}\text{C}$ and b) $\delta^{15}\text{N}$ ratios of *Solemya velum* and *Mya arenaria* (Whole tissue and endosymbiont fraction values from Chapter 3)

a) $\delta^{13}\text{C}$ ratios of animals from Little Buttermilk Bay

Species	Tissue values	THAA	Free amino acids
<i>Solemya velum</i>	-31.5 to -33.9‰	-30.4‰	-27.6‰
<i>S. velum</i> (Endosymbiont fraction)	-32.3 to -33.6‰		
<i>Mya arenaria</i>	-17.1 to -17.8‰	-16.6‰	-18.7, -18.8‰

b) $\delta^{15}\text{N}$ ratios of animals from Little Buttermilk Bay

Species	Tissue values	THAA	Free amino acids
<i>Solemya velum</i>	-7.7 to -9.8‰	-8.0‰	-9.2‰
<i>S. velum</i> (Endosymbiont fraction)	-6.9 to -8.6‰		
<i>Mya arenaria</i>	8.2 to 8.5‰	9.7‰	7.8, 7.9‰

Table 5.2 Total Hydrolysable Amino Acids of *Solemya velum* and *Mya arenaria*
(Mean Molar Percent +/- S.D., N=5)

Amino Acid	<i>S. velum</i>						<i>M. arenaria</i>					
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
	Gill		Foot		Visceral	Mass	Gill		Foot		Visceral	Mass
Asp	9.9	2.5	10.1	1.8	10.7	2.2	9.9	0.1	8.7	0.8	8.1	0.1
Glu*	10.5	1.2	11	1.2	10.8	1.4	9.2	1.7	9.7	1.5	9.2	1.1
Oh-P	0.4	0.2	0.4	0.2	0.3	0.1	0.3	0.1	0.8	0.1	0.4	0.1
Asn (1)	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Ser*	0.8	0.1	1.3	0.7	1.2	0.6	2.5	0.2	2.6	0.3	2.8	0.5
Gln (1)	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Gly	11.0	2	8.3	0.9	9.1	1.7	17.7	1	14.9	0.5	18.1	2.5
His	2.1	0.4	1.8	0.3	2.1	0.1	2.2	0.2	1.9	0.2	2.1	0.1
Tau	12.1	2.1	12.5	1.7	12.5	2.5	4.3	1.2	3.5	0.8	3.6	1
Arg	6.0	1.2	6.4	0.3	6.6	1	5.9	1.1	5.8	0.7	6.1	0.5
Thr*	2.0	0.5	3	2.5	2.2	0.8	2.6	0.2	3.2	0.7	3.2	0.9
Ala	9.9	0.7	10.8	2.3	9.7	0.5	11.9	1.2	15.3	1.6	13.4	1.3
Pro	4.3	0.5	3.8	0.1	3.8	0.4	4	0.2	4.4	0.2	3.9	0.2
Tyr	1.8	1	2	1	1.8	0.9	1.8	0.8	1.4	0.8	1.9	1.2
Val	5.9	0.6	6.1	0.2	6.4	0.6	6.5	0.2	6.6	0.3	6.3	0.4
Met	1.4	0.7	1.8	0.5	2	0.4	0.7	0.6	0.6	0.3	0.7	0.5
Cys	0.2	0.1	0.4	0.2	0.2	0.3	0.7	0.5	0.8	0.5	0.7	0.2
Ile	4.2	0.4	4.4	0.3	4.5	0.1	4.4	0.4	4.4	0.4	4.5	0.4
Leu	6.0	0.9	6.3	0.7	6.8	0.1	6	0.1	6	0.1	6	0.4
Phe	2.7	0.2	2.8	0.1	2.7	0.4	3.2	0.1	2.9	0.2	3.1	0.1
Trp*	0.1	0.1	0.1	0.1	0.2	0.1	1	1	0.1	0.1	0.1	0.1
Orn	1.0	0.9	0.3	0.1	0.3	0.1	0		0		0	
Lys	6.8	0.4	6.7	0.7	6.3	1.2	6.8	0.1	6.5	0.1	5.9	1
%Hydrophobic	34.6		36.0		36.1		36.7		40.2		38.0	
%Polar unc.	15.7		15.0		14.4		25.2		22.9		26.7	
%Polar +ve	15.7		14.8		15.0		14.9		14.2		14.1	
%Polar -ve#	20.4		21.0		21.5		19.1		18.5		17.3	
%Essential	38.0		39.2		39.8		38.4		37.9		37.7	

(1) No data, these amino acids are converted to Asp and Glu during acid hydrolysis.

* Yields of these amino acids are reduced during acid hydrolysis

Does not include taurine

Underlined sections indicate amino acids that were significantly different in the two species
(P<0.05, Student's T-test).

Figure 5.1 Total hydrolyzable amino acids of a) *Solemya velum* and b) *Mya arenaria*, expressed as molar percent of the total amino acids.

Table 5.3 Protein Amino Acid Composition of *Solemya velum* and *Mya arenaria*(1)
(Mean molar percent $\pm$ s.d., N=5)

Amino Acid	<i>S. velum</i>				<i>M. arenaria</i>							
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
	Gill		Foot		Visceral Mass		Gill		Foot		Visceral Mass	
Asp	10.3	0.9	13.2	2.4	12.7	2.4	12.2	1.1	12.2	0.7	11.8	1.9
Glu*	12.3	0.1	16.2	1.7	16.2	0.9	12.9	2.3	15.7	1.2	13.8	2.6
Oh-P	0.7	0.1	0.2	0.1	0.3	0.3	0.3	0.1	0.9	0.1	0.4	0.3
Asn (2)	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Ser#	1.4	0.7	0.7	0.1	0.8	0.1	2.5	0.3	2.3	0.7	2.4	1.4
Gln (2)	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Gly*	7.1	0.7	4.9	0.1	4.6	0.3	5.0	1.3	4.9	0.5	3.6	2.4
His	2.6	0.3	2.6	0.3	2.9	0.1	2.7	0.2	2.3	0.2	2.5	0.3
Tau	0.0		0.0		0.0		0.0		0.0		0.0	
Arg	10.0	0.8	8.9	2.4	11.6	1.1	10.1	1.9	10.0	1.9	10.1	1.8
Thr#	4.4	2.9	2.4	0.3	2.6	0.7	3.0	0.6	3.4	1.0	4.2	1.1
Ala*	8.4	1.8	5.7	0.7	5.3	0.5	4.9	0.3	4.4	0.7	2.4	1.1
Pro	4.7	0.5	4.0	0.1	3.8	0.2	4.5	0.2	5.1	0.5	4.9	0.3
Tyr	3.2	2.0	3.1	1.6	2.5	1.5	3.5	1.4	2.4	1.8	3.0	2.5
Val	6.7	0.4	6.9	1.0	6.7	0.2	7.5	0.2	7.7	0.6	8.1	0.4
Met	2.0	0.5	1.9	1.1	2.7	0.6	1.0	0.7	0.9	0.6	1.1	1.0
Cys	0.1	0.1	0.0		0.2	0.3	0.8	0.5	1.1	0.7	0.7	0.5
Ile	4.9	0.1	5.5	0.1	5.6	0.3	5.9	0.5	6.1	0.5	6.8	0.9
Leu	6.9	0.4	7.7	0.7	8.5	0.4	7.9	0.3	8.2	0.7	9.0	0.3
Phe	4.5	0.1	4.3	0.1	4.1	0.5	5.4	0.3	5.1	0.4	5.9	0.3
Trp#	0.1	0.1	0.0		0.4	0.4	0.1	0.1	0.1	0.1	0.0	
Lys	9.4	2.5	11.9	1.3	9.1	2.0	10.1	0.2	7.3	2.7	9.4	1.5
%Hydrophobic	38.4		35.9		36.9		37.0		37.6		38.3	
%Polar unc.	16.1		11.1		10.6		14.8		14.2		14.0	
%Polar +ve	22.0		23.4		23.7		22.9		19.6		21.9	
%Polar -ve	22.6		29.4		28.9		25.1		27.7		25.5	
%Essential	51.8		52.1		54.1		53.6		51.1		57.1	

(1) Values determined by subtracting FAA from THAA.

(2) No data, these amino acids are converted to Asp and Glu during acid hydrolysis.

Underlined sections indicate amino acids that were significantly different in the two species
($P < 0.05$, Student's T-test).

* Significant differences between tissues ($P < 0.05$, Student's T-test)

Yields of these amino acids are reduced during acid hydrolysis

Figure 5.2 Protein-bound amino acids of a) *Solemya velum* and b) *Mya arenaria*, expressed as molar percent of the total protein-bound amino acids.

Table 5.4 Free AA Composition of *Solemya velum* and *Mya arenaria*
(Mean molar percent $\pm$ s.d., N=5)

Amino Acid	<i>S. velum</i>						<i>M. arenaria</i>					
	Mean	s.d	Mean	s.d	Mean	s.d	Mean	s.d	Mean	s.d	Mean	s.d
	Gill		Foot		Visceral	Mass	Gill		Foot		Visceral	Mass
Asp	3.4	1.0	3.1	1.9	2.4	1.0	1.3	0.5	0.8	0.6	0.6	0.4
Glu*	4.6	0.9	1.0	0.3	1.4	0.4	3.1	0.3	2.7	0.2	2.4	0.4
Oh-P	0.8	0.4	0.8	0.3	0.4	0.1	0.5	0.2	0.4	0.1	0.3	0.1
Asn	1.7	0.7	1.2	1.3	1.3	1.4	0.5	0.3	0.4	0.2	0.6	0.1
Ser*	0.4	0.3	5.8	6.8	4.8	5.4	0.5	0.4	0.4	0.1	0.5	0.1
Gln	0.5	0.2	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.3	0.2
Gly*	1.0	0.5	1.0	1.2	6.3	9.0	39.9	2.7	30.5	1.9	42.9	5.1
His	0.9	0.4	0.4	1.3	0.6	0.3	2.4	0.5	2.0	0.3	2.0	0.5
Tau*	73.7	7.5	70.0	11.0	63.1	8.6	23.8	1.9	18.8	1.2	15.7	3.5
Arg	1.3	2.1	1.3	0.6	1.5	3.0	1.3	0.8	3.5	0.5	3.1	1.8
Thr	1.8	2.7	0.7	0.7	0.7	0.5	0.3	0.3	0.1	0.1	0.3	0.1
Ala*	5.9	2.2	12.8	4.0	15.4	4.7	23.8	2.5	38.6	2.9	28.5	3.1
Pro	1.1	1.7	0.4	0.5	0.3	0.2	0.4	0.3	0.2	0.1	0.4	0.1
Tyr	0.3	0.2	0.7	0.4	0.5	0.4	0.2	0.1	0.1		0.2	0.1
Val	0.5	0.2	0.4	0.1	0.4	0.1	0.4	0.3	0.4	0.3	0.3	0.1
Met	0.2	0.1	0.2	0.1	0.1	0.1	0.5	0.4	0.2	0.1	0.3	0.2
Cys	0.3	0.2	0.2	0.3	0.0		0.0		0.0		0.0	
Ile	0.3	0.2	0.1		0.1		0.2	0.2	0.1	0.1	0.2	0.1
Leu	0.4	0.3	0.2	0.1	0.2	0.1	0.3	0.2	0.2	0.1	0.3	0.1
Phe	0.3	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Trp	0.2	0.2	0.0		0.0		0.1	0.1	0.1	0.1	0.1	
Lys	1.7	1.0	0.6	0.4	0.4	0.2	0.4	0.2	0.3	0.2	0.5	0.1
Total Pool	317.0	23.0	393.0	13.0	327.0	85.0	191.0	27.0	311.0	80.0	206.0	49.0
%Essential	7.4		4.1		4.3		6.0		7.0		7.3	

Underlined sections indicate amino acids that were significantly different in the two species
($P < 0.05$, Student's T-test).

*Significant differences between tissues ($P < 0.05$, Student's T-test)

Figure 5.3 Free amino acids of a) *Solemya velum* and b) *Mya arenaria*, expressed as molar percent of the free amino acids.

Table 5.5 Relative Abundances of the Major Free Amino Acids in the Experimental Animals in Comparison With Representative Marine Bivalves

A) Values Presented as Mean Molar Percentages

	<i>S. velum</i> (1) Salinity = 34ppt. (Whole animal)	<i>M. arenaria</i> (1) Salinity = 34ppt. (Whole animal)	<i>M. arenaria</i> (1) Salinity = 34ppt (Foot tissue)	<i>M. arenaria</i> (2) Salinity = 30ppt (Adductor)	<i>Venus gallina</i> (3) Salinity = 37ppt. (Whole)	<i>Mytilus edulis</i> (4) Salinity = 30ppt. (Whole)	<i>Scapharca cornea</i> (3) Salinity = 37ppt. (Whole)	<i>Crassostrea virginica</i> (5) Salinity = 31-34ppt (Mantle)
Taurine	66.8	16.4	18.8	6.7	40.0	40.0	65.0	65.0
Alanine	11.7	28.4	38.6	45.4	15.0	7.0	3.0	8.0
Glycine	4.2	42.5	30.5	26.9	22.0	40.0	7.0	5.0
Aspartic Acid	2.7	0.6	0.8	0.5	8.0	6.0	7.0	11.0
Glutamic Acid	2.2	2.5	2.7	4.3	7.0	10.0	8.0	5.0
Proline	0.8	0.4	0.2	0.8	.	2.0	1.0	3.0

(1) Animals measured in this study.

(2) Values from Virkar and Webb (1970).

(3) Values from tables of Somero and Bowler (1983), and references therein.

(4) Values from Livingstone et al, 1979)

(5) Values from Heavers and Hammen, 1985)

B) Values Presented as $\mu\text{mol/g}$ Wet Weight

	<i>S. velum</i> (1) Salinity = 34ppt (Whole animal)	<i>S. velum</i> (1) Salinity = 34ppt (Foot)	<i>M. arenaria</i> (1) Salinity = 34ppt (Whole animal)	<i>M. arenaria</i> (1) Salinity = 34ppt (Foot)	<i>M. arenaria</i> (2) Salinity = 30ppt (Adductor)	<i>Mytilus edulis</i> (3) Salinity = 30ppt. (Whole)	<i>Crassostrea virginica</i> (4) Salinity = 31-34ppt. (Mantle)
Taurine	225.9	276.9	33.6	59.2	18.7	63.0	94.8
Alanine	39.5	49.0	58.0	120.3	127.8	5.1	12.2
Glycine	14.3	3.6	87.0	45.0	75.6	30.4	7.4
Aspartic Acid	9.3	11.1	1.3	2.8	1.5	7.8	16.4
Glutamic Acid	7.5	3.0	5.1	8.4	12.2	4.6	6.9
Proline	2.8	2.1	0.8	0.6	2.1	2.2	4.6
Total FAA pool $\mu\text{mol/g wet wt.} \geq$	329.5	392.8	204.4	311.7	280.6	143.4	145.9

(1) Animals measured in this study

(2) Values from Virkar and Webb, 1970. Converted from dry weight to wet weight using a value of 80% tissue water per gram tissue.

(3) Values from Livingstone et al., 1979. Converted from dry weight to wet weight using a value of 80% water per gram tissue.

(4) Values from Heavers and Hammen, 1985.

5.4 Discussion

5.4.1 Protein-bound amino acids

The protein-bound amino acid composition of *S. velum* differed little from previously described species; all the essential amino acids were present, and no amino acid appeared to occur in either unusually large or unusually small amounts. The dissimilarities between the protein-bound and free amino acids of both species suggest that neither organism is amino acid limited, as the study of Riley (1980) demonstrates that the free amino acid composition of marine bivalves will become more similar to the protein-bound amino acid composition during starvation.

Most molluscan species have a requirement for the ten essential amino acids common to most metazoans (arginine, histidine, valine, threonine, phenylalanine, tryptophan, methionine, lysine, leucine and isoleucine). Additionally, some species may also require serine, glycine, cysteine and proline (Bishop *et al.*, 1983). Marine invertebrates exhibit a fairly constant distribution of the essential amino acids in their tissues; however, as most invertebrates cannot synthesize these amino acids they must be obtained in the diet in approximately the same relative proportions as they occur in the animal (Allen and Kalgore, 1975; Lasser and Allen, 1976). In both species examined here, essential amino acids accounted for approximately 50% of the total bound amino acids (Table 5.3), whereas they only accounted for ~7% of the free amino acids (Table 5.4).

The large digestive system of *M. arenaria* could process enough phytoplankton, protozoans and bacteria to provide the necessary essential amino acid requirements of this species. However, the highly reduced gut of *S. velum* suggests that ingestion via the gut is unlikely to provide large amounts of essential amino acids (the experiments of Appendix III demonstrate minimal uptake of phytoplankton particulate matter), although it is important to note here that small amounts of amino acids are probably derived from the ambient seawater. Gallager (unpub. results) has demonstrated the uptake of dissolved alanine by the *S. velum* symbiosis (Figure 5.4). Although the alanine concentrations used in

these experiments are unrealistically large, the tissues of *S. velum* (particularly the gills) do appear to be capable of active uptake of small dissolved molecules. In addition, Fiala-Médioni *et al.*, (1986a) have demonstrated uptake of amino acids by *Bathymodiolus* spp. It is unlikely, however, that this trophic pathway accounts for a large proportion of the amino acid inventory of *S. velum*.

Figure 5.4. Dissolved amino acid uptake by *Solemya velum* in comparison with representative marine bivalves (Data courtesy of S. Gallager, WHOI).

Thiomicrospira crunogena, a chemoautotrophic sulfur-oxidizing bacterium, is capable of synthesizing the essential amino acids when grown in the absence of amino acids (Chapter 6). Trytek and Allen (1980) have provided evidence to demonstrate the synthesis of essential amino acids by the procaryotic symbionts of *Bankia setacea* and the inability of animal tissue alone to synthesize these amino acids. In this experiment, only the gills of *B. setacea*, which contain the symbionts incorporated radiolabelled CO₂ into essential amino acids. Similar experiments by Fiala-Médioni *et al.* (1986) demonstrated that 41-57% of radioactivity was incorporated into small molecules such as amino acids and carbohydrates, and 43-59% was incorporated into proteins when *Bathymodiolus* was incubated in radiolabelled bicarbonate. The individual compounds were not identified in *Bathymodiolus*.

If one considers that the protein composition of *S. velum* is approximately 70% (Table 2) on a dry weight basis, then essential amino acids probably account for 35% of the animal's dry weight. If we then assume that an "average" amino acid has 4-5 carbons and a molecular weight of approximately 120, then 33% to 50% of the amino acid is carbon. Consequently, 11% to 17% of the animal's dry weight is essential amino acid carbon. The carbon and nitrogen isotope ratios in *S. velum* are extremely negative demonstrating that the endosymbiotic bacteria probably provide the bulk of the carbon and nitrogen budgets of this species (Chapter 3; Table 6). In fact, almost 100% of the hosts carbon may be endosymbiont derived. The stable isotope ratios of the THAA pool of *S. velum* support this hypothesis. The carbon and nitrogen isotope ratios of the THAA pool (-30.4‰ and -8.2‰ respectively) reflect a chemoautotrophic signature, suggesting that much of the carbon and nitrogen of the amino acids of this species are either derived from the endosymbionts, or are synthesized using carbon and nitrogen derived from the endosymbionts.

5.4.2 Free amino acids

The stable isotope ratios of the free amino acid pool of *S. velum* show a strong bacterial signature. Stable isotope ratios of the FAA pools of *S. velum* are very different from those of *M. arenaria*, resembling those of intact *S. velum* tissues and enriched endosymbiont preparations. The apparent $\delta^{13}\text{C}$ enrichment in the free amino acids of *S. velum* ($\sim +4$ to $+6\text{‰}$ relative to whole tissue values) is most likely a result of 1) the ingestion of small amounts of exogenous amino acids, diluting the bacterial-derived carbon with plant carbon; and 2) differences in the chemical pathways involved in the production of specific amino acids, altering the $\delta^{13}\text{C}$ ratios. It is probably a mixture of both processes. As just discussed, *S. velum* is capable of epithelial dissolved amino acid uptake, and this species does have a small but functioning gut. In addition, the $\delta^{13}\text{C}$ signatures of different amino acids can differ markedly with respect to the starting carbon source. For example, Arginine, glycine, serine, and threonine are often more enriched in ^{13}C , while leucine, isoleucine, phenylalanine, and tryptophan are usually more ^{13}C depleted (Prahl and Muehlhausen, 1989).

The free amino acid composition of *S. velum* suggests that the amino acid taurine may serve an important physiological function not found in typical marine clams, as the taurine concentrations found in this symbiosis are equal to the total free amino acid concentrations of most marine bivalves. The relative FAA profiles of *S. velum* and *M. arenaria* are similar to those reported for other bivalves (Table 5.5 a). We can be confident that the unusually high taurine levels in *S. velum* are not an artifact of the relatively new derivitization technique, as the values we report here for *M. arenaria* compare favorably with published FAA values for this species (Table 5.5a, b). The FAA pool size of the adductor muscles of *M. arenaria* reported by Virkar and Webb (1970) and converted to wet weight values assuming a value of 80% tissue water for this species is $281 \mu\text{mol g}^{-1}$. This value is similar to the range we report for whole tissues of *M. arenaria*; however, a better comparison is between the FAA pools of the foot tissue of *M. arenaria* reported here and the adductor muscle values reported by Virkar and Webb

(1970), as predominantly muscular tissues generally have larger FAA pools (This has been found in *S. velum* and *Mya arenaria* in this study and in *Mytilus edulis* (Zurburg and DeZwann, 1981). This comparison yields values of $319 \mu\text{mol g}^{-1}$ and $281 \mu\text{mol g}^{-1}$ for foot and adductor muscles respectively (Table 5.5b). The relative amounts of FAA are also similar when FAA values for the foot tissue of *M. arenaria* are compared with published values of adductor muscle (Table 5.5a). The increased concentrations of taurine in the foot of *S. velum* could explain the higher sulfur content of the foot of this species. The ratio of foot:gill total sulfur in *S. velum* is 1.21:1. The ratio of foot:gill taurine is 1.17:1. Consequently, differences in taurine content may be partially responsible for these differences in tissue sulfur content.

5.4.3 Sources of taurine

The unusually large amounts of taurine and very negative FAA stable isotope ratios in *S. velum* raise questions as to the origin and function of this amino acid in *S. velum*. The normal pathway for taurine biosynthesis in higher vertebrates appears to be via the conversion of cysteine to taurine through the cysteine sulfonic acid-hypotaurine pathway (Pathway I, Figure 5.5 [Bishop *et al.*, 1983; Van Waarde, 1988]). This pathway may occur in *Rangia cuneata* and cephalopods. In *Mya arenaria* and *Mytilus edulis*, taurine biosynthesis may occur via decarboxylation of cysteic acid (Pathway V, Figure 5.5, See review of Bishop *et al.*, 1983). However, the ability of these organisms to synthesize taurine appears limited (Bishop *et al.*, 1983; Van Waarde, 1988). Taurine biosynthesis has not been found in fish, and in general, the rates of taurine biosynthesis found in marine bivalves are too low to result in the high amounts of taurine found in most species. In most marine bivalves it is probable that taurine is obtained in the diet, or by the action of gut flora on ingested food (See review of Bishop *et al.*, 1983). This appears to be the case for *M. arenaria* (Allen and Garrett, 1972); however, *Crassostrea gigas* has some capacity for taurine biosynthesis (Heavers and Hammon, 1985).

In *S. velum*, the large amounts of taurine may be derived from a variety of sources. The large amounts of taurine present are unlikely to be synthesized by the animal tissues, although this possibility cannot be ruled out. The taurine levels could be maintained by active absorption of dissolved taurine from surrounding seawater. Uptake of taurine has been found in *Mytilus edulis* (Wright and Secomb, 1984; 1986) which is capable of uptake at ambient taurine concentrations as low as 10 nML^{-1} . The concentration of taurine in natural waters is between 20 nM and $2 \text{ }\mu\text{M}$ (Wright and Secomb, 1986; Braven *et al.*, 1984); thus this species is equipped to take up taurine at environmental concentrations. Taurine is also lost from tissues to the surrounding seawater (Wright and Secomb, 1984; 1986); in fact in *Crassostrea gigas*, taurine regularly constitutes 5-20% of the nitrogenous compounds released in the excretory water (Hammen, 1969). Interestingly, taurine is also the major amino acid excreted by *S. velum* (Chapter 6). Consequently, active uptake may be insufficient to account for the large levels found in *S. velum*. Taurine could also be obtained from ingested food stuffs. Considering the small digestive system of *S. velum* it is unlikely that gut ingestion provides the animal with significant amounts of taurine.

The stable isotope ratios of both intact *S. velum* and the amino acid pools of this species suggest that most of the animal's energy requirements are being provided by the bacterial endosymbionts. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios of the FAA pool of *S. velum* bear a strong endosymbiont signature (-27.6 and -9.2‰ , for carbon and nitrogen respectively). These isotope ratios probably reflect those of taurine, which can account for greater than 70% of the FAA pool of *S. velum*. Thus, taurine is probably synthesized by the bacterial endosymbionts of this species. Many bacteria are capable of synthesizing and catabolizing taurine and in many animals associated bacteria are presumed to be responsible for both the synthesis and catabolism of any taurine present in the tissues (cockroaches grown axenically are incapable of taurine synthesis while species with symbiotic bacteria produce taurine, suggesting the bacteria are responsible for taurine synthesis [Block and Henry,

1961)). There is evidence to suggest that some sulfur-oxidizing chemoautotrophs are capable of taurine synthesis. Large amounts of cysteine and an HPLC peak which appeared to be taurine was found in *Thiomicrospira crunogena* (Chapter 6); perhaps the conversion of cysteine to taurine occurs in these bacteria.

5.4.4 Functions of taurine

Taurine may serve many functions in invertebrates (See Jacobsen and Smith, 1968), with its osmoregulatory role well described for marine bivalve species (See review by Somero and Bowlus, 1983 and references therein). It is generally accepted that most marine molluscs use nitrogenous solutes as the major intracellular osmotic effectors (Somero and Bowlus, 1983). In most molluscs alanine, glycine, proline, glutamic acid, aspartic acid, and taurine are the dominant solutes found and taurine appears to be particularly important as an osmotic effector (Lange, 1963; Jacobsen and Smith, 1968; Bedford, 1971; Giles, 1972; Jeffries, 1972). Strongly hydrophobic amino acids such as leucine, isoleucine, and valine are usually found in low concentrations, as are the essential amino acids, although these may be utilized during periods of starvation (Riley, 1980). In both *S. velum* and *M. arenaria* taurine present in the free amino acids probably serves an osmoregulatory purpose. However, *S. velum* would appear to have much more taurine than necessary for osmoregulation alone, suggesting additional functions for this amino acid.

Pathway I - Methionine-Cysteine-Cysteinesulfinic acid-hypotauroine-taurine
 Pathway II - Methionine-cysteine-cysteinesulfinic acid-cysteic acid-taurine
 Pathway III - Cysteamine/cystamine intermediates-hypotauroine-taurine
 Pathway IV - Sulfate-sulfite intermediates-cysteic acid-taurine
 Pathway V - Cystine-cystine disulfoxide-cystamine disulfoxide-hypotauroine-taurine
 (After Jacobsen and Smith, 1972)

Figure 5.5. Possible pathways of taurine synthesis (From Jacobsen and Smith, 1968).

Taurine may be used in energy storage by certain annelids and sipunculids (Thoai and Robin, 1965; Thoai and Roche, 1960). These animals phosphorylate the guanidino derivatives of taurine and hypotaurine. Taurine may also function to regulate the excitability of a variety of cell types (Jacobsen and Smith, 1968; Huxtable, 1978; Pasantes-Morales, 1982). In this context, taurine may affect the permeability of cardiac membranes to potassium and the shape of the action potential. In some archaeogastropods and brachiopods, pyruvic acid and taurine may undergo reductive condensation to yield tauropine (Sato *et al.*, 1985; Gade, 1986; Doumen and Ellington, 1987). There is no evidence available in the literature to suggest that any of these processes are operating in marine bivalves; however, the existence of such functions for the excess taurine of *S. velum* cannot be ruled out. In teleost fish and higher vertebrates, bile acids, the end products of cholesterol metabolism, are conjugated with taurine, resulting in the formation of bile salts (Haslewood, 1962). This also occurs in terrestrial mammals (Lehninger, 1979). Bile salts stimulate lipolysis in the gut and esterification of long-chain fatty acids in mucosal cells during absorption. Their production leads eventually to the elimination of excess cholesterol. Bile salt formation does not appear to operate in invertebrates.

One putative role for the excess taurine may be to serve as a sink for the sulfites and sulfates produced by the bacterial endosymbionts. The symbionts of *S. velum*, by analogy with free-living sulfur-oxidizing bacteria, convert reduced sulfur compounds to sulfites and sulfates. If taurine served as a sink for these metabolic end-products, sulfate excretion would not be required and the animal would be provided with an efficient osmotic effector.

Biochemical pathways do exist for the incorporation of sulfite and sulfate into taurine (Pathway IV, Figure 5.5). Although this pathway is not considered to be widespread in invertebrates, it is found in bacteria, and interestingly, the symbionts of cockroaches utilize this pathway for taurine synthesis (Jacobsen and Smith, 1968 and references therein). This pathway also operates in chicken embryos. Machlin and coworkers (1955) showed that injection of

^{35}S -sulfate into 24h old chicken embryos results in a 65% recovery of the radiolabel in the taurine of the embryo 21 days later. Conversion of ^{35}S -sulfate to taurine has also been demonstrated in young chickens and hens. Sulfate may also be incorporated to cysteine (which can then be converted to taurine) in chicken embryos (Machlin *et al.*, 1956, Martin and Patrick, 1966; Martin *et al.*, 1966). In chickens the incorporation of sulfate into taurine proceeds via conversion of sulfate to sulfite, and fixation of sulfite to a three carbon compound derived from L-cysteine (Figure 5.5), leading to the formation of cysteic acid. The enzyme catalyzing this reaction, cysteinylase, requires pyridoxin. Decarboxylation of the cysteic acid then yields taurine (Jacobsen and Smith, 1968, and references therein). Thus, it is possible that taurine may function as a sink for oxidized sulfur compounds in the *S. velum* symbiosis, or be involved in some other aspect of the internal sulfur cycle of this symbiosis.

To summarize, the THAA and protein amino acid composition of *M. arenaria* and *S. velum* differed little from each other and from the values reported for other species; 50% of the amino acids of both species were essential amino acids. Stable isotope ratios suggest that both the total hydrolyzable and free amino acids of *S. velum* appear to be synthesized directly by the bacterial endosymbionts or from bacterial metabolites. The free amino acid composition of *S. velum* was unusual. Taurine concentrations exceeded the total free amino acid concentrations of most marine bivalves suggesting that taurine may serve an additional purpose in the *S. velum* symbiosis. We suggest that taurine may function as a sink for oxidized sulfur compounds produced by the endosymbionts. This possibility is explored further in Chapter 6.

5.5 References

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CHAPTER 6

PRELIMINARY INVESTIGATIONS OF THE TAURINE-SINK
HYPOTHESIS

6.1 Abstract

The potential role of taurine as a sink for oxidized sulfur compounds produced by bacterial endosymbionts in *Solemya velum* was explored using a variety of indirect measurements. $\delta^{34}\text{S}$ ratios of the free amino acid pool of *S. velum* were very negative (-17.2‰), suggesting that the sulfur of the taurine in *S. velum* is derived from reduced sulfur sources, such as porewater sulfides. The results of experiments designed to show this directly were ambiguous. Taurine levels in *S. velum* appeared to be affected by the availability of reduced sulfur sources in the seawater medium, decreasing in the absence of a sulfur-source, although the relationship was not linear. Taurine is the only amino acid which can be measured in the excretory water of *S. velum*.

6.2 Introduction

In chapter 5 the amino acid composition of *Solemya velum* was investigated in detail and it became apparent that *S. velum* appeared to have significantly higher concentrations of the sulfur-containing amino acid, taurine ($^+_3\text{HN}-\text{CH}_2-\text{CH}_2-\text{SO}_3\text{H}$), than most marine bivalves studied to date. Taurine may serve a variety of functions in marine invertebrates. Taurine may be used in energy storage by certain annelids and sipunculids (Thoai and Robin, 1965; Thoai and Roche, 1960); taurine may function to regulate the excitability of a variety of cell types (Jacobsen and Smith, 1968; Huxtable, 1978; Pasantes-Morales, 1982); in some archaeogastropods and brachiopods, pyruvic acid and taurine may undergo reductive condensation to yield tauropine (Sato *et al.*, 1985; Gade, 1986; Doumen and Ellington, 1987).

There is no evidence available in the literature to suggest that any of these processes are operating in marine bivalves. In teleost fish and higher vertebrates, bile acids, the end products of cholesterol metabolism, are conjugated with taurine, resulting in the formation of bile salts (Haslewood, 1962). Bile salts stimulate lipolysis in the gut and esterification of long-chain fatty acids in mucosal cells during absorption. Their production leads eventually to the elimination of excess cholesterol. Bile salt formation does not appear to operate in invertebrates.

A putative role for the excess taurine in *S. velum* may be to serve as a sink for the sulfites and sulfates produced by the bacterial endosymbionts (see chapter 5). The symbionts of *S. velum*, by analogy with free-living sulfur-oxidizing bacteria, are thought to convert reduced sulfur compounds to sulfites and sulfates. If taurine serves as a sink for these metabolic end-products, sulfate excretion requirements would be reduced and the animal would be provided with an efficient osmotic effector.

Biochemical pathways do exist for the incorporation of sulfite and sulfate into taurine (Chapter 5, Figure 5.5). Although this pathway is not considered to be widespread in invertebrates, it is found in bacteria, and interestingly, the symbionts of cockroaches

utilize this pathway for taurine synthesis (Jacobsen and Smith, 1968 and references therein). This pathway also operates in chicken embryos. Sulfate may also be incorporated to cysteine (which can then be converted to taurine) in chicken embryos (Machlin *et al.*, 1956, Martin and Patrick, 1966; Martin *et al.*, 1966).

In this chapter the possibility that taurine may serve as a sink for oxidized sulfur compounds was explored in a variety of experiments. In order to investigate the taurine-sink hypothesis directly, it would be necessary to trace sulfur molecules from an external reduced sulfur source, directly into sulfite or sulfate and then taurine. This type of study was beyond the scope of this thesis as the potential pathways and possible sulfur intermediates that might be involved are not known. Thus, in this chapter, a variety of experiments were conducted in order to test the hypothesis that taurine may function as a sink for oxidized sulfur compounds in *S. velum*.

6.3 Materials and methods.

Experiments were designed to test the following sub-hypotheses:

- 1) The sulfur molecules in the taurine of *S. velum* are ultimately derived from external reduced sulfur sources.

If taurine serves as a sink for oxidized sulfur compounds produced by the endosymbiotic bacteria, then the sulfur molecules in the taurine of *S. velum* would be derived from external reduced sulfides. Both stable isotope ratios and radioisotopes were used to determine whether the sulfur molecules of the taurine in *S. velum* are ultimately derived from external reduced sulfides.

$\delta^{34}\text{S}$ ratios

The $\delta^{34}\text{S}$ ratios of *S. velum* (Chapter 3) are extremely negative ($\sim -30\text{‰}$) and similar to the values reported in the literature for porewater sulfides (~ -15 to -70‰). The very high concentrations of

taurine in *S. velum* suggest that large amounts of the sulfur in this species is tied up in taurine, consequently it is likely that the sulfur of the taurine of *S. velum* is also derived from porewater sulfides. If this is the case, one would expect the $\delta^{34}\text{S}$ ratios of the taurine in *S. velum* to be very negative. In order to examine the $\delta^{34}\text{S}$ ratios of the taurine in *S. velum*, the free amino acids of five specimens of *S. velum* were extracted as described in chapter 2, and combined. The combined extracts were eluted through a minicolumn containing activated copper in order to remove any elemental sulfur present. Reagents were evaporated under nitrogen, and the $\delta^{34}\text{S}$ ratio of the sample was determined as outlined in Chapter 2. Although taurine was not separated from the other free amino acids, taurine is the only sulfur-containing amino acid to occur in measurable concentrations (see Chapter 5), and accounts for approximately 70% of the total free amino acid pool, so we can assume that any sulfur in the sample is localized in the taurine.

^{35}S uptake

The incorporation of radiolabelled reduced sulfides from the external seawater medium into the taurine of *S. velum* was investigated in order to verify the results of the $\delta^{34}\text{S}$ measurements and demonstrate a potential link between bacterial sulfide-oxidation and taurine in *S. velum*. Ideally, $\text{Na}_2^{35}\text{S}_2\text{O}_3$ would have been the radiolabelled sulfur source of choice as it does not rapidly oxidize under aerobic conditions; however, this was unobtainable, so Na_2^{35}S was used instead. The major problem with using Na_2^{35}S is that this compound will rapidly oxidize chemically to S^0 , SO_3^{2-} , and $\text{S}_2\text{O}_3^{2-}$, thus constant sulfide concentrations are impossible to obtain, and uptake of radiolabelled external oxidized sulfur compounds may occur, obscuring any incorporation of reduced sulfides into taurine. In fact, preliminary experiments using the Cline technique (Cline, 1969) of H_2S determination demonstrated that Na_2S has a half-life of approximately 2 hours in the absence of animals, and approximately 1-1.5 hours in the presence of *S. velum* using similar animal concentrations to those anticipated for the experiment. Despite these drawbacks, the experiment was conducted as follows.

Twenty one specimens of *S. velum*, and 17 specimens of the control bivalve *Mya arenaria*, were incubated for 3 hours in 500 ml 0.22 μm filtered seawater containing Na_2^{35}S and Na_2^{32}S at a final concentration of 0.1 mM and specific activity of 2.0×10^6 dpm/ml. After the three hour pulse, three specimens of each species were separated from the shell, rinsed, weighed, and placed in scintillation vials containing 4 ml of the free amino acid (FAA) extraction medium (5% TCA in $\text{EtOH:H}_2\text{O} = 50:50$). The remaining animals were placed in clean, 0.22 μm filtered seawater, and unradiolabelled Na_2S added to a final concentration of 0.1 mM. Three, six, and twelve hours after the pulse period, three specimens of each species were removed, weighed and placed in 4 ml of the FAA extraction medium. The seawater was changed and new Na_2S added to a final concentration of 0.1 mM every three hours. Fifteen hours after the experiment was started, the animals were placed in unradiolabelled seawater containing 0.1 mM thiosulfate, the seawater was exchanged and 3 animals were removed and extracted every twelve hours until 48 h had elapsed since the end of the pulse period.

The FAAs of each species were extracted for 36 h. The TCA extract was filtered through 0.22 μm syringe filters into scintillation vials, and Scintiverse LC scintillation cocktail added. The remaining animal tissue was digested in 4 ml of Protosol at 60°C for 24-36 h. The Protosol was neutralized with 6N HCL, the tissue digest was bleached with 30% hydrogen peroxide, and Scintiverse LC was added. Radioactivity was measured with a United Technologies Packard Minaxi β Tri-carb 4000 series scintillation counter.

2) In the absence of an external sulfur source, taurine levels will be reduced, in the presence of a reduced sulfur source, taurine levels should increase or taurine excretion should increase.

If taurine is involved in the sulfur-oxidation cycle in *S. velum*, there should be a link between bacterial sulfur oxidation and taurine levels. In order to test this hypothesis, specimens of *S. velum* were incubated for 24 h in seawater containing varying

amounts of sodium thiosulfate as follows. Specimens of *S. velum* were collected and divided into 5 groups of 3 animals each. The FAAs of one group were immediately extracted and pooled. The animals from the other groups were incubated for 24 h in either 0.00 mM, 0.05 mM, 0.5 mM, or 5.0 mM sodium thiosulfate. At the end of the incubation period, animals from each treatment were removed and pooled, soft tissue weight was determined and FAAs were extracted. The FAA composition of all the samples was determined as outlined in Chapter 2.

3) If taurine serves as a sink for oxidized sulfur sources, the animal should be able to excrete taurine.

Taurine is unlikely to serve as a sink for oxidized sulfur compounds in the *S. velum* symbiosis if taurine could not be excreted. To test this hypothesis, 20 ml samples of seawater were removed at the start of the experiments just outlined in sub-hypothesis 2, and again at 12 h and 24 h. Control treatments containing seawater, sodium thiosulfate but no animals were also sampled concurrently. GABA and norleucine were added to the seawater aliquots to serve as internal standards and the samples were frozen until analysis. The FAA and ammonium concentrations of the seawater samples were then determined as outlined in Chapter 3.

6.4 Results and discussion

1) The sulfur molecules in the taurine of *S. velum* are ultimately derived from external reduced sulfur sources

$\delta^{34}\text{S}$ ratios

$\delta^{34}\text{S}$ ratios for the FAAs of *S. velum* were extremely negative (-17.2‰). The $\delta^{34}\text{S}$ ratios for intact *S. velum* specimens range from -26.7‰ to -31.1‰ (Chapter 3). Dissimilatory sulfur reduction in marine sediments occurs with a large isotope fractionation effect (-30 to -70‰, Goldhaber and Kaplan, 1975; Fry *et al.*, 1986) and results in sediment sulfides characterized by very negative sulfur isotope ratios (-15 to -30‰). The sulfur isotope ratios resulting from bacterial sulfur reduction are quite distinct from those of seawater sulfate which has $\delta^{34}\text{S}$ ratios of $\sim +20$ ‰. The $\delta^{34}\text{S}$ ratios in the reducing sediments characteristic of *S. velum* habitats could be in the -15 to -30‰ range, and the low $\delta^{34}\text{S}$ ratios in the tissues and FAAs of *S. velum* are probably due to utilization of these external sulfides by the endosymbionts and subsequent translocation of sulfur-containing compounds to the host.

Taurine is the predominant sulfur-containing FAA in *S. velum* (Chapter 5), accounting for 63-73% of the total FAA pool, and is the only sulfur-containing amino acid in the FAA pool (apart from trace amounts of cysteine and methionine). Thus, the low $\delta^{34}\text{S}$ ratios of the FAA pool almost certainly reflect the sulfur isotope values of the taurine of *S. velum*. If this is the case, it suggests incorporation of external reduced pore-water sulfides into the taurine of the symbiosis. Reduced pore water sulfides may serve as an energy source for the endosymbiotic bacteria, and are probably oxidized into sulfite and sulfate. Thus it is conceivable that taurine is involved in the internal sulfur cycling of the symbiosis. Interestingly, high taurine levels have also been noted in *Solemya borealis*, a newly identified animal-bacteria symbiosis (Chapter 7), suggesting that unusual roles for taurine are also found in this species.

The difference in $\delta^{34}\text{S}$ ratios between taurine and the whole tissues of *S. velum* is intriguing and may be the result of internal isotope fractionation events. In *S. reidi*, $\delta^{34}\text{S}$ ratios of the foot tissue (-30.4‰) are considerably more negative than corresponding values for the symbiont-containing gills (-21.7‰; Vetter *et al.*, in prep.). $\delta^{34}\text{S}$ measurements of the free sulfides in the sediments where *S. reidi* is found are -17.7 to -19.4‰ (Vetter *et al.*, in prep.). Thus, the values for the gills of *S. reidi* are close to those of the porewater sulfides where this species lives. In order for the foot tissue to have even lower $\delta^{34}\text{S}$ ratios, an additional unknown fractionation must occur during internal sulfur oxidation by the symbiosis. Perhaps a similar internal fractionation takes place in *S. velum*, and the $\delta^{34}\text{S}$ ratios of the taurine are similar to porewater values, but $\delta^{34}\text{S}$ ratios of the whole tissues differ as isotope fractionation occurs during further assimilatory sulfur pathways in the animal tissues.

^{35}S incorporation

Radiolabel incorporation into the FAA pool and remaining tissue fractions of each species is outlined in Figures 6.1 and 6.2 respectively. It can be seen that in both tissue fractions and in both species, there was a high initial incorporation of radiolabel, followed by a gradual decrease in radioactivity during the chase period. In *S. velum*, dpm/mg tissue extracted had levelled off by about 12 h post-pulse in both tissue fractions; however, in *M. arenaria*, ^{35}S had only begun to reach low levels by about 36 h post-pulse. It is also interesting to notice that the variability in radiolabel incorporation was extremely high in *M. arenaria*, often exceeding $\pm 100\%$, while in *S. velum*, variability was almost negligible by 12 h after the pulse. In addition, in both the radiolabel fractions of *S. velum*, initial dpm/mg was higher than in *M. arenaria*, and was followed by a rapid decrease in radioactivity until 12 h post-pulse when relatively steady levels were obtained. In *M. arenaria*, dpm/mg was variable, and gradually decreased, although steady levels had not been attained by 36 hours after the pulse.

These results are difficult to interpret, as a variety of forms of ^{35}S would have been present in the pulse medium, as Na_2^{35}S would

rapidly oxidize to $^{35}\text{S}^0$, $^{35}\text{SO}_3^{2-}$, $^{35}\text{S}_2\text{O}_3$, and $^{35}\text{SO}_4^{2-}$. In addition, the FAA extraction medium would also remove most of the contaminating radiolabel adhering to the outside of the animal, and may extract dissolved sulfates and sulfites in the animal tissues. The initial high levels of radiolabel found in the extraction medium probably reflects external contamination with radiolabelled seawater. The high amounts of radiolabel taken up into both the FAA pool and tissue residue of *S. velum* and *M. arenaria*, the non-symbiont control, may be the result of sulfate, sulfite, or hydrogen sulfide uptake. The results for *M. arenaria* demonstrate that radiolabel incorporated by *S. velum* may have little to do with endosymbiont metabolic processes. It is also possible that *M. arenaria* is capable of H_2S uptake. This species inhabits the same sediments as *S. velum* and would encounter H_2S *in situ*. Sulfide uptake and detoxification mechanisms may operate in this species. Many marine animals appear to thrive in sulfide-rich habitats, and in some species, unusual heme compounds called hematins are involved in *in vivo* sulfide oxidation processes (Powell and Arp, 1989).

Overall, the results of this experiment are ambiguous, and neither support nor disprove the taurine-sink hypothesis.

Figure 6.1 Radiolabel incorporation into the FAA of the animal. (a) Total radiolabel incorporated into the FAA of the animal. (b) Radiolabel incorporated into the FAA of the animal. The pool would also include surface contamination and dissolved sulfur species.

Figure 6.1 Radiolabel incorporation into the FAA of the animal tissues of a) *Solemya velum* and b) *Mya arenaria* (mean of three animals $\pm$ s.d.). This pool would also include surface contamination and dissolved sulfur species.

Figure 6.2 Radiolabel incorporation into the remainder of the animal tissues of a) *Solemya velum* and b) *Mya arenaria* (mean of three animals $\pm$ s.d.).

2) In the absence of an external sulfur source, taurine levels will be reduced, in the presence of a reduced sulfur source, taurine levels should increase or taurine excretion should increase.

Although this experiment is also preliminary in nature, there does appear to be an effect of sulfur-availability on the taurine levels in *S. velum*, although the effect is by no means clear cut. Taurine levels expressed as a percentage of the total amino acids present dropped from 69% to 61% in animals incubated in the absence of thiosulfate, and increased to 77% in animals provided with 0.05 mM thiosulfate (Figure 6.3). The taurine levels in the higher thiosulfate treatments increased by only a small extent after 24 h (71.5% and 72.6% for the 0.5 and 5.0 mM treatments respectively). However, when the absolute levels of taurine are examined (Figure 6.4) the results are ambiguous. In all the treatments absolute taurine levels declined, although the smallest drop was exhibited by the animals incubated in 0.05 mM thiosulfate, and the largest drop in animals incubated without any reduced sulfur source (Figure 6.4).

The similarity between the absolute amounts at the start of the experiment, and the taurine concentrations of animals incubated in 0.05 mM thiosulfate may indicate that this concentration is most similar to the field sulfide levels, or that this concentration is closer to the optimum reduced sulfur concentration for this symbiosis. In *S. reidi*, the maximum autotrophic balance with respect to CO₂ occurs in ambient sulfide concentrations of ~ 0.1 mM/L (Anderson *et al.*, 1987; Table 6.1). CO₂ uptake declines above and below this concentration (Table 6.1). In addition, gill ciliary activity is maintained after 30 min in 100 µM sulfide concentrations, but declines before 20 min have elapsed in 0.5 mM sulfide concentrations in *S. reidi* (Anderson *et al.*, 1987). In *S. velum*, both absolute and relative levels of taurine were greatest in the 0.05 mM thiosulfate treatments after 24 h, suggesting that reduced sulfur levels in this range are probably optimal for *S. velum*, and should be

used in future experiments with this species. This conclusion is supported when the ammonium excretion rates of *S. velum* in the different treatments are calculated (see later discussion).

The results of this experiment suggest that the hypothesis that taurine might serve as a sink for oxidized sulfur compounds cannot be rejected; the large drop in both the absolute and relative taurine concentrations of animals incubated without thiosulfate does suggest a link between sulfur oxidation and taurine concentrations.

However, from this experiment we cannot tell whether taurine is directly involved in the sulfur cycle, or whether the drop in taurine levels is the result of changes in the overall metabolic status of the symbiosis. After all, the rates of endosymbiont CO_2 fixation in *S. velum* are reduced in the absence of an external energy source (Cavanaugh, 1983, Table 6.1) consequently, it is conceivable that the reduction in the food supply to the symbiosis results in altered taurine levels.

Figure 6.3 Taurine concentration (expressed as a molar percent of the amino acids present) for pooled *Solemya velum* incubated in seawater containing different amounts of a reduced sulfur source.

Figure 6.4 Taurine concentration (expressed as $\mu\text{mol taurine g wet wt}^{-1}$ of animal) for *Solemya velum* incubated in seawater containing different amounts of a reduced sulfur source.

Table 6.1 Metabolite fluxes in *Solemya reidi* (From Anderson *et al.*, 1987)

Sulfide concentration (mM)	CO ₂ Flux*	O ₂ Flux*	Sulfide Flux*
0.0	+2.17	-1.97	
0.05-0.08	-0.22	-2.99	-1.5
0.1	-0.89	-4.3	-3.1
0.25	+0.35	-3.2	-2.8
0.5	+1.84	-2.6	-2.1

* $\mu\text{mol g}^{-1} \text{ wet wt./h}$

3) If taurine serves as a sink for oxidized sulfur sources, the animal must be able to excrete taurine.

Taurine would only be useful as a storage form, or sink, for oxidized sulfur compounds if it can ultimately be excreted. From the results of the taurine excretion analyses (Figure 6.5), it can be seen that *S. velum* does appear to excrete taurine, in fact this was the only measurable net amino acid excreted in these experiments.

Figure 6.5 Taurine excretion by *Solemya velum* incubated in seawater containing differing thiosulfate concentrations.

Again, there appeared to be no strict correlation between sulfur concentration and taurine excreted. The results do agree with those of Figure 6.3, however, as the animals incubated in seawater

containing 0.00 mM thiosulfate excreted the highest amounts of taurine. The taurine levels of these animals consequently dropped dramatically. The next highest excretion rates were found in the 0.05 mM thiosulfate treatments, while higher thiosulfate concentrations appeared to result in only low levels of taurine excretion.

It is interesting to examine the ammonium excretion rates of *S. velum* during these experiments (Figure 6.6). The highest excretion rates were found in animals incubated in 0.05 mM thiosulfate, further suggesting that this was the optimum thiosulfate concentration used in these experiments, and animal metabolic activities were greatest in this treatment.

Figure 6.6 Ammonia excretion by *Solemya velum* incubated in seawater containing differing thiosulfate concentrations (determined by subtracting blank control values from levels found in the experimental vials to counteract the effects of ammonia oxidation on ammonium levels in the experimental vials).

To summarize, the results of these preliminary experiments were tantalizing but difficult to interpret. It may just be a coincidence that high levels of taurine are found in Solemyid bivalves with sulfur-oxidizing symbionts. Yet, the the FAA $\delta^{34}\text{S}$ ratios suggest that the sulfur from external porewater sulfides may ultimately end up in taurine molecules, although this could not be shown experimentally. In addition, external reduced sulfur concentrations may affect internal taurine levels. However, demonstrating a direct link between sulfide oxidation and taurine synthesis is difficult. The use of radiolabelled thiosulfate in tracer experiments would be preferable to hydrogen sulfide, as it does not oxidize chemically in seawater. A detailed biochemical analyses for potential intermediates in the pathway may demonstrate a link between sulfur oxidation and taurine synthesis, yet it would be inappropriate to begin these types of studies until there is more evidence to suggest that a link even exists.

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CHAPTER 7

A COMPARATIVE STUDY OF THE BIOCHEMICAL
COMPOSITION OF TWO ADDITIONAL ANIMAL-BACTERIA
SYMBIOSES, *SOLEMYA BOREALIS* AND *INANIDRILUS*
LEUKODERMATUS, AND *THIOMICROSPIRA CRUNOGENA*, A
SPECIES OF SULFUR-OXIDIZING BACTERIUM.

7.1 Abstract

In this chapter, additional invertebrate-chemoautotroph symbioses were examined in order to determine whether the biochemical markers used in the study of *Solemya velum* were appropriate for the study of other species. Stable isotope ratios, lipid, and amino acid composition was analyzed in two additional animal-bacteria symbiosis, *Solemya borealis* and *Inanidrilus leukodermatus*, and compared to *S. velum* and the sulfur-oxidizing bacterium, *Thiomicrospira crunogena*. The carbon and nitrogen stable isotope ratios of all the species examined were similar and very negative ($\delta^{13}\text{C} = -26\text{‰}$ to -34.6‰ , $\delta^{15}\text{N} = 0.4$ to -10.1‰). These low stable isotope ratios suggest that the diets of each of these symbioses are based on bacterial chemoautotrophy, and it is likely that large amounts of the carbon and nitrogen pools of each host species are derived from the bacterial endosymbionts. The amino acid composition of *S. borealis* was very similar to that of *S. velum*, despite differences in the site and season of collection. High concentrations of taurine were found in *S. borealis*. This suggests that similar metabolic pathways are probably utilized in each solemyid. The amino acids of *I. leukodermatus* were typical of marine invertebrates. All the symbioses examined contained large amounts of the essential amino acids. The bacterium *Thiomicrospira crunogena* contained all the physiological amino acids and appears to be capable of essential amino acid synthesis *de novo*.

The fatty acids of both *S. borealis* and *I. leukodermatus* were similar to those of *S. velum* and characterized by high concentrations of *cis*-vaccenic acid. This lipid is presumed to be bacterial in origin as it is common in many Gram-negative bacteria, and is probably utilized by the animal host for nutritional purposes. Thin layer and gas chromatography of the fatty acid composition of the lipid classes of *S. borealis* demonstrates utilization of *cis*-vaccenic acid in all the lipid classes of this species, particularly the neutral lipid pools. Both *S. borealis* and *I. leukodermatus* had very low concentrations of the polyunsaturated fatty acids characteristic of most marine organisms. *Cis*-vaccenic acid would appear to be an important biomarker for the presence of bacterial endosymbionts in animal-bacteria symbioses.

7.2 Introduction

In the preceding chapters the protobranch clam, *Solemya velum*, has been used as a general model of invertebrate-chemoautotrophic bacteria symbioses. The aim of this research has been to elucidate endosymbiont "markers" in *S. velum* which may be used in studies of other symbioses where only a few specimens are available. In this context, particular attention has been placed on the study of chemical parameters, such as stable isotope ratios and lipid composition, which can be measured in preserved specimens. Yet we do not know whether this bivalve is representative of other animal-bacteria symbioses, particularly species from other phyla. Will the biochemical "markers" found in *S. velum* be useful in the study of other symbioses?

In this chapter, stable isotope ratios, lipid composition, and amino acid ratios have been measured in an additional known animal-bacteria symbioses, *Inanidrilus* (= *Phallodrilus*) *leukodermatus* (which is mouthless and gutless), collected from calcareous sediments in Bermuda (Appendix II). In addition, the same parameters were measured in a new putative symbiosis - *Solemya borealis*, collected from Buzzards Bay, MA (Appendix I). A species of sulfur-oxidizing bacterium, *Thiomicrospira crunogena* cultured from samples obtained by Holger Jannasch at a hydrothermal vent near the East Pacific Rise has also been examined.

Comparative biochemical studies of this type allow basic similarities between different animal-bacteria symbioses to be determined. In addition, it may be possible to identify whether the endosymbiont "markers" found in *S. velum* are ubiquitous phenomena in animal-bacteria symbioses. The methods used in these analyses are identical to those used in the previous chapters, thus the results obtained are ideal for comparative purposes. Materials and methods are outlined in Chapter 2.

7.3 Results and discussion

The results of all the species examined are outlined below. Because the results for the symbioses and bacteria were very similar, the data are only outlined and discussed in detail for the first species examined, in order to avoid excessive repetition.

7.3.1 Stable isotope ratios.

The stable isotope ratios for all the symbioses studied (including *S. velum* values from Chapter 3), are outlined in table 7.1, along with comparative values for symbiont-free animals (*Mya arenaria* and *Tellina agilis*, Chapter 3).

Table 7.1 Stable isotope ratios of *Solemya borealis*, *Inanidrilus leukodermatus*, and *Thiomicrospira crunogena* in comparasion with *Solemya velum* and representative symbiont-free bivalves.

Species	$\delta^{13}\text{C}$ ratios	$\delta^{15}\text{N}$ ratios
<i>Solemya velum</i> (gill)	-32.4 to -33.9‰	+0.4 to -9.8‰
<i>Solemya velum</i> (foot)	-30.9 to -32.1‰	+4.4 to -4.6‰
<i>Solemya velum</i> "bacteria"	-32.3 to -33.6	-7.9 to -8.6
<i>Solemya borealis</i> (gill)	-32.3 to -33.6‰	-7.9 to -8.6‰
<i>Solemya borealis</i> (foot)	-32‰	-8.9‰
<i>Inanidrilus leukodermatus</i>	-26‰	-2.1‰
<i>Thiomicrospira crunogena</i>	-27.3‰	-10‰
<i>Mya arenaria</i>	-17.2 to -17.8‰	+8.3 to +8.5‰
<i>Tellina agilis</i>	-14.2 to -15.6‰	+6.3 to +8.2‰

$\delta^{13}\text{C}$ ratios

The theory behind the use of isotope ratios has been discussed extensively in Chapter 3, and will not be treated here in any detail. To summarize, stable isotope ratios can be used to identify trophic pathways and processes as a few well characterized reactions are responsible for the stable isotope compositions of most organic matter (Fry and Sherr, 1984; Rounick and Winterbourn, 1986; Spiro

et al., 1986). The isotope signatures resulting from these "root" reactions are generally maintained during food-web processes with relatively little change. Thus the stable isotope ratios of an organism can sometimes provide information regarding the 'root' reaction responsible for its formation. This is important in the study of animal-bacteria symbioses, as the fixation of carbon during chemoautotrophy often results in low bacterial $\delta^{13}\text{C}$ ratios. Thus, animals utilizing bacterial carbon will have lower $\delta^{13}\text{C}$ ratios than organisms utilizing phototrophically-fixed carbon sources.

The carbon stable isotope ratios of all the organisms studied are similar and very negative (-26‰ to -34.6‰; Table 7.1). Fixation of carbon by chemoautotrophic organisms may result in larger isotope fractionation effects than those which occur during photosynthetic carbon fixation (Ruby *et al.*, 1987). The low $\delta^{13}\text{C}$ ratios found in all the symbioses examined here, and both types of bacteria (*T. crunogena* and enriched *S. velum* endosymbiont fraction) suggest a chemoautotrophic source for the carbon of each species. This is particularly true for the *S. borealis* specimen, which had the most negative $\delta^{13}\text{C}$ ratios. The extremely negative $\delta^{13}\text{C}$ ratios of *S. borealis*, coupled with the morphological similarities this species shares with *S. velum* (very reduced digestive system, large gills) suggest that this species has bacterial chemoautotrophic symbionts and that the bacterial contribution to host nutrition in *S. borealis* is large. The results of the stable isotope studies are supported by the lipid composition of this species, see later discussion, and by electron micrographs of gill tissue (Appendix I). If the endosymbionts of *S. borealis* have stable isotope ratios similar to the endosymbionts of *S. velum* (Table 7.1), then almost all of the organism's carbon and nitrogen could be derived from the symbionts (Figure 7.1).

Figure 7.1 Bacterial contribution to a) the carbon and b) the nitrogen nutrition in *Solemya borealis* and *Inanidrilus leukodermtus* calculated from the two-source mixing model described in Chapter 3. $\Delta\delta$ values denote isotopic differences between the foot tissue of the control bivalves *Mya arenaria* and *Tellina agilis* (■, bacterial contribution assumed to be = 0%; Chapter 3), the bacterial pellet of *S. velum* (*, bacterial contribution = 100%), and animal foot tissue (whole tissue in the case of *I. leukodermtus*). Although the end-member values are speculative, the results are probably similar to those that would be calculated if the endosymbionts of each species were separated and measured.

a) $\delta^{13}\text{C}$ b) $\delta^{15}\text{N}$

The $\delta^{13}\text{C}$ ratios of *I. leukodermatus* and *T. crunogena* were the most positive (most ^{13}C enriched) found in this study, yet there is still considerable evidence to suggest that a large proportion of the carbon for both species is derived from chemosynthesis. In the case of *T. crunogena*, the bacteria were cultured in the laboratory in the absence of any organic carbon compounds, so any organic carbon molecules must have been synthesized *de novo* by the bacteria from external inorganic carbon sources. The bacteria were cultured in 4 l flasks with approximately 0.3 l of head space at the top for air-water gas exchange, so it is possible that the bacteria may have been in conditions of CO_2 limitation towards the end of the incubation period. Limitation of inorganic carbon sources will result in reduced isotope discrimination effects. Alternatively, the differences between $\delta^{13}\text{C}$ ratios of *T. crunogena* and the animal-bacteria symbioses may just be the result of differences in enzyme pathways or in the $\delta^{13}\text{C}$ ratios of the carbon being provided to the bacteria. For example, the free-living *T. crunogena* will be taking up inorganic carbon directly from seawater, while endosymbionts will be utilizing inorganic carbon that has been taken up, and perhaps respired, by the animal host, and ultimately passed on to the symbionts. This may involve a greater number of enzymatic steps, and could affect the $\delta^{13}\text{C}$ ratios of the final fixed-carbon.

In the case of *I. leukodermatus*, there is considerable evidence to suggest chemoautotrophic carbon fixation is occurring. RuBPCase activity is found in this species, and Gram-negative bacteria are found in the sub-cuticular spaces of the host (Giere, 1985). In this organism the more positive $\delta^{13}\text{C}$ ratios may be due to 1) the ingestion of exogenous organic carbon with more positive isotope ratios; although the animal is entirely gutless, uptake of DOM could occur; 2) differences in CO_2 fixation enzymatic pathways; 3) differences in the $\delta^{13}\text{C}$ ratios of the starting inorganic carbon source. *I. leukodermatus* has been shown to utilize dissolved sugars and amino acids in laboratory studies (Giere, *et al.*, 1984), and it has been suggested that in the overall metabolism of the worms, "the bacterial contribution is held to be of subordinate rank" (Giere *et al.*, 1984). Consequently, intake of exogenous organic matter with more

positive $\delta^{13}\text{C}$ ratios may account for the isotope ratios found in this annelid. However, enzyme studies demonstrate the potential for chemoautotrophy in *I. leukodermatus*, and the $\delta^{13}\text{C}$ ratios of this species suggest the utilization of some chemoautotrophically-fixed carbon. If it is assumed that the isotope ratios of the "bacterial" fraction of *S. velum* (-32.3 to -33.6‰), are representative of the values for the symbionts of *I. leukodermatus*, and that exogenous carbon will have $\delta^{13}\text{C}$ ratios of ~-18‰ (See Figure 3.2, Chapter 3), the animal values of -26‰ would represent a symbiont nutritional contribution of about 50% (Figure 7.1). Obviously, these values are speculative at best, but a symbiont carbon contribution of ~ 50% would fit in well with the known biology of this species (Giere, 1981; Giere *et al.*, 1984). One problem with trying to determine the bacterial contribution to the nutrition of *I. leukodermatus* is that we are unable to sample tissue which does not contain endosymbionts (such as the foot tissue of the bivalves), as the bacteria are found throughout the sub-cuticular layers of the animal. Thus, any tissue we measure will also include bacterial biomass, making the two source mixing model difficult to apply. Giere *et al.* (Appendix II) suggest that the endosymbionts of *I. leukodermatus* account for about 25% of the organism by volume. If these endosymbionts have a $\delta^{13}\text{C}$ ratio of ~-33‰, as in the case of the *S. velum* symbionts, then in order for the intact symbiosis to have a $\delta^{13}\text{C}$ ratio of -26‰, the "pure" animal tissue of *I. leukodermatus* must have a value of ~-23 to -24‰. If we apply the two source mixing model using this value we estimate a bacterial contribution of ~40% for this annelid (Figure 7.1). This is probably closer to the actual value for this species.

$\delta^{15}\text{N}$ ratios

The nitrogen isotope ratios of all the species examined are also unusual, and apart from some of the values for *S. velum* foot tissues discussed in Chapter 3, all the nitrogen isotope ratios are <0‰. This suggests the utilization of 1) a large non-limiting N source by the bacteria and symbioses; 2) a nitrogen source with unusually low $\delta^{15}\text{N}$ ratios; or 3) the presence of N_2 fixation pathways in the symbioses. At this point it is not possible to rule out possibilities 1)

or 3); however, it is quite likely that the initial N source does not have unusually low $\delta^{15}\text{N}$ ratios as sedimentary nitrogen usually has isotope ratios similar to sea water nitrate and ammonium which are usually $>0\text{‰}$. If the isotope ratios of the symbioses are due to nitrogen fixation or utilization of non-limiting nitrogen sources, such as pore-water ammonium, then the nitrogen is probably initially utilized or fixed by the endosymbionts. Nitrogen is then probably passed to the animal component of the symbiosis by translocation mechanisms or bacterial death.

If it is again assumed that the isotope ratios of the *S. velum* "bacteria" are representative of the endosymbionts of *S. borealis* and *I. leukodermatus*, then it would appear that almost all of the nitrogen of *S. borealis* is symbiont-derived, while only about 40-50% of the nitrogen in *I. leukodermatus* is symbiont-derived (Figure 7.1). These values although speculative are consistent with the biology of both animal host species.

7.3.2 Amino acid compositions

Solemya borealis

The total hydrolyzable amino acid, protein-bound amino acid, and free amino acid composition of *S. borealis* very closely resembled that of *S. velum* (Tables 7.2, Figures 7.2, 7.3, 7.4). The essential amino acids (EAAs) are present in the THAA of *S. borealis* and account for 26-40% of the THAA and about 40% of the protein-bound amino acids of this species. It is likely that a large proportion of these EAAs are synthesized *de novo* by the symbiosis, either directly by the bacteria, or using carbon and nitrogen derived from the bacteria. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios of *S. borealis* tissues are very negative, suggesting a symbiont source, and if it is assumed that *S. borealis* has a similar protein content to that of *S. velum* (~60-70%) then a large proportion of the carbon and especially the nitrogen of *S. borealis* is contained in EAAs and probably has very negative stable isotope ratios.

There appeared to be extremely large amounts of taurine in the THAA fraction of the foot of *S. borealis*. Because we only had the opportunity to examine one specimen, it is not possible to tell

whether this is typical or a feature of this one animal. Apart from the differences in the amount of taurine between the gill and foot of this specimens, THAA profiles are very similar to those of *S. velum*. This is also the case when the free amino acids (FAA) of *S. borealis* are examined.

Taurine accounted for most of the FAA present (66-71%, Table 7.3) and occurred in very high concentrations (100-200 $\mu\text{mol/g}$ wet wt.), although concentrations did not appear to be as high as in *S. velum* (Chapter 5, Table 5.5b). Again, because only one specimen could be examined it is difficult to conclude much about *S. borealis* in general, particularly when it comes to the absolute amounts of amino acids, as these can vary considerably between individuals. However, the close similarities between the amino acids of *S. borealis* and *S. velum* suggest the metabolic processes operating in the two cogenetic species are similar, particularly with respect to taurine metabolism. It is interesting to note that in both *S. borealis* and *S. velum* the foot tissue contains the highest levels of taurine of the tissues examined.

Table 7.2 Total hydrolyzable amino acid of *Solemya borealis*.

Amino Acid	Foot		Gill	
	$\mu\text{mol/g wet wt.}$	% of total	$\mu\text{mol/g wet wt.}$	% of total
Asp	38	2.8	71	5.8
Glu	110	8.2	129	10.6
Oh-P	5	0.4	8	0.7
Asn*	0	0.0	0	0.0
Ser#	18	1.3	16	1.3
Gln*	0	0.0	0	0.0
Gly	214	15.9	118	9.8
His	12	0.9	28	2.3
Tau	398	29.5	177	14.6
Arg	23	1.7	61	5.0
Thr#	48	3.6	39	3.2
Ala	183	13.6	140	11.5
Pro	18	1.3	49	4.1
Tyr	1	0.1	3	0.2
Val	103	7.6	99	8.2
Met	0	0.0	9	0.7
Cys	8	0.6	11	0.9
Ile	46	3.4	69	5.7
Leu	66	4.9	108	8.9
Phe	4	0.3	43	3.5
Trp#	0	0.0	0	0.0
Om	0	0.0	0	0.0
Lys	53	3.9	34	2.8
% Essential AA		26.3		40.5
Total	1348		1212	

* Converted to asp and glu during hydrolysis

Yields of these amino acids are reduced during hydrolysis

Figure 7.2 Total hydrolyzable amino acid composition of a) *Solemya borealis* in comparison with b) *S. velum* (Data presented as molar percentages).

Table 7.3 Protein-bound amino acid composition of *Solemya borealis*.^o

Amino Acid	Foot		Gill	
	$\mu\text{mol/g wet wt.}$	% of total	$\mu\text{mol/g wet wt.}$	% of total
Asp	30	3.5	69	5.7
Glu	103	11.7	116	9.6
Oh-P	4	0.5	8	0.6
Asn*	0	0.0	0	0.0
Ser#	16	1.9	14	1.2
Gln*	0	0.0	0	0.0
Gly	213	24.3	117	9.6
His	10	1.2	26	2.1
Tau	0	0	0	0
Arg	23	2.6	59	4.9
Thr#	46	5.3	38	3.1
Ala	140	16.1	122	10.1
Pro	17	1.9	48	3.9
Tyr	0	0.0	0	0.0
Val	100	11.4	98	8.1
Met	0	0.0	9	0.7
Cys	8	0.9	11	0.9
Ile	45	5.1	69	5.7
Leu	64	7.3	106	8.7
Phe	4	0.4	42	3.4
Trp#	0	0.0	0	0.0
Orn	0	0.0	0	0.0
Lys	51	5.9	32	2.6
% Essential AA		39.2		39.4
Total	874		1212	

^o Values determined as THAA-FAA, Taurine values assumed to be zero, as taurine is not found in proteins.

* Converted to asp and glu during hydrolysis.

Yields of these amino acids are reduced during hydrolysis.

Figure 7.3 Protein-bound amino acid composition of a) *Solemya borealis* in comparison with b) *S. velum*. Data presented as molar percentages.

Table 7.4 Free amino acid composition of *Solemya borealis*.

Amino Acid	Foot		Gill	
	$\mu\text{mol/g wet wt.}$	% of total	$\mu\text{mol/g wet wt.}$	% of total
Asp	7	2.8	1	0.7
Glu	7	2.7	13	8.2
Oh-P	1	0.2	1	0.4
Asn	1	0.3	1	0.9
Ser	1	0.5	1	0.7
Gln	0	0.0	1	0.5
Gly	1	0.4	2	1.2
His	1	0.5	2	1.3
Tau	192	71.3	104	66.5
Arg	0	0.0	2	1.3
Thr	2	0.7	1	0.8
Ala	43	16.0	17	11.1
Pro	1	0.4	1	0.9
Tyr	2	0.7	1	0.6
Val	3	1.3	1	0.9
Met	1	0.3	0	0.1
Cys	0	0.0	1	0.6
Ile	1	0.5	1	0.5
Leu	2	0.7	2	1.1
Phe	1	0.3	1	0.6
Trp	0	0.0	0	0.0
Orn	0	0.0	0	0.0
Lys	2	0.7	2	1.6
Total	270		156

Figure 7.4 Free amino acid composition of a) *Solemya borealis* in comparison with b) *S. velum*. (Data presented as molar percentages).

Inanidrilus leukodermatus

The THAA composition of the pooled *I. leukodermatus* sample is shown in Table 7.5 and Figure 7.5. The free amino acids of this species could not be measured because of the small amount of sample available for study.

Table 7.5 Total hydrolyzable amino acid composition of *Inanidrilus leukodermatus*.

Amino Acid	$\mu\text{mol/g dry wt.}$	% of Total
Aspartic Acid	144	3.9
Glutamic Acid	322	8.8
Hydroxy-Proline	15	0.4
Asparagine#	5	0.1
Serine*	54	1.5
Glutamine#	0	0.0
Glycine	640	17.5
Histidine	43	1.2
Taurine/Arginine	122	3.3
Threonine*	136	3.7
Alanine	560	15.3
Proline	41	1.1
Tyrosine	28	0.8
Valine	472	12.9
Methionine	3	0.1
Cystine/Cysteine	21	0.6
Isoleucine	290	7.9
Leucine	396	10.8
Phenylalanine	186	5.1
Tryptophan*		0.0
Lysine	193	5.3
Total AA	3671	
Ammonia	980	

These amino acids are largely converted to aspartic and glutamic acid

* Yields of these amino acids are reduced during acid hydrolysis

Essential amino acids = 46.8-50.1% of total

depending on whether taurine/arginine peak is all taurine or all arginine

Approximately 50% of the amino acids of this species are essential amino acids; sources for these molecules include synthesis by the bacterial symbionts, and epithelial uptake of dissolved organic matter. The sediments where *I. leukodermatus* is found contain very high concentrations of amino acids ($\sim 150 \mu\text{M}$ amino acids/l, Giere *et al.*, 1984) and *I. leukodermatus* has been shown to utilize dissolved sugars and amino acids in laboratory studies (Giere, *et al.*, 1984). Taurine does not appear to be important in the physiology of this species. Unfortunately, because of the small amount of material it was not possible to resolve the taurine and arginine peaks in this sample; however, even if the taurine/arginine peak was assumed to be taurine, amounts would not be as high as those found in the *Solemya* symbioses. The major amino acids found in *I. leukodermatus* were glycine, alanine, valine, leucine and isoleucine. These are frequently the most common amino acids in marine invertebrates.

Figure 7.5 Total hydrolyzable amino acid composition of *Inanidrilus leukodermatus*.

Thiomicrospira crunogena

The amino acid composition of *T. crunogena* is only reported as molar percentages because the dry weights of the bacterial culture used could not be measured due to the large amounts of elemental sulfur excreted externally by this bacterium (Table 7.6).

Table 7.6 Amino acid composition of *Thiomicrospira crunogena* (data presented as molar percentages).

Amino Acid	Total Hydrolyzable Amino Acids % of Total	Free Amino Acids % of Total
Aspartic Acid	0.4	13.2
Glutamic Acid	1.1	6.0
Hydroxy-Proline	0.7	0.0
Asparagine*	0.6	4.8
Serine#	0.6	2.6
Glutamine*	0.0	1.3
Glycine	10.1	4.5
Histidine	0.0	6.5
Taurine	1.0	9.4
Arginine	4.1	8.7
Threonine#	3.0	0.0
Alanine	16.1	7.7
Proline	19.4	1.1
Tyrosine	0.0	0.0
Valine	15.9	0.0
Methionine	1.0	3.3
Cystine/Cysteine	2.7	15.7
Isoleucine	6.9	10.0
Leucine	9.3	4.2
Phenylalanine	4.3	1.2
Tryptophan#	0.0	0.0
Lysine	2.9	0.0
% Essential AA	47.5	24.9

* Converted to asp and glu during acid hydrolysis

Yields of these amino acids are reduced during acid hydrolysis

Thiomicrospira crunogena THAA *Thiomicrospira crunogena* FAA

Figure 7.6 Amino acid composition (THAA and FAA) of the sulfur oxidizing bacterium *Thiomicrospira crunogena*. Data presented as molar percent.

The amino acid composition of this species of chemoautotrophic sulfur-oxidizing bacteria is interesting as it demonstrates the complete *de novo* synthesis of all the physiological amino acids, including the essential amino acids (Table, 7.6, Figure 7.6). *Thiomicrospira crunogena* also appeared to be capable of taurine synthesis (Table 7.6). The ability of *T. crunogena*, a sulfur-oxidizing chemoautotroph, to synthesize all the physiological amino acids demonstrates the possibility of similar amino acid synthesis capabilities in the endosymbionts of animal-bacteria symbioses. This could be a vital nutritional role for the endosymbionts, particularly in the case of animal-host species which lack digestive systems. In the shipworm *B. setacea*, bacterial endosymbionts are thought to be important in providing the mollusc host with nitrogenous compounds, including essential amino acids (Trytek and Allen, 1980). In *B. setacea*, the animal component of the symbiosis is incapable of synthesizing the essential amino acids, and the wood-based diet of this species is deficient in nitrogenous compounds. The sulfur-oxidizing bacterium *Thioploca* sp., which is may be chemoautotrophic (McCaffrey, pers. comm.) also contains all the essential amino acids, suggesting that this species can also synthesize these amino acids *de novo*. The ability of the sulfur-oxidizing endosymbionts of the animal-bacteria symbioses described here to synthesize amino acids is not known, but *in vivo* synthesis by the endosymbionts is a possibility.

The THAA composition of *T. crunogena* was unusual, in that in addition to the common amino acids such as alanine, glycine, valine and leucine, there were considerable amounts of proline. In fact, this was the most common amino acid found. Proline accounted for almost 20% of the amino acids measured in the THAA pool, although only trace amounts were found in the FAA pool. The FAA pool of *T. crunogena* was also unusual in that there was a wide variety of amino acids present as opposed to the usual bacterial pattern of a few dominant amino acids. In most bacteria the dominant free amino acids present are alanine, aspartic acid, glutamic acid, proline and Arginine (Brown and Stanley, 1972). Taurine was found in the free amino acid pool of *T. crunogena*; however, because of the

extremely small amounts of sample available for analysis, the resolution of taurine and arginine peaks was not always clear cut. Interestingly, the most common amino acid in the FAA pool was cysteine, which also contains a sulfur group.

The first descriptions of bacterial free amino acid pools appeared in the 1940's (Gale and Taylor, 1947). In these studies it was noted that bacterial FAA pools were formed largely as a result of the transport and exogenous accumulation of amino acids. In many bacteria the FAA pools act as a precursor pool for protein synthesis, although there is usually little similarity between protein-bound and FAA pool compositions. More recent work on the growth of bacteria on inorganic carbon sources has shown, that in the marine environment, a large amount of FAAs are accumulated within the cells by *de novo* synthesis from inorganic carbon. This must certainly be the case in *T. crunogena*, as the culture media used (made with distilled water in autoclaved glassware) is unlikely to have provided the bacteria with appreciable concentrations of amino acids. In bacteria, just as in eucaryotes, environmental conditions such as salinity and temperature will affect the composition of FAA pools.

7.3.3 Fatty acid compositions

Solemya borealis

The fatty acid composition of the gills of *S. borealis* are shown in Table 7.7 and compared with those of *S. velum* in Figure 7.7. Three different sections of the gill tissue were extracted and analyzed as outlined in chapter 2. The most obvious characteristic of the lipids of *S. borealis* are the high concentrations of *cis*-vaccenic acid (18:1 ω 7). As discussed in Chapter 4, this fatty acid is the most common lipid in *S. velum*, and *T. crunogena*, and is predominantly a bacterial fatty acid which is synthesized using the bacterial anaerobic MUFA synthesis pathway (Goldfine, 1972; Fulco, 1983). In the gills of *S. borealis* this lipid occurred in concentrations of 2.6-15.0 $\mu\text{g} \cdot \text{mg}^{-1}$ dry wt. The extreme variability between the *cis*-vaccenic acid composition of different gill segments is highly unusual (Table 7.7).

The high variability in the data may be due to either analytical error, or extreme differences in the fatty acid composition of different sections of the gills. Analytical error cannot be ruled out, however; in each of these extractions the percentage recovery of the internal recovery standards was very high, exceeding 95% for both the C₁₉ FA and the C₂₁ FAME, suggesting that sample loss during the lipid extraction procedure is unlikely. Oxidation, or conversion of the *cis*-vaccenic acid to other lipids is also unlikely, as no other saturated lipids showed an increase in concentration correlating with a decrease in *cis*-vaccenic acid levels. The most likely explanation is differences in tissue composition. The endosymbionts of many animal-bacteria symbiosis are often localized in specific sections of the gills and this does appear to be the case for *S. borealis* (Appendix I); the variability in lipid composition of the gills of *S. borealis* may reflect differences in the bacterial content of the gill sections examined. In *S. velum*, intertissue differences in fatty acid concentrations may also be present; however, because of the relatively small size of *S. velum* samples, gill tissues are generally analyzed whole and such differences would not be seen. The large amounts of *cis*-vaccenic acid in *S. borealis*, along with the extremely negative $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope ratios provide strong evidence to suggest that this bivalve contains bacterial endosymbionts, similar to the *S. velum* symbiosis. Electron micrographs of the gill tissue of *S. borealis* support this theory (Appendix I).

Table 7.7 Gill fatty acids of *Solemya borealis*

Fatty acid	Extraction 1 ng mg dry wt. ⁻¹	Extraction 2 ng mg dry wt. ⁻¹	Mean ng mg dry wt. ⁻¹
14:0	4510	4340	4420
14:1 ω 5	23	200	110
a-15:0	25	23	24
15:0	58	59	58
16:0	13070	13160	13110
16:1 ω 7	4820	1040	2930
a-17:0	53	0	27
17:0	190	64	130
18:0	5550	4910	5230
i-18:0	0(b)	0	0
18:1 ω 9	1800	1620	1710
18:1 ω 7	14980	2570	8770
18:2 ω 6	480	19	250
18:3 ω 3	80	0	38
18:3 ω 6	0	0	0
18:4 ω 3	200	0	99
20:0	73	10	41
20:1* (a)	0	120	58
20:1 ω 9	1710	110	910
20:1 ω 7	1290	220	750
20:2*	140	0	700
20:2 ω 6	810	120	460
20:3*	360	0	180
20:3 ω 3	590	150	370
20:4 ω 6	1130	0	560
20:5 ω 3	0	0	0
22:0	28	0	14
22:1 ω 9	60	0	30
22:2 ω 6	0	0	0
22:2*	0	0	0
22:3 ω 3	0	0	0
22:4 ω 6	1900	2560	2230
22:6 ω 3	36	0	18
24:0	38	0	19
Total	55250	31250	43250

(a) Position of double bonds unknown; (b) below the detection limit of 1 ng mg dry wt.⁻¹

Figure 7.7 Fatty acid composition of the gills of a) *Solemya borealis* in comparison with b) *S. velum*. Fatty acid concentrations of individual fatty acids presented as a percentage of the total fatty acids present.

Thin layer chromatographic analysis of the fatty acid composition of the lipid classes of *S. borealis* demonstrate that, just as in *S. velum*, the *cis*-vaccenic acid found in *S. borealis* is located in all the major lipid classes (Table 7.8, Figure 7.7). This lipid would appear to be involved in a variety of physiological processes in *S. borealis* and is probably important in both membrane structure maintenance and energy production. In *S. velum* *cis*-vaccenic acid is most localized in the phospholipid pool (Figure 7.7, See discussion of Chapter 4); interestingly, in *S. borealis*, *cis*-vaccenic is more localized in the mono-, di-, and triglyceride pools. This is probably due to differences in the ambient site temperature at the time of sample collection. *S. velum* specimens were collected during November when triglyceride pools would be expected to be reduced as a result of reduced exogenous food source availability, spawning, and/or reduced endosymbiont metabolic rates. In addition, the reduced site temperatures may result in an increased utilization of *cis*-vaccenic acid for membrane fluidity maintenance purposes. On the other hand, the specimen of *S. borealis* was collected during July, when food availability is probably increased, animals may be in a reproductive state, and ambient temperatures are higher. In this situation, it is possible that greater amounts of the *cis*-vaccenic acid are stored as tri- and diglycerides and less of the bacterial fatty acid is mobilized for membrane structural requirements.

In *S. borealis*, only low concentrations of PUFAs were found, and these predominantly belonged to the $\omega 6$ series of PUFAs as opposed to the typical $\omega 3$ PUFAs found in most marine bivalves (See Joseph, 1982). The source of the 20:4 $\omega 6$ and 22:4 $\omega 6$ PUFAs in *S. borealis* are unknown, but possibilities include *in vivo* synthesis by the host or the putative endosymbionts, ingestion of small amounts of marine algae or epithelial uptake of DOM.

The major sterol found in *S. borealis* was cholesterol which accounted for almost 80% of the total sterols of this species, and over 90% of the esterified sterols (Table 7.8.). This again was similar to the pattern found in *S. velum*, and suggests limited input of plant-derived material.

Table 7.8 Fatty acid composition of the major lipid classes of *Solemya borealis* (fatty acids of each lipid class presented as a percentage of the total fatty acids of that class).

Fatty Acid	Phospholipids	Monoglycerides	Diglycerides, Triglycerides + FFAs	Steryl Esters + Methyl Esters
14:0	4.9	5.8	6.4	5.0
16:0	19.7	16.1	18.7	25.1
16:1 ω 7	17.1	8.8	10.6	8.2
18:0	8.9	7.8	7.9	13.2
18:1 ω 9	2.1	4.5	4.4	6.1
18:1 ω 7	27.7	42.1	38.8	28.4
18:2 ω 6	2.5	1.9	1.2	0.9
20:1 ω 9	1.7	2.3	0.6	2.8
20:1 ω 7	1.8	4.0	4.0	4.0
20:2 ω 6	4.3	0.0	0.7	1.1
20:3 ω 3	3.0	0.0	1.0	1.7
20:4 ω 6	2.0	3.6	2.4	2.6
22:2 ω 6	2.3	1.8	2.4	0.0
Total	97.9	98.9	99.0	99.2
	Free Sterols (ng/mg dry wt.)	Percent of Total	Steryl esters (ng/ng dry wt.)	Percent of Total
Cholesterol	1722	78.4	939	97.5
Other Sterols	475	21.6	24	2.5

Figure 7.8 Fatty acid composition of the major lipid classes of a) *S. borealis* and b) *S. velum*. Fatty acids of each lipid class presented as a percentage of the total fatty acids of each lipid class.

Inanidrilus leukodermatus

The fatty acid composition of *I. leukodermatus* (Table 7.7, Figure 7.9) were similar to those of the Solemyid bivalves and *T. crunogena*. There were large amounts of *cis*-vaccenic acid (18:1w7) and low concentrations of ω 3 and ω 6 PUFAs in *I. leukodermatus*. This suggests that the endosymbionts of this species play a role in the lipid nutrition of the host.

Figure 7.9. Fatty acid composition of *Inanidrilus leukodermatus*, fatty acid concentrations of individual fatty acids presented as a percentage of the total fatty acids present.

Table 7.9 Fatty acid concentrations of *Inanidrilus leukodermatus*.

Fatty Acid	Average of 3 extractions	
	ng/mg dry wt.	% of Total
14:0	1040	2.2
16:0	6430	13.5
16:1w7	2770	5.8
18:0	4430	9.3
18:1w9	3020	6.3
18:1w7	20510	43.0
18:2w6	2360	4.9
18:3 w3	230	0.5
20:0	730	1.5
20:1 w7	400	0.8
20:3 w3	330	0.7
20:4 w6	260	0.6
20:5 w3	0	0.0
22:0	910	1.9
22:1 w9	0	0.0
22:2 w6	480	1.0
22:3 w3	610	1.3
22:4/22:6	2220	4.7
24:0	960	2.0
Total	47690	

Summary

The stable isotope ratios of all the species examined are very negative suggesting that marine phytoplankton-based primary production does not play a significant role in the nutrition of these species. The annelid *I. leukodermatus*, appears to rely on the endosymbionts for only about 40-50% of its nutritional needs, while the putative *S. borealis* symbiosis appears to obtain most of its nitrogenous and carbon requirements from endosymbiont chemoautotrophy.

The amino acid composition of the species studied was similar to that of most marine invertebrates. *S. borealis*, in common with *S. velum*, had large concentrations of the sulfur-containing amino acid

taurine. It is possible that this amino acid is involved in an internal sulfur-cycling pathway in this species.

The lipid composition of the species examined were similar to those of *T. crunogena* and other sulfur-oxidizing bacteria. High concentrations of *cis*-vaccenic acid were found and are probably derived from the bacterial endosymbionts of the species studied. This lipid may serve as a highly useful screening tool for the presence of symbionts in other species.

7.4 References

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CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

8.1 Summary

The overall goal of this dissertation was to increase our understanding of the nutritional physiology of animal-bacteria symbioses and to determine the importance of endosymbiotic bacteria in the nutrition of the host. The protobranch bivalve *S. velum*, was chosen as a model symbiosis for a number of reasons. (1) This shallow water species is found in a variety of sites along the east coast of the continental U.S., and is available for collection throughout the year on Cape Cod. (2) Relatively large (~20-30) numbers of specimens can be collected at a time, permitting the use of statistical analyses, and 'trial and error' experiments. For example, the bacterial enrichment technique used in Chapter 3, was refined on a number of specimens, prior to use in estimating the stable isotope signature of the bacterial endosymbionts of *S. velum*. This would not have been possible if only a few specimens were available for analysis. Because many specimens can be examined, new techniques can be 'calibrated' on the *S. velum* symbiosis, and applied to the study of other animals when the opportunity arises. (3) A preliminary data base on the *S. velum* symbiosis was available (Cavanaugh, 1985).

The soft-shelled clam *Mya arenaria* was used as a non-symbiont control throughout this dissertation as this species is found in the same sediments as *S. velum* at the Little Buttermilk Bay site, thus environmental conditions are similar for both species. In addition, this species is of commercial importance and is well characterized at the physiological and biochemical levels. Results obtained during the research outlined in this thesis could be compared with published data on this species, providing a check on the methodologies used here.

The results of this thesis were subdivided as follows:

1. The nutritional importance of the endosymbionts to the overall carbon and nitrogen budgets of *S. velum* was estimated through the use of stable isotope ratios and application of a two-source mixing model.
- 2 The lipid and amino acid composition of *S. velum*, *M. arenaria*, and the sulfur-oxidizing bacterium, *Thiomicrospira crunogena*, was examined in order to isolate potential biomarkers for endosymbiont activity, and to examine basic differences between symbiont-containing and symbiont-free bivalves.
- 3 The techniques used in the analyses of *S. velum* were applied to two additional animal-bacteria symbioses, *Solemya borealis* and *Inanidrilus leukodermatus*, in order to determine similarities between species, and to provide initial data on the nutritional role of endosymbiotic bacteria in these species.

The results of the investigation can be summarized as follows. In Chapter 3, C, N, and S stable isotope compositions were investigated in *S. velum* and symbiont-free containing bivalves. Photosynthetic and chemosynthetic organisms often have different isotope signatures, and these signatures are passed along food webs with only minor changes (Fry and Sherr, 1984). Thus, the stable isotopes of an organism may reveal the predominant nutritional sources utilized by the animal. C and N isotope ratios of both foot and gill tissues were examined in *S. velum*. This allowed differences between tissues with and without endosymbionts to be determined. C and N isotope values were also examined in an enriched bacterial fraction, separated from intact gills by differential centrifugation. Although the bacteria were not cultured, the resulting bacterial fraction was enriched in bacteria in comparison with the original gill tissue. The bacterial fraction and host tissues had similar $\delta^{13}\text{C}$ values (-30.9 ‰ to -33.9 ‰) which were very different from those of the control bivalves *Mya arenaria* and *Tellina agilis* (-14.2 to -17.8‰). Application of a simple two-source mixing model suggests that endosymbiotic bacteria provide as much as 98% of the carbon requirements of *Solemya velum*. The $\delta^{15}\text{N}$ values of both *S. velum*

and the endosymbiont fraction were comparable (4.4‰ to -9.8‰ and considerably lower than those of the non-symbiont controls (*Mya arenaria* and *Tellina agilis*, $\delta^{15}\text{N} = 6.3\text{‰}$ to 8.5‰). Uptake of pore-water ammonium by the endosymbionts and subsequent translocation of nitrogenous compounds to the host may account for the low $\delta^{15}\text{N}$ values of *S. velum*. In this context, the symbionts may provide 100% of the host nitrogen. The contribution of the bacteria to both the carbon and nitrogen budgets appeared to differ between sites. This could be due to differences in environmental conditions or bacterial populations, or be an artifact resulting from limitations of the stable isotope techniques. The $\delta^{34}\text{S}$ values of *S. velum* (-26.7‰ to -31.1‰) suggest the use of biogenically produced H_2S as a sulfur source, as porewater H_2S , produced as a result of bacterial dissimilatory sulfur reduction, usually has a very negative $\delta^{34}\text{S}$ signature, (~ -15 to -70‰ , Goldhaber and Kaplan, 1975; Fry *et al.*, 1986) while seawater sulfate $\delta^{34}\text{S}$ values are positive ($\sim +30\text{‰}$). Both the sulfur and nitrogen stable isotope ratios are among the lowest reported for animal tissues.

The lipids of marine bivalves generally reflect a dietary signature (Pollero *et al.*, 1979; Moreno *et al.*, 1980; Piretti *et al.*, 1987), and the lipids of marine algae are usually very different from those of marine bacteria. Thus, it is likely that the lipids of a bivalve utilizing bacteria as a main nutritional source will differ from those of marine bivalves whose diet is based on marine algal primary production. In Chapter 4, a detailed analysis of the lipid composition of *Solemya velum*, the symbiont-free clam *Mya arenaria*, and the sulfur-oxidizing bacterium, *Thiomicrospira crunogena* was undertaken. $\delta^{13}\text{C}$ ratios of the fatty acids and sterols of each species were also measured in order to determine potential lipid carbon sources.

Both fatty acid and sterol composition were found to differ markedly between the two bivalves. The lipids of *S. velum* were characterized by large amounts of 18:1 ω 7 (*cis*-vaccenic acid), 16:0, 16:1 ω 7, and low concentrations of the highly unsaturated plant-derived fatty acids characteristic of most marine bivalves. The predominant sterol found in *S. velum* was cholesterol, although

small amounts of 24-ethylcholesterol were found in some specimens. *Mya arenaria* had fatty acid and sterol compositions similar to typical marine bivalves and were characterized by large amounts of the highly unsaturated fatty acids 20:5 ω 3 and 22:6 ω 3 and a variety of plant-derived sterols. The fatty acids of *T. crunogena* were dominated by 16:1 ω 7, 16:0 and 18:1 ω 7, and were similar to those of *S. velum*.

The *cis*-vaccenic acid found in *S. velum* is presumed to be symbiont-derived, as this lipid is the predominant fatty acid in many gram-negative marine bacteria (Fulco, 1983). This lipid could serve as a biomarker for symbiont-lipid incorporation by the animal host. The high concentrations of *cis*-vaccenic acid in the tissues of *S. velum* (up to 35% of the total fatty acid content) suggest an important role for the endosymbionts in the lipid metabolism of this bivalve. In addition, the presence of *cis*-vaccenic acid in all the major lipid classes of *S. velum* demonstrates both incorporation and utilization of this fatty acid. The reduced amounts of polyunsaturated fatty acids found in *S. velum*, along with the absence of sterols of plant origin, provide further evidence to suggest that this symbiosis relies on endosymbiont chemoautotrophy to fulfill the majority of its nutritional requirements. *Cis*-vaccenic acid may play an important role in maintaining membrane integrity in the *S. velum* symbiosis, as it is predominantly localized in the phospholipid pool. This lipid may function in lieu of the high concentrations of PUFAs usually found in the phospholipid pool of marine bivalves. *Cis*-vaccenic acid may be translocated to the animal tissues by bacterial cell digestion and 'blebbing', or pinching off, of sections of the bacterial cell membrane.

The $\delta^{13}\text{C}$ ratios of the fatty acids and sterols of *S. velum* were significantly lighter (-38.4‰ to -45.3‰) than those of *M. arenaria* (-23.8‰ to -24.2‰) and similar to the values found for the fatty acids of *T. crunogena* (-45‰) suggesting that the lipids of *S. velum* are either derived directly from the endosymbionts, as would appear to be the case for the large concentrations of *cis*-vaccenic acid found, or are synthesized using carbon derived from the endosymbionts.

Total and free amino acids were determined for both *S. velum* and *M. arenaria* in Chapter 5. Both the relative and absolute

amounts of free amino acids differed significantly between the two species. In *S. velum*, the absolute concentrations of the sulfur amino acid taurine were greater than the total free amino acid concentrations typically found in bivalves. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotope ratios of both the free and total hydrolyzable amino acids of *S. velum* and *M. arenaria* were very different. Those of *M. arenaria* reflected utilization of photosynthetically-derived organic matter for amino acid synthesis. In *S. velum* the very negative amino acid isotope ratios suggest that the amino acids are either synthesized directly by the endosymbionts, or are synthesized by the host using carbon and nitrogen originally derived from the endosymbionts.

The possible roles for taurine as a sink for oxidized sulfur compounds in the *S. velum* symbiosis were examined in Chapter 6. These preliminary experiments suggest that taurine levels may increase in the presence of reduced sulfur compounds, demonstrating a possible link between sulfur-oxidation and taurine synthesis in this symbiosis. $\delta^{34}\text{S}$ ratios of the FAA pool of *S. velum* were very negative (-17.2‰), suggesting incorporation of sulfur derived from porewater sulfides into the taurine of the host. Perhaps the H_2S is oxidized by the symbionts and the oxidized sulfur compounds are utilized in taurine synthesis. Attempts to demonstrate a link between the H_2S uptake and taurine synthesis were unsuccessful.

In Chapter 7 the stable isotope and lipid composition profiles of *Solemya borealis*, a newly symbiosis (Appendix I), and *Inanidrilus leukodermatus* a symbiotic annelid (Appendix II), were examined in order to determine whether the biomarker approach was useful in the study of other symbioses. Isotope ratios and lipid profiles were very similar in all the symbioses examined, and quite different from those of typical marine bivalves. This demonstrates the usefulness of stable isotopes and lipid composition studies in the analysis and characterization of these types of symbioses. In particular, both techniques are valuable as initial screening tools for the presence of symbiotic sulfur-oxidizing bacteria. The amino acid pool of *S. borealis* closely resembled that of *S. velum*, suggesting related taurine metabolic pathways. All the symbioses examined contained

the essential amino acids, which presumably are synthesized by the autotrophic symbionts.

Overall, the results of this study demonstrate the importance of bacterial endosymbionts in the nutrition of *S. velum*. Biochemical analyses (Chapters 3-5) and feeding experiments (Appendix III) demonstrate that marine photosynthetic algae are probably of only minor importance. The bacteria appear to provide much of the overall C and N budgets, lipid, and amino acid pools. Moreover, examining the biochemical composition of the *S. velum* symbiosis, and comparing the results with similar data for more typical bivalves, revealed basic differences in physiology between symbiotic and non-symbiotic species. Unusual characteristics in the biochemical composition of *S. velum* may be diagnostic of animal-bacteria symbioses of the type discussed here. In this context, *cis*-vaccenic acid, which was found in high concentrations in *S. velum*, may be a valuable screening tool for determining the existence of other animal-bacteria symbioses.

Publications that were generated during the dissertation are listed below. These publications formed the basis for chapters 3-7 and Appendices I and II, although more extensive discussions of the data were included in the dissertation chapters.

- Conway, N., J. McDowell Capuzzo, and B. Fry (1989). The role of endosymbiotic bacteria in the nutrition of *Solemya velum*: Evidence from a stable isotope analysis of endosymbionts and host. *Limnology and Oceanography* 34(1): 249-255.
- Conway, N. and J. McDowell Capuzzo (1990). The use of biochemical indicators in the study of trophic interactions in animal-bacteria symbioses: *Solemya velum*, a case study. In: *Trophic relationships in the marine environment* (M. Barnes and R.N. Gibson, Eds.). Proceedings, 24th Europ. Mar. Biol. Symp. Aberdeen University Press, pp. 553-564.
- Conway, N. and J. McDowell Capuzzo (1990). Incorporation and utilization of bacterial lipids by the *Solemya velum* symbiosis. (In Press, *Marine Biology*).
- Conway, N. and J. McDowell Capuzzo. Excess taurine in the *Solemya velum* symbiosis, possible sources and functions. (To be submitted to *Mar. Ecol. Prog. Ser.*).
- Conway, N., B. Howes, C. Cavanaugh, R. Turner, and J. McDowell Capuzzo. Initial characterization and site description of *Solemya borealis*, a newly described symbiosis. (To be submitted to *Mar. Ecol. Prog. Ser.*).
- Giere, O., N. Conway, G. Gastrock, and C. Schmidt (1990) Regulation of the ecology of a gutless annelid by endosymbiotic bacteria. (Submitted to *Mar. Ecol. Prog. Ser.*).

8.2 Suggestions for further research

As with most dissertations, this study raised more questions than it answered. This research provides a broad data base on the physiology and biochemistry of the *S. velum* symbiosis, along with preliminary data on the *S. borealis* symbiosis, which can be used as a building block for further studies of these symbioses. If only I could have had these data when I was starting my thesis!

Some potential research projects which could utilize and build on the data presented in this thesis are summarized below.

- 1) *Determination of the source of the 20:4 ω 6 fatty acid;*
 - 2) *Lipid and amino acid synthesis in *S. velum*;*
 - 3) *Taurine synthesis question;*
 - 4) *Incorporation of endosymbiont carbon into different biochemical fractions of the hos;*
 - 5) *Follow-up *S. borealis* studies;*
 - 6) *Seasonal studies of *S. velum* symbiosis;*
 - 7) *Sediment geochemistry at sites where *S. velum* occurs;*
 - 8) *POM utilization via external production of digestive enzymes;*
 - 9) *Detailed DOM utilization studies;*
 - 10) *Investigation of carbonic anhydrase activity of animal-bacteria symbioses.*
- 1) *Determination of source of 20:4 ω 6 and 2) Lipid and amino acid synthesis in *S. velum*.*

The question of essential nutrient synthesis in animal-bacteria symbioses could be addressed directly. These types of experiments would involve incubating the symbiosis in radiolabelled bicarbonate

and analyzing the incorporation of radiolabel into specific biochemicals by coupling HPLC and GC apparatus to in-line scintillation counters. In this way, the radioactivity of individual chromatography peaks can be detected. This would be an extremely powerful way of looking at the metabolism of these symbioses, and would permit us to determine whether the symbiosis is capable of synthesizing the 20:4 ω 6 fatty acid that was found in *S. velum*, along with other essential lipids and amino acids. The 20:4 ω 6 question is particularly intriguing as this lipid is not thought to be synthesized by either bacteria or animals. Photosynthetic organisms are generally assumed to be the only source for the ω 3 and ω 6 lipids.

3) *Taurine synthesis question*

There may be a link between the taurine levels of this species, and bacterial sulfur oxidation. Taurine is a non-protein amino acid, which contains an oxidized sulfur group. It is probably more than a coincidence that high concentrations of a sulfur-containing amino acid are found in the symbiosis between *S. velum* and sulfur-oxidizing bacteria. The types of radiolabelling experiments alluded to in the preceding section could be used to determine whether the symbiosis is capable of synthesizing this amino acid. Bivalves have little or no capacity for taurine synthesis (Bishop *et al.*, 1983); the endosymbionts may be the source of this amino acid. It would also be interesting to determine the role of this amino acid in the metabolism of this species (interestingly, very high levels of this amino acid were also found in *S. borealis*). Is it only involved in osmoregulation, or is it part of the internal sulfur cycle in this symbiosis? The preliminary experiments discussed in Chapter 6 need elaboration. In general, it did appear as if there is a relationship between sulfur oxidation and taurine levels, although we know little about the processes involved. Incubating the symbiosis in radiolabelled thiosulfate, as opposed to hydrogen sulfide, as carried out in Chapter 6, might demonstrate a link between sulfur oxidation and taurine synthesis. If further experiments do demonstrate a link, biochemical investigations to

determine potential pathways and intermediates would be warranted.

4) Incorporation of endosymbiont carbon into different biochemical fractions of the host.

We know that the endosymbionts are vital to the nutrition of *S. velum*, in terms of C and N budgets and lipid metabolism. In order to better understand the role of the endosymbionts in the overall nutrition of the host, it would be interesting to follow radiolabelled bicarbonate from an external seawater medium, through the endosymbionts into the different biochemical fractions of the bivalve host. This would provide data on the partitioning of symbiont nutrients by the animal host. In these types of experiments, specimens of *S. velum* would be incubated with $\text{NaH}^{14}\text{CO}_3$ and dissected. Radiolabel incorporation into various biochemical fractions can be determined by fractionating the tissues into the following biochemical fractions and determining radioactivity.

Fraction	Treatment
Free amino acids, sugars	70% TCA extraction
Lipids	95% ethanol/ether extraction
Nucleic acids and polysaccharides	10% TCA at 60°C
Alkaline soluble proteins	1N NaOH
Acid hydrolyzable proteins	6N HCL at 110°C

Examining radiolabel incorporation after various pulse and chase experiments should provide information on the types of compounds initially translocated (very short pulses), and on the types of compounds subsequently synthesized by the host from endosymbiont nutrients (e.g. following the radioactivity of biochemical fractions in the foot tissue [no bacteria] after short pulse periods and long chase periods.

5) *Follow-up S. borealis studies.*

During two box-coring cruises last summer Brian Howes (WHOI) found two specimens of *Solemya borealis*, which we have now identified as a new animal-bacteria symbiosis (Appendix I). We know very little about the general physiology of this species. Respiration rates, CO₂ fixation rates and sulfur-oxidation rates of intact tissues, or intact animals need to be determined. Ribosomal RNA analyses could be carried out to determine possible relationships between the symbionts of this species and the other two Solemyids, *S. velum* and *S. reidi*, characterized to date. Colleen Cavanaugh now has an immunological probe to the RuBPCase of *S. velum* (Cavanaugh *et al.*, 1988); this probe could be used to localize carbon fixation activity in *S. borealis*.

6) *Seasonal studies of S. velum symbiosis*

During this dissertation research I found very high concentrations of the bacterial fatty acid *cis*-vaccenic acid in *S. velum*. This lipid was found in all the lipid classes, but particularly in the phospholipid pools. It would be very interesting to quantify the amounts of *cis*-vaccenic acid present, and the location of the *cis*-vaccenic acid in different lipid classes, during the seasonal cycle, as this lipid is probably involved in membrane structure and function, and consequently should exhibit variable levels throughout the year, with levels greatest in the winter months. If levels are adjusted seasonally it would demonstrate the existence of a tight metabolic coupling between endosymbiont and host. This would open a whole new aspect of studies of animal-bacteria symbioses, specifically the controlling mechanisms involved in the symbiont-host interactions. There must be a variety of signals or messengers involved in ensuring symbiosis homeostasis.

7) *Sediment geochemistry at sites where S. velum occurs.*

S. velum is distributed very sporadically in the sites that we have examined, suggesting that this species requires a specific sediment geochemistry. Apart from initial analysis of sediment grain size and TOC content, we know little about the sediment

geochemistry of sites inhabited by this species. This knowledge will become increasingly important if questions of POM and DOM utilization are addressed, as we need to know what concentrations and types of these food sources are available to the symbiosis *in situ*. We know little about sulfate reduction rates, sediment oxygen demand, sedimentation rates, organic matter availability, pore water nutrient concentrations, etc. in the sediments where *S. velum* is found. It would be interesting to analyze the habitat of *S. velum* in detail. This will not only provide information about the ecological niche of this species, but may provide the information required for better laboratory maintenance of this species.

8) POM utilization in *S. velum* via external production of digestive enzymes

Although *S. velum* is almost gutless, particulate organic matter (POM) could conceivably be utilized in a variety of ways. In general, a diet based on a very restricted food source, such as a single species of endosymbiont, might lead to dietary deficiencies. Consequently, it would be advantageous for animal-bacteria symbioses to maintain the ability to utilize exogenous food sources, even if this ability is limited. The host may only require trace amounts of specific essential nutrients to subsidize the endosymbiont-based diet. In this context, the production and secretion of digestive enzymes by the gill and mantle epithelial surfaces of *S. velum* remains unexplored. A wide variety of bivalves secrete digestive enzymes into the mantle cavity, which may function to initialize digestion of POM in the mantle cavity (Conway, 1987). This predigestion may result in the production of dissolved organic matter, allowing the epithelial uptake of DOM to occur. DOM uptake is exhibited by the gills and mantle of *S. velum* (Gallager, unpub. results).

9) Detailed DOM utilization studies by *S. velum* and gutless animals.

The possibility of uptake of essential dietary nutrients such as essential amino acids, fatty acids, and sterols by animal-bacteria

symbioses warrants further study. Previous studies with other bivalves have demonstrated the capacity for active uptake of these nutrients from dilute concentrations (Stewart, 1979). Moreover, S. Gallager (WHOI) has demonstrated the uptake of dissolved amino acids by *S. velum* (See Figure 5.4, Chapter 5). Studies with gutless animals could extend our knowledge of these trophic mechanisms, as it may be possible to study the absorption of DOM without having to take into account ingestion of particulate material. In fact, as John Farrington has commented, gutless invertebrates, such as many animal-bacteria symbioses, may be useful models for the study of contaminant uptake by marine bivalves, as uptake of contaminants in the dissolved phase might be separated from uptake of particulate contaminants with appropriate experiments.

10) Investigation of carbonic anhydrase activity of animal-bacteria symbioses.

We do not know how animal-bacteria symbioses ensure uptake of CO_2 from the seawater bicarbonate pool. The uncatalyzed conversion of HCO_3^- to CO_2 is slow. In addition, intake of inorganic carbon compounds may be limited by unstirred boundary layers and passage across the several membrane layers separating bacteria from host. Some algae and cyanobacteria possess a CO_2 -concentrating mechanism that involves the enzyme carbonic anhydrase (Lucas and Berry, 1985). CA activity has also been reported in both the algal and animal fractions of cnidarian symbioses (Graham and Smillie, 1976; Isa and Yamazato, 1984). Recently, Weis *et al.* (1989) provided evidence to show that CA in the animal host may function as a CO_2 supply mechanism in 22 species of tropical cnidarian symbioses. It is conceivable that a similar mechanism may operate in animal-bacteria symbioses.

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APPENDIX I

**CHARACTERIZATION AND SITE DESCRIPTION OF *SOLEMYA*
BOREALIS, A NEWLY DISCOVERED ANIMAL-BACTERIA
SYMBIOSES.**

Noellette Conway¹, Brian Howes¹, Colleen Cavanaugh², Ruth Turner²,
and Judith McDowell Capuzzo¹

¹ Department of Biology
Woods Hole Oceanographic Institution
Woods Hole
MA 02543

² Department of Organismic and Evolutionary Biology
Biological Laboratories
Harvard University
Cambridge
MA 02138

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Abstract

Solemya borealis was collected from reducing sediments in Buzzards Bay, Cape Cod, Massachusetts and examined for the presence of symbiotic, chemoautotrophic bacteria. In addition, sediment cores collected seasonally at the same site were analyzed to provide a detailed description of the *S. borealis* habitat. Here we present microscopic, enzymatic, biochemical, and stable isotope data which suggests that *Solemya borealis*, like the related species *Solemya velum* and *Solemya reidi*, contains high concentrations of symbiotic chemoautotrophic bacteria in gill bacteriocytes which play a significant role in nutrition. Transmission electron micrographs reveal the presence of rod shaped putative bacteria within gill bacteriocytes. Ribulose-1,5-biphosphate carboxylase activity in cell-free extracts of *S. borealis* gill tissue was comparable with that found in other animal-bacteria symbioses. Very negative $\delta^{34}\text{S}$ ratios (-32.6 to -15.7‰) suggest the utilization of porewater sulfides as an energy source for the symbionts. Carbon and nitrogen stable isotope ratios were also extremely negative ($\delta^{13}\text{C}$ = -32 to -34.6‰, $\delta^{15}\text{N}$ = -9.7 to -8.6‰) and very similar to those of other bivalve-chemoautotroph symbioses, suggesting that most of the animals carbon and nitrogen is derived from the putative symbionts. High concentrations of *cis*-vaccenic acid, a fatty acid previously found in invertebrate-chemoautotroph symbioses, were found in the gills of *S. borealis*. This lipid is localized in all the major lipid classes of *S. borealis*, further suggesting a significant role for the endosymbionts in the lipid nutrition of this bivalve. High levels of taurine in the free amino acid pool of *S. borealis* suggest the existence of unusual amino acid metabolic pathways which may be the result of endosymbiont activity. The *S. borealis* specimens were found in relatively shallow water sediments dominated by silts and clays. The sediments contain high concentrations of organic carbon and nitrogen, exhibit limited oxygen penetration, and have high rates of ammonium and sulfide input from the anaerobic microbial community.

Introduction

During the last decade, symbiotic associations involving chemoautotrophic sulfur-oxidizing bacteria and marine invertebrates have been described in four invertebrate phyla from a diverse range of habitats (Cavanaugh *et al.*, 1981; Felbeck, 1983; Giere *et al.*, 1984; Southward *et al.*, 1986). In general, these symbioses involve chemoautotrophic bacteria which are endosymbiotic within specific hypertrophied host tissues (such as the gills of bivalve hosts and the trophosome of vestimentiferan and pogonophoran worms) and which are often intracellular. Physiological and biochemical studies of these types of invertebrate-chemoautotroph symbioses have shown that in many of the habitats involved, such as hydrothermal vent areas, reducing sediments, and sewage outfall areas, a key feature is simultaneous access to both oxygen and reduced inorganic sulfur compounds. Energy present in the sulfur compounds appears to be exploited by the bacteria via oxidative pathways, and the ATP and NAD(P)H produced used to drive the reductive reactions of carbon fixation (Felbeck *et al.*, 1981; Cavanaugh, 1983; Southward *et al.*, 1986). In many invertebrate-chemoautotroph symbioses (hereafter referred to as animal bacteria symbioses) the digestive system of the host is small or absent entirely (Reid, 1980; Cavanaugh, 1983), suggesting the host may obtain much of its nutritional requirements from the bacterial endosymbionts.

Two of the best characterized animal-bacteria symbioses belong to the protobranch mollusc genus *Solemya*. *Solemya reidi* is found along the west coast of the U.S and is gutless (Reid, 1980), while *S. velum* is predominantly an east coast species and has a very small gut (Yonge, 1939). Both bivalve species have large fleshy gills and harbor high concentrations of symbiotic, Gram-negative, sulfur-oxidizing chemoautotrophic bacteria within specialized gill bacteriocytes (Cavanaugh, 1983; Felbeck, 1983). The symbionts appear to be of importance in the nutrition of these solemyids. In *S.*

velum, stable isotope analyses suggest that the symbionts can provide almost 100% of the C and N requirements of the host at some sites (Conway *et al.*, 1989), while *S. reidi* may also be able to meet its carbon needs through autotrophy (Anderson *et al.*, 1987). Other members of the genus *Solemya* have not been examined for the presence of symbionts.

We recently discovered two specimens of *Solemya borealis* in sediment samples being collected during a multicomponent project to assess the ecological impacts of nutrient input alterations in New Bedford Outer Harbor providing us with the opportunity to examine this relatively unknown species for the presence of bacterial symbionts. *S. borealis*, is an east coast species, but is less common than *S. velum* (Morse, 1913). Morse (1913) described *S. borealis* as resembling an enlarged *S. velum*, suggesting that *S. borealis* also has large fleshy gills, while Bernard (pers. comm. in Reid, 1980) suggests the *S. borealis* may be gutless. The morphological similarities between *S. borealis* and other solemyids known to contain endosymbiotic chemoautotrophic bacteria led us to speculate that a similar symbiosis may exist in *S. borealis*. In this paper we provide microscopic, enzymatic, stable isotope, and biochemical evidence to suggest that this species, in common with *S. velum* and *S. reidi*, harbors chemoautotrophic bacterial symbionts. In addition we describe in detail the sediments where the specimens were found in order to provide information regarding the ecological niche of *S. borealis*.

Materials and methods.

(1) Animal and sediment collection

Single specimens of *Solemya borealis* were collected from 2 sites in Buzzards Bay (Figure 1), Station R at 41°29.27N, 70°53.34W (Sanders, 1960) and an inshore site at 41°32.29N, 70°52.77W, during April and July 1989 respectively, with a box corer (50x50x70 cm) deployed from the Woods Hole Oceanographic Institution RV *Asterias*. Core sub-samples were taken from the grabs at each

station for subsequent sediment analyses. Both sites are in relatively shallow water (13-18 m) where infauna are subjected to large seasonal temperature variations (Figure 1). The April *S. borealis* specimen was preserved whole and donated to the MCZ mollusc collection at Harvard University, Cambridge, Mass., USA. The July specimen was dissected into gills, foot tissue and remaining soft-parts, and constituent tissues were sub-divided. Tissue fractions were either frozen at -70°C for enzyme and biochemical analyses, dried at 60°C for stable isotope analyses, or preserved in 2% glutaraldehyde in 0.1M phosphate buffer for electron microscopy.

(2) Symbiosis Characterization

Electron microscopy

Sections of freshly dissected gill tissue were fixed and stored in 2% glutaraldehyde in 0.1M phosphate buffer solution. Tissues were post-fixed in 1% osmium, dehydrated through an ethanol series, and embedded in Araldite 6005. Thin sections were stained with lead citrate and viewed with a Hitachi 7000 transmission electron microscope operating at 60 kV.

Ribulose -1,5-biphosphate carboxylase (RuBPcase) activity

RuBPcase activity was determined using the methods of Beudeker *et al.* (1980) and Williams *et al.* (1988). Approximately 0.1 g gill and foot sections which had been stored at -70°C were homogenized on ice in about 5 ml complete assay buffer and passed through a French Press to thoroughly disrupt the cells. A cell free extract (CFE) was obtained by centrifuging the tissue homogenate at 12,000 RCF for 20 minutes. After a preincubation of 300 μl of the CFE at 30°C , 20 μl of $^{14}\text{CNaHCO}_3$ were added and the sample was mixed. Twenty μl samples were removed and added to scintillation vials containing 0.3 ml hyamine hydroxide and 10 ml Packard Ultima Gold scintillation fluid for determination of specific activity. The assay was started by the addition of RuBP to a final concentration of 1.0 mM. 50 μl samples were removed every

minute for six minutes and added to scintillation vials containing 400 μ l of glacial acetic acid at 60°C and left for 20 min to remove any unfixed CO₂. Samples were counted in a United Technologies Packard Minaxib Tricarβ 4000 series scintillation counter and corrected for quenching. The protein content of the CFE was determined using the Biuret protein assay, and the RuBPCase activity was calculated as nmol CO₂ fixed (mg CFE protein)⁻¹ minute⁻¹. RuBPCase activity of fresh spinach was determined as a positive control, while background fixation rates were determined in controls to which no RuBP had been added.

Stable isotope ratios

$\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ stable isotope ratios were determined in dried samples of *Solemya borealis* gill and foot tissue in Dr. Brian Fry's laboratory (Ecosystems Center, Marine Biological Laboratory, Woods Hole, Mass.) as outlined in detail in Conway *et al.* (1989). Samples were combusted in sealed Vycor tubes at 900°C for 1 h, followed by combustion of CO₂ and N₂ gases. All isotope measurements were determined using a Finnigan 251 ratio mass spectrometer. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values are reported relative to Pee Dee Belemnite (PDB), atmospheric N₂, and Canyon Diablo Troilite respectively using the standard delta notation

$$\delta X = [(R_{\text{sample}}/R_{\text{std}}) - 1] \times 10^3$$

where X = ¹³C, ¹⁵N, or ³⁴S and R = ¹³C:¹²C, ¹⁵N:¹⁴N, or ³⁴S:³²S

Lipid analyses

Lipids were analyzed as described in Conway and McDowell Capuzzo (1990a). Lipids were extracted by successive sonication with isopropyl alcohol, chloroform:methanol (1:1), and chloroform:methanol (3:1) after the addition of two internal recovery standards. Half the lipid extract was used for total fatty acid determination and was saponified by heating with 0.5N KOH in methanol. Fatty acids were converted to fatty acid methyl esters (FAMES) by reaction with 10% BCl₃-methanol and separated from other lipid groups by fractionation on a silica gel column. The

remaining lipid extract was separated into lipid classes by thin layer chromatography (TLC) on Whatman LK5D Linear K Silica Gel TLC plates. The solvent system consisted of dichloroethane:chloroform:acetic acid:acetone:isopropyl alcohol (92:8:0.1:0.03:0.03). The fatty acids of each lipid class were saponified and free fatty acids converted to FAMES as described above. All FAME samples were analyzed by gas chromatography after coinjection on a J&W Scientific Durabond DB-5 30 m fused silica column (0.32 mm i.d., 0.25 mm film thickness) and a J&W Scientific Durabond DB-Wax 30 m fused silica column (0.25 mm i.d., 0.25 mm film thickness). FAME identifications were confirmed by electron-impact gas chromatography mass spectrometry (GC-MS).

Amino acid analyses

Total hydrolyzable amino acids (THAA) and free amino acids (FAA) were analyzed in gill and foot tissues using a modification of the phenylisothiocyanate (PITC) derivitization technique (Conway, 1990). For THAA analyses, tissues were hydrolyzed under nitrogen for 24-36 h in sealed ampoules containing 6N HCL. For FAA determination, tissues were extracted with 5% trichloroacetic acid in ethanol:water (50:50). Amino acids were derivitized with PITC solution (ethanol:water:triethylamine:PITC = 7:1:2:1) and separated by gradient elution high pressure liquid chromatography on a C₁₈ reverse phase column at 48°C.

(3) Sediment analyses

Most analyses were determined on triplicate 6.5 cm (i.d.) subcores from box cores at each station during each of five cruises. Subcores were then subdivided into 2 cm depth fractions over a depth range of 0-16 cm.

Nutrient pools

Dissolved and total available ammonium, organic nitrogen and carbon, and porosity and dry density were determined. Porewater for dissolved ammonium and sulfate was collected by centrifugation;

total available ammonium was determined after extracting fresh sediment (10 cm³) with 20 ml of 2N KCL (pH 2) for 18 h. Ammonium concentrations of the porewater and KCL extracts were measured using the Indophenol method (Scheiner, 1976) after filtration (0.45µm, Millipore). Corrections were made for porosity.

Total carbon and nitrogen were determined using a Perkin Elmer CHN analyzer on dried sediment which had been treated with 2N HCL to remove particulate carbonate. Sediment porosity and dry density were determined by drying known volumes of fresh sediment to constant weight at 60°C.

Sulfate reduction rates and reduced sulfur pools

Sediment sulfur pools were fractionated into total reduced sulfur, acid volatile sulfur (AVS; contains hydrogen sulfide and ferrous sulfide), and chromium reducible sulphur pools (CRS; contains elemental sulphur and iron pyrite). Total sulphur was determined using a Leco SC132 sulphur analyzer. AVS and CRS were determined as part of the sulfate reduction experiments outlined below.

Sediments subcores (4.5cm i.d.) were collected from the boxcorer, held and incubated at *in situ* temperature. Rates of reduced sulfur input through microbial sulfate reduction were determined using direct injection of tracer levels of ³⁵S-SO₄ (carrier free, New England Nuclear) into sediment cores (Jorgensen, 1977). Activity was stopped by freezing and cores were held at -40°C until analysis. Recovery of reduced sulfur into acid volatile sulfur (AVS), and chromium reducible sulfur (CRS) fractions was preformed by active distillation (argon passed over hot copper) of 2 cm core fractions during sequential treatment with 1N HCL and hot CrCL₂ (Howes *et al.*, 1984). The AVS pool and its ³⁵S content were analyzed by anaerobic acidification of sediments and collection of liberated H₂S in traps containing ZnCl (10%) and excess ZnOH. The remaining sediments were digested anaerobically for 2h with hot (~60°C) reduced chromium (Zhabina and Volkov, 1976) with subsequent H₂S release trapped as above.

Subsamples (0.6 ml) of the zinc traps were analyzed for $[H_2S]$ using a modification of the procedure of Cline (1969) to yield estimates of AVS and CRS pools. The radioactivity of the trap samples was determined using a Minaxi β Tri-Carb 4000 liquid Scintillation Counter after the addition of 10 ml Aquasol II. These analyses were carried out on 4 cores over 24 h time course experiments on each of the five sampling dates.. SO_4 concentrations were determined on porewater from 2 cm depth fractions of each of three parallel cores using a modification of the turbidimetric assay of Tabatabai (1974).

Results

(1) Symbiosis characterization

Animal description

Both specimens of *Solemya borealis* were approximately 6.5 cm in length and 2.5 cm in width. This species is considerable larger than *Solemya velum* (~2 cm in length and 0.7 cm in width for adults) which occurs in similar sediments. The dissected specimen lacked an observable digestive system and had extremely hypertrophied gills which were red/purple in color with white flecks at the edges; characteristics typical of the other *Solemya* species which have been found to contain endosymbiotic bacteria (Cavanaugh, 1983; 1985). The foot was large and pinkish in color. The periostracum of *S. borealis*, like that of *S. velum*, repelled water as described by Morse (1913).

Electron Microscopy - endosymbiont description

The gills of *Solemya borealis* contain numerous subcellular inclusions which resemble procaryotic cells (Figure 2) and will be referred to as symbionts in this discussion. The symbionts are rod-shaped and have cell envelopes similar to those of Gram-negative

bacteria. Symbionts are variable in length (up to μm) and width (μm to μm) and are intracellular (Figure 2), contained within bacteriocytes bounded by a membrane which is probably host-derived. The overall organization of the gill symbionts in *S. borealis* parallels that observed for *S. velum* and other bivalve-chemoautotroph symbioses (Cavanaugh, 1983; 1985). The symbionts are typically concentrated in the apical regions of the bacteriocytes, and bacteriocytes are confined to the region of the gill filament just distal to the ciliated edge of the gill (Figure 2). Generally, the bacteriocytes are separated by thin goblet-shaped intercalary cells which are symbiont-free. The microvillar surface of the gill filaments appears to be comprised of the apical portion of intercalary cells. Myeline-like inclusions, resembling lysosomal digestion products are typically observed in the apical regions of the bacteriocytes.

Carbon fixation

RuBP-dependent CO_2 fixation activity in the cell free extract (CFE) of the gills of *Solemya borealis*, where the symbionts are located, was significant, while CO_2 fixation in CFE of the foot tissue was not apparent (Figure 3). Carbon fixation rates in the gills of *S. borealis* are between 7.0 and 7.9 nmol CO_2 fixed (mg CFE protein) $^{-1}$ minute $^{-1}$ based on the slope of CO_2 fixation versus activity (Figure 3).

Stable isotope ratios

The stable isotope ratios measured in *Solemya borealis* (Table 1) were extremely negative ($\delta^{13}\text{C} = -32$ to -34.6‰ ; $\delta^{15}\text{N} = -8.6$ to -9.7‰ , $\delta^{34}\text{S} = -15.7$ to -32.6‰), very similar to those previously reported for *S. velum*, and very different from those of typical marine bivalves which are part of the typical phytoplankton-based marine trophic system.

Lipid composition

The fatty acid composition of the gills of *Solemya borealis* are shown in Table 2 and Figure 4. Two different sections of the gill tissue were extracted and analyzed. The most obvious characteristic of the lipids of *S. borealis* are the high concentrations of *cis*-vaccenic acid (18:1 ω 7) present which occurred in concentrations of 2.6-15.0 μ g mg dry wt⁻¹. Analysis of the fatty acid composition of the lipid classes of *S. borealis* demonstrate that, just as in *S. velum* (Conway and McDowell Capuzzo, 1990a), the *cis*-vaccenic acid found in *S. borealis* is located in all the major lipid classes (Table 3, Figure 4).

In *S. borealis*, only low concentrations of PUFAs were found, and these predominantly belonged to the ω 6 series of PUFAs as opposed to the typical ω 3 PUFAs found in most marine bivalves (cf. Joseph, 1982). Levels of plant-derived sterols were negligible. Low concentrations of PUFAs and plant-derived sterols have been noted in other invertebrate-chemoautotrophic bacteria symbioses (Conway and McDowell Capuzzo, 1990a; Giere *et al.*, 1990).

Amino acid composition

The total hydrolyzable amino acid (THAA) and free amino acid (FAA) composition of *Solemya borealis* (Figure 5) very closely resembles that of *S. velum* (Conway, 1990; Conway and McDowell Capuzzo, in prep.). Essential amino acids (EAAs)¹ are present in the THAAs of *S. borealis* and account for 26-40% of the THAA (Table 4). There were extremely large amounts of taurine in the THAA fraction of the foot of *S. borealis* (Figure 5). Because we only had the opportunity to examine one specimen, it is not possible to tell

¹ Essential amino acids are those amino acids that cannot be synthesized by most metazoans including molluscs and include arginine, histidine, valine, threonine, phenylalanine, tryptophan, methionine, valine, leucine, and isoleucine; Lehninger, 1975; Bishop *et al.*, 1983). Although it is not known whether *Solemya borealis* can synthesize these amino acids it is unlikely that this species will differ from most metazoans studied and we will assume that these amino acids are essential dietary requirements for both bivalves throughout the following discussion.

whether this is typical or a feature of this one animal. Apart from the differences in the amount of taurine between the gill and foot THAA profiles are very similar to those of *S. velum*. Taurine accounted for most of the FAA present in *S. borealis* (66-71%, Figure 5) and occurred in very high concentrations (100-200 $\mu\text{mol g wet wt}^{-1}$. (Figure 5), although concentrations were not as high as in *S. velum* (Conway and McDowell Capuzzo, in prep.).

2) Sediment characteristics

The sediments of the *S. borealis* sites in New Bedford Outer Harbor are predominantly composed of fine silts and clays and contain significant levels of organic carbon and nitrogen which increase with depth (Figure 6); porosity decreases with depth from 0.82 to 0.73 ml cm^{-3} . Porewater sulfate levels decreased with depth from a value of 21.1 $\mu\text{mol cm}^{-3}$ in the top 2 cm to 17 $\mu\text{mol cm}^{-3}$ by 14-16 cm. The decrease in porewater sulfate is a result of sulfate reduction by sulfur reducing bacteria. Surface sulfate reduction activity showed a strong seasonal trend ranging from 266 $\mu\text{mol m}^{-2} \text{ h}$ in summer to 29 $\mu\text{mol m}^{-2} \text{ h}$ during the winter (Figure 6). This variability is most likely due to temperature changes, although secondary effects resulting from changes in organic matter availability cannot be ruled out. Acid volatile sulfur species (AVS; includes H_2S and ferrous sulfide) also increased with depth from 0.1 $\mu\text{mol cm}^{-3}$ at 0-2 cm to 3.4 $\mu\text{mol cm}^{-3}$ at 14-16 cm, with a maximum of 5.0 $\mu\text{mol cm}^{-3}$ at 8-10 cm (Figure 6). Sediment total sulfur content increased with depth from a level of 42 $\mu\text{mol cm}^{-3}$ in the top two cm to 163 $\mu\text{mol cm}^{-3}$ in the 14-16 cm depth section (Figure 6). Over 50% of the sediment sulfur content is accounted for by the chromium reduced sulfur species (CRS) which include elemental sulfur and pyrites (Figure 6).

Porewater ammonium concentrations increase from 0.06 to 0.14 $\mu\text{mol cm}^{-3}$ over the 16 cm depth range analyzed (compared to 1 nmol ml^{-1} in the overlying water column ; Howes, Unpub. data). Many of the animal-bacteria symbioses described to date inhabit similar fine-grained reducing-sediments (Dando *et al.*, 1985; Dando

and Southward, 1986; Conway, 1990). The predominant reduced inorganic compounds available for bacterial chemoautotrophy appear to hydrogen sulfide and ferrous sulfides (AVS pool) and ammonium. At present, it is not possible to ascertain the extent to which FeS may be available for utilization.

Discussion

The results presented here suggest that 1) The gills of the protobranch bivalve *Solemya borealis* contain intracellular symbiotic bacteria; 2) The symbiotic bacteria are chemoautotrophic sulfur-oxidizing bacteria which use sediment sulfides to fuel carbon fixation; and 3) The symbiotic bacteria provide a major nutritional source for the bivalve host.

1) *Solemya borealis* contains intracellular symbiotic bacteria

Solemya borealis appears to house numerous subcellular inclusions, which resemble procaryotic cells, in specific gill cells which we will refer to as bacteriocytes. The overall organization of the gill symbionts in *S. borealis* parallels that observed for *S. velum* and other bivalve-chemoautotrophic bacteria symbioses (Cavanaugh, 1983; 1985), suggesting that these symbiotic inclusions are probably bacteria.

It is not possible to tell whether more than one type of bacterial symbiont exists in *S. borealis*. Some bivalve-chemoautotroph symbioses have been reported to harbor more than one type of symbiont (Southward, 1986; Soyer *et al.*, 1989). However, the variation in size and ultrastructure of the symbionts described here is identical to the pattern observed for the chemoautotrophic bacterial endosymbionts of *Solemya velum* (Cavanaugh, 1983; Cavanaugh *et al.*, 1988). Phylogenetic analyses of ribosomal RNA sequences indicate that only one type of symbiont exists in *S. velum* and many other bivalve-chemoautotroph symbioses (Distel *et al.*, 1988). Thus, it is possible that the bacterial

pleomorphism observed in *S. borealis* represents varying developmental stages of the symbionts.

2) *The symbionts of Solemya borealis are chemoautotrophic sulfur-oxidizers.*

Two lines of evidence support our hypothesis that the symbionts described here are chemoautotrophic. The results of the RuBPCase assays (Figure 3) demonstrate that the putative bacterial symbionts of *S. borealis* are almost certainly autotrophic. While the very negative $\delta^{34}\text{S}$ ratios of *S. borealis* tissues (Table 1) provides indirect evidence to suggest that the symbionts are chemoautotrophic, utilizing porewater sulfides as an energy source.

RuBPCase activity is a strong indicator for the presence of autotrophic symbionts in an animal as it is only found in autotrophic organisms. Carbon fixation rates in the gills of *S. borealis* are comparable with those of the bacteria-containing tissues of other invertebrate-chemoautotroph symbioses. No RuBPCase activity was detected in the foot tissue (which contains no endosymbionts). Using similar assay procedures, Williams *et al.* (1988) reported carbon fixation rates of $6.3 \text{ nmol CO}_2 (\text{mg CFE protein})^{-1} \text{ minute}^{-1}$ for the trophosome tissue of the vent tube-worm *Riftia pachyptila* at 30°C . RuBP-dependent CO_2 -fixation activity has also been determined in the *Solemya velum* symbiosis (Cavanaugh, 1983) and is equivalent to $810 \text{ nmol CO}_2 \text{ fixed } (\text{mg CFE protein})^{-1} \text{ minute}^{-1}$ in cell free extracts of gill tissue. In order to compare our data with that of Cavanaugh (1983) it is necessary to convert the rate/g tissue reported for *Solemya velum* to rates/mg protein. The gills of *S. velum* consist of about 78 % water, while protein accounts for approximately 60 % of the dry weight of the animal (Conway, 1990). Thus, the rates reported for *S. velum* by Cavanaugh (1983) correspond to roughly $6.1 \text{ CO}_2 \text{ fixed } (\text{mg CFE protein})^{-1} \text{ minute}^{-1}$, rates very similar to those reported here for the gills of *S. borealis*.

Although we did not have the opportunity to examine *Solemya borealis* for sulfur-oxidation ability, the highly negative $\delta^{34}\text{S}$ ratios found (-15.7‰ for gill tissue and -32.6‰ for foot tissue; Table 1)

strongly suggest that isotopically light porewater sulfides, produced as a result of dissimilatory sulfate reduction processes, is utilized by the symbiosis. Dissimilatory sulfur reduction in marine sediments occurs with a large isotope fractionation effect (-30 to -70‰, Goldhaber and Kaplan, 1975; Fry *et al.*, 1986) and results in sediment sulfides characterized by very negative sulfur isotope ratios. The sulfur isotope ratios resulting from bacterial sulfur reduction are quite distinct from those of seawater sulfate which has $\delta^{34}\text{S}$ ratios of $\sim +20\text{‰}$. Consequently, the $\delta^{34}\text{S}$ ratios in the reducing sediments characteristic of *S. velum* habitats could be in the -15 to -30‰ range. It is probably that these sulfides are oxidized by the bacterial symbionts to fuel carbon fixation and sulfur incorporated by the bacterial symbionts is ultimately utilized by the bivalve host. The differences in $\delta^{34}\text{S}$ ratios between gill and foot tissue is intriguing. Similar differences between foot and gill sulfur isotope ratios have also been noted in *Solemya reidi* (Fry, Pers. Comm.), and to a lesser extent in *Solemya velum* (Conway *et al.*, 1989). The differences are probably due to some as yet unknown internal isotope fractionation process.

It should be noted here that the symbionts of *Solemya borealis* are typically concentrated in the apical regions of the bacteriocytes, and bacteriocytes are confined to the region of the gill filament just distal to the ciliated edge of the gill. This suggests that the symbionts may be provided with nutrients for chemoautotrophy, such as sulfide, oxygen and carbon dioxide, across the gill surface.

3) *The symbiotic bacteria provide a major nutritional source for the bivalve host.*

Analysis of the C and N isotope ratios and the lipid composition of *Solemya borealis* suggests the utilization of endosymbiont nutrients by the host. Stable isotope ratios are often used to identify trophic pathways and processes as a few well characterized reactions are responsible for the stable isotope compositions of most organic matter (Fry and Sherr, 1984; Rounick and Winterbourn,

1986; Spiro *et al.*, 1986). The isotope signatures resulting from these "root" reactions are generally maintained during food-web processes with relatively little change. Thus the stable isotope ratios of an organism can sometimes provide information regarding the "root" reaction responsible for its formation. This is important in the study of animal-bacteria symbioses, as the fixation of carbon during chemoautotrophy often results in low bacterial $\delta^{13}\text{C}$ ratios (Degens, 1969; Ruby *et al.*, 1987; Conway *et al.*, 1989). Thus, animals utilizing bacterial carbon may have more negative $\delta^{13}\text{C}$ ratios than organisms utilizing phototrophically-fixed carbon sources.

The very negative carbon stable isotope ratios found in *S. borealis* suggest that much of the animal's carbon is derived from bacterial chemoautotrophy; marine bivalves utilizing photosynthetically-derived carbon generally have $\delta^{13}\text{C}$ ratios similar to those of marine phytoplankton (-18 to -24‰, Gearing *et al.*, 1984 and references therein). The unusually low $\delta^{15}\text{N}$ isotope found in *S. borealis* (-8.6 to -9.7‰) are similar to those found in *S. velum* (4.4 to -9.8‰, Conway *et al.*, 1989; Table 1) and suggest that the animal may be utilizing nitrogen derived from the endosymbionts, as non-symbiont bivalves generally have $\delta^{15}\text{N}$ values similar to that of seawater nitrate (~6 to 10‰). It is conceivable that the very negative nitrogen isotope values are due to the utilization of non-limiting supplies of pore water ammonium (concentrations of 0.06 to 0.14 $\mu\text{mol cm}^{-3}$ were found over the 16 cm depth range analyzed) as has been proposed for the *S. velum* symbiosis (Conway *et al.*, 1989).

The lipid composition of marine bivalves is usually influenced by dietary sources (Pollero *et al.*, 1979; Moreno *et al.*, 1980; Piretti *et al.*, 1987) particularly with respect to the polyunsaturated fatty acid (PUFA) and sterol composition, as many of these lipids cannot be synthesized by animals *de novo* (Lehninger, 1975; Goad, 1976). Consequently, the lipids of animal-bacteria symbioses may reflect the utilization of endosymbiont nutrients if endosymbionts are utilized for nutritional purposes (Conway and McDowell Capuzzo, 1990a;b).

Cis-vaccenic acid is the most common lipid in *S. velum* (Conway and McDowell Capuzzo, 1990a;b), the *Inandirilus* (= *Phalloedrillus*) *leukodermatus* invertebrate-chemoautotroph symbiosis (Giere *et al.*, 1990) and many Gram-negative sulfur-oxidizing bacteria (Fulco, 1982, Conway and McDowell Capuzzo, 1990a;b and references therein), and is predominantly a bacterial fatty acid which is synthesized using the bacterial anaerobic monounsaturated synthesis pathway (Goldfine, 1972; Fulco, 1983). The high concentrations of this lipid in *Solemya borealis* suggests the incorporation of bacterial lipids by the host.

The extreme variability between the *cis*-vaccenic acid composition of different gill segments is unusual (Table 2). The most likely explanation is differences in tissue composition. The endosymbionts of many animal-bacteria symbiosis are often localized in specific sections of the gills, the variability in lipid composition of the gills of *S. borealis* may reflect differences in the bacterial content of the gill sections examined. In this context, the bacteriocytes of *S. borealis* appear to be confined to the region of the gill filament distal to the ciliated edge of the gill, demonstrating intertissue differences bacteriocyte concentrations. In *S. velum*, intertissue differences in fatty acid concentrations may also be present; however, because of the relatively small size of *S. velum* samples, whole gill tissues are generally analyzed (Conway and McDowell Capuzzo, 1990a) obscuring such differences.

The location of *cis*-vaccenic acid in all the lipid classes of *S. borealis* suggest that this lipid is probably involved in a variety of physiological processes in *S. borealis*, and may be important in both membrane structure maintenance and energy production. In *S. velum* *cis*-vaccenic acid is predominantly found in the phospholipid pool (Conway and McDowell Capuzzo, 1990a), interestingly, in *S. borealis*, *cis*-vaccenic is more localized in the mono-, di-, and triglyceride pools. This is probably due to differences in the ambient site temperature and the reproductive state of the specimens at the time of sample collection. The specimen of *S. borealis* was collected during July, when ambient temperatures are

higher (Figure 1). In this situation, it is possible that greater amounts of the *cis*-vaccenic acid are stored as tri- and diglycerides and less of the bacterial fatty acid is mobilized for membrane structural requirements. On the other hand, *S. velum* specimens were collected during November (Conway and McDowell Capuzzo, 1990a), when triglyceride pools would be expected to be reduced as a result of reduced metabolic rates.

The low levels of plant derived PUFAs and sterols in *S. borealis* demonstrate that marine algae are not an important nutritional source for this species. Low levels of plant-derived lipids have also been noted in other invertebrate-chemoautotroph symbioses (Conway and McDowell Capuzzo, 1990a;b; Giere *et al.*, 1990). The source of the small amounts of 20:4 ω 6 and 22:4 ω 6 PUFAs in *S. borealis* are unknown, but possibilities include *in vivo* synthesis by the host or the putative endosymbionts, ingestion of small amounts of marine algae, or epithelial uptake of dissolved organic matter. The high levels of *cis*-vaccenic acid and low levels of PUFAs found in *S. borealis* provide further evidence to suggest that the diet of this bivalve is based on bacterial chemosynthesis. Moreover, the presence of high concentrations of *cis*-vaccenic acid in marine invertebrates may be diagnostic for the presence of chemoautotrophic bacterial symbionts.

The small gut of *Solemya borealis*, and the likelihood that bacterial endosymbiotic bacteria are the main nutritional source for this species suggest that a large proportion of the essential amino acids (EAAs) found in *S. borealis* are synthesized *de novo* by the symbiosis, either directly by the bacteria, or using carbon and nitrogen derived from the bacteria. In both *S. borealis* and *S. velum* (Conway, 1990) the foot tissue FAA pool contains the highest levels of taurine of the tissues examined. While only one specimen of *S. borealis* was examined, and amino acid profiles may vary between individuals, the close similarities between the amino acids of *S. borealis* and *S. velum* suggest the metabolic processes operating in the two congeneric species are similar, particularly with respect to taurine metabolism. Overall, the biochemical composition of *S.*

borealis and *S. velum* (Conway, 1990; Conway and McDowell Capuzzo, 1990a) is strikingly similar, suggesting strong similarities between the types of bacterial symbiont present and host metabolic processes in these two related species.

The appearance of myelin-like inclusions in the bacteriocytes (Figure 2) of *Solemya borealis* suggest that it is possible that in addition to the direct translocation of soluble organic compounds (Fisher, 1986) and lipids (Conway and McDowell Capuzzo, 1990) observed in other solemyids, the symbionts of *S. borealis* may be autolyzing or being actively digested. Subsequent transfer of nutrients such as lipids and amino acids to the host may then occur via the adjacent blood space. The symbionts may thus be actively 'farmed'.

The detailed analysis of the sediments where both specimens of *S. borealis* were found allows us to place limits on the concentrations of reduced sulfur sources available for use by the putative chemoautotrophic symbionts of this bivalve. Although detailed whole animal metabolic studies were not conducted, the similarities between *S. borealis* and other solemyids, coupled with our sediment sulfur measurements, allows us to place limits on the animal-sediment relationships. Given the evidence presented here, it would appear that symbiotic sulfur-oxidizing bacterial symbionts form the main nutritional source for *S. borealis*. The significant input of reduced sulfur to the sediments ($0.86 \text{ mol S m}^{-2} \text{ yr}^{-1}$) from *in situ* microbial sulfate reduction and the highly negative $\delta^{34}\text{S}$ ratios of *S. borealis* supports the use of sediment sulfide as the main energy source for the *S. borealis* symbiosis, as has been found for *S. velum* (Cavanaugh, 1983; 1985; Chen *et al.*; 1987) and *S. reidi* (Anderson *et al.*, 1987).

Using available data on the sulfur cycle of the sediments supporting *S. borealis* and literature values for conversion efficiencies and respiration rates of cogeneric species it is possible to determine the scales of animal sulfide requirements versus sulfide availability. We will concentrate on summer months when sediment

temperatures are warmest (20°C, Figure 1) and respiration rates highest. Since whole animal O₂ consumption rates were not determined for *S. borealis*, the metabolic rates of related species were used in the calculations. Felbeck (1983) calculated the O₂ consumption of *S. reidi* at 2.1 μmol O₂ g wet wt⁻¹ h⁻¹, while Cavanaugh (1985) estimates that *S. velum* uses 9.6 μmol O₂ g dry wt⁻¹ h⁻¹. The specimen of *S. borealis* analyzed weighed 2.3 g, therefore an approximate respiration rate of 4.8 mmol O₂ individual⁻¹ h⁻¹ was estimated as an approximate summer respiration rate for this species. The sulfur demand needed to support this metabolic rate was calculated using the calculation of Kelly and Keunen (1984) that for each mole of reduced sulfur oxidized by a bacterial symbiont 0.2 mol of organic carbon would be fixed by the bacteria, yielding approximately 0.04 mol carbon for the host (if we assume 20% efficiency). Although these values are only approximate, they suggest that between 24-120 μmol S²⁻ individual⁻¹ h⁻¹ must be used for carbon fixation (assuming O₂:C = 1:1) to support the calculated respiration rates (the range is 100% to 0% of respiration by bacteria versus host). The calculated sulfide uptake requirement by *S. borealis* can be compared to sediment sources of reduced S. The measured reduced S input in summer was 220 μmol m⁻² h⁻¹ (0-16cm) versus a sedimentary pool of AVS of ~5 μmol cm⁻³.

It is likely that *S. borealis* is satisfying its sulfide requirement by "mining" the sediments, as its demand is between 10-50% of the daily S input. The relatively low concentration of soluble sulfides suggest that either large volumes of porewater are being extracted, or more likely that soluble partially oxidized sulfur species (thionates, Jorgensen, 1990) or "particulate" sulfide, most likely iron sulfides are being utilized. The mechanism for such uptake is as yet unclear but given the potentially large sulfide demand of this organism, even at low densities its effect on sediment sulfur cycling may be significant. The seeming disparity between soluble sulfide availability and the sulfide demand needed to support animal-bacteria symbioses has been observed by other researchers (Dando *et al.*, 1985; Dando and Southward, 1986).

To summarize, the microscopic, enzymatic, stable isotope, and biochemical data presented here provides strong evidence for the existence of bacterial endosymbionts in the gills of *Solemya borealis*. The gills of *Solemya borealis* contain numerous subcellular inclusions which resemble procaryotic cells. High levels of RuBPCase activity are found only in the symbiont-containing gills of *S. borealis* suggesting that the symbionts are autotrophic, while the very negative $\delta^{34}\text{S}$ ratios of *S. borealis* suggest that porewater sulfides may fuel bacterial chemosynthesis. The very negative $\delta^{13}\text{C}$ and $\delta^{14}\text{N}$ isotope ratios of *S. borealis* indicate that almost all of the animals carbon and nitrogen is provided by a bacterial diet, while the lipid composition of this species demonstrates incorporation and utilization of bacterial lipids. *S. borealis* is remarkably similar to *S. velum* with respect to stable isotope ratios and biochemical composition (Conway *et al.*, 1989; Conway, 1990; Conway and McDowell Capuzzo, 1990a;b). When the morphology of the animal is considered (very small digestive system, large gills containing endosymbiotic bacteria) it seems most likely that the endosymbionts provide the dominant food source for *S. borealis*. This conclusion is consistent with the availability of reduced sulfur species in sediments colonized by *S. borealis*, although uptake from reduced sulfur pools other than soluble S^{2-} may be important.

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Figure legends

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a) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios

Species	$\delta^{13}\text{C}$ ratios	$\delta^{15}\text{N}$ ratios
<i>Solemya borealis</i> (gill)	-34.6‰	-9.7‰
<i>Solemya borealis</i> (foot)	-32‰	-8.6‰
<i>Solemya velum</i> (gill) ¹	-32.4 to -33.9‰	+0.4 to -9.8‰
<i>Solemya velum</i> (foot) ¹	-30.9 to -32.1‰	+4.4 to -4.6‰
<i>Solemya velum</i> "bacteria" ¹	-32.3 to -33.6‰	-7.9 to -8.6‰
<i>Mya arenaria</i> ¹	-17.2 to -17.8‰	+8.3 to +8.5‰
<i>Tellina agilis</i> ¹	-14.2 to -15.6‰	+6.3 to +8.2‰

b) $\delta^{34}\text{S}$ ratios

Species	$\delta^{34}\text{S}$ ratios
<i>Solemya borealis</i> (gill)	-15.7‰
<i>Solemya borealis</i> (foot)	-32.6‰
<i>Solemya velum</i> (gill) ¹	-26.7 to -28.2‰
<i>Solemya velum</i> (foot) ¹	-29.2 to -31.1‰

¹ Conway *et al.*, 1989.

Table 2. Fatty acid composition of *Solemya borealis* gills.

Fatty acid	Extraction 1 ng mg dry wt. ⁻¹	Extraction 2 ng mg dry wt. ⁻¹	Mean ng mg dry wt. ⁻¹
14:0	4510	4340	4420
14:1 ω 5	23	200	110
a-15:0	25	23	24
15:0	58	59	58
16:0	13070	13160	13110
16:1 ω 7	4820	1040	2930
a-17:0	53	0	27
17:0	190	64	130
18:0	5550	4910	5230
i-18:0	0(b)	0	0
18:1 ω 9	1800	1620	1710
18:1 ω 7	14980	2570	8770
18:2 ω 6	480	19	250
18:3 ω 3	80	0	38
18:3 ω 6	0	0	0
18:4 ω 3	200	0	99
20:0	73	10	41
20:1* (a)	0	120	58
20:1 ω 9	1710	110	910
20:1 ω 7	1290	220	750
20:2*	140	0	700
20:2 ω 6	810	120	460
20:3*	360	0	180
20:3 ω 3	590	150	370
20:4 ω 6	1130	0	560
20:5 ω 3	0	0	0
22:0	28	0	14
22:1 ω 9	60	0	30
22:2 ω 6	0	0	0
22:2*	0	0	0
22:3 ω 3	0	0	0
22:4 ω 6	1900	2560	2230
22:6 ω 3	36	0	18
24:0	38	0	19
Total	55250	31250	43250

(a) Position of double bonds unknown; (b) below the detection limit of 1 ng mg dry wt.⁻¹

Table 3 Fatty acid composition of the major lipid classes of *Solemya borealis* (Fatty acids of each lipid class presented as a percentage of the total fatty acids of that class.

Fatty Acid	Phospholipids	Monoglycerides	Diglycerides, Triglycerides + FFAs	Steryl Esters + Methyl Esters
14:0	4.9	5.8	6.4	5.0
16:0	19.7	16.1	18.7	25.1
16:1 ω 7	17.1	8.8	10.6	8.2
18:0	8.9	7.8	7.9	13.2
18:1 ω 9	2.1	4.5	4.4	6.1
18:1 ω 7	27.7	42.1	38.8	28.4
18:2 ω 6	2.5	1.9	1.2	0.9
20:1 ω 9	1.7	2.3	0.6	2.8
20:1 ω 7	1.8	4.0	4.0	4.0
20:2 ω 6	4.3	0.0	0.7	1.1
20:3 ω 3	3.0	0.0	1.0	1.7
20:4 ω 6	2.0	3.6	2.4	2.6
22:2 ω 6	2.3	1.8	2.4	0.0
Total	97.9	98.9	99.0	99.2
	Free Sterols (ng/mg dry wt.)	Percent of Total	Steryl esters (ng/ng dry wt.)	Percent of Total
Cholesterol	1722	78.4	939	97.5
Other Sterols	475	21.6	24	2.5

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APPENDIX II

REGULATION OF GUTLESS ANNELID ECOLOGY BY
ENDOSYMBIOTIC BACTERIAO. Giere⁺, N. Conway^{*}, G. Gastrock⁺ and C. Schmidt⁺⁺Zoological Institute

University of Hamburg

Martin-Luther-King-Platz 3

D-2000

Hamburg 13

Germany

^{*}Woods Hole Oceanographic Institution

Woods Hole

Ma. 02543

USA.

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Regulation of gutless annelid ecology by endosymbiotic bacteria**O. Giere⁺, M.M. Conway^{*}, G. Gastrock⁺, C. Schmidt⁺**

⁺ Zoological Institute, University of Hamburg, Martin-Luther-King-Platz 3,
D-2000 Hamburg 13, Federal Republic of Germany

^{*} Woods Hole Oceanographic Institution, Coastal Research Lab, Woods Hole,
MA 02543, U.S.A.

ABSTRACT: In studies on invertebrates from sulphidic environments (thiobios) exploiting reduced substances through symbiosis with bacteria, ecological results are underrepresented and experimental work is generally lacking. The gutless oligochaete *Inanidrilus leukodermatus* contains endosymbiotic sulphur-oxidizing bacteria and inhabits the sediment layers around the redoxpotential discontinuity (RPD) where there is access to both microoxic and sulphidic conditions. This annelid species is highly suitable for ecological studies, notably on the distributional effects of symbiotic associations with sulphur bacteria as it is very mobile and locally abundant.

By experimental manipulation of physico-chemical gradients we have shown for the first time that the distribution pattern of these worms directly results from active migrations towards the variable position of the RPD, demonstrating the ecological relevance of the concomitant chemical conditions for these worms. Their distributional behaviour probably helps to optimize the metabolic conditions for the endosymbiotic bacteria coupling the needs of symbiont physiology with host behaviour and ecology. The prevailing bacterial role in the ecophysiology of the symbiosis was confirmed by biochemical analyses (stable isotope ratios for C and N; assays of lipid and amino acid composition) which showed that a

dominating portion of the biochemical fluxes in the symbiosis is bacteria-based.

It appears that the distributional and nutritional ecology of the gutless annelids is regulated by their symbiosis with sulphur bacteria. These ecological and biochemical data supplement and confirm our earlier structural, microbiological and physiological results underlining the importance of the prokaryotes in this highly evolved and much integrated mutualistic bacteria - animal association.

Running head page:

Regulation of gutless annelid ecology

INTRODUCTION

Many symbioses between invertebrates and chemoautotrophic bacteria are documented at the structural, and to some extent physiological and microbiological levels (for reviews see Cavanaugh 1985, Southward 1987). However, the ecological effects resulting from this close partnership between bacteria and animal host are generally less recognized.

Thiobiotic animals, both meio- and macrobenthic, tend to live around the redox chemocline (Powell & Bright 1981, Powell et al. 1983, Scherer 1985, Southward 1987, Meyers et al. 1987, 1988, Ott & Novak 1989). In this preferred layer they have access to both mild concentrations of reduced sulphur and oxygen. Initial analyses of populations of the gutless tubificid *Inanidrilus (Phallodrilus) leukodermatus* by Giere et al. (1982) suggested that this pattern is also found in bacteria-symbiotic annelids from sulphidic sediments. The present paper describes in more detail their vertical field distribution as related to the physico-chemical gradient system.

To date, this apparently general distributional pattern in thiobiotic animals has not been experimentally tested, and it is not known whether the symbiotic system can adjust to redox and sulphide gradients in the habitat. Understanding the environmental behaviour of the host is important in determining the extent to which the metabolic demands of the symbiotic endobacteria regulate the host's activities, distributional patterns and migratory behaviour within the physico-chemical gradients of the sedimentary habitat.

Here, we have attempted to understand the distribution of *Inanidrilus (Phallodrilus) leukodermatus* by manipulation of the sediment gradient system with respect to oxygen and reduced sulphur compounds. Among the "sulphide fauna", one of the few animals suitable for experimental ecological work

is *I. leukodermatus* due to its sufficient size, high mobility, locally rich populations and its well documented scientific background (Giere & Langheld 1987, Giere 1989). This meiobenthic worm lives symbiotically with chemoautotrophic endobacteria which oxidize preferentially thiosulfate in order to obtain their metabolic energy (Giere et al. 1988 a).

In order to further characterize the relationships between endosymbiont and host we also studied the nutritional interactions in this symbiosis. The role of the bacterial symbionts as a nutritional source for their gutless hosts is difficult to assess because of the intricate structural and physiological interactions between the symbiotic partners (Giere & Langheld 1987). Analysis of biochemical composition and measurement of stable isotope ratios (C and N) can give indications about the relevance of bacterial chemoautotrophy for the diet of the worms (Conway & McDowell Capuzzo 1990 a).

In horizons with good access to reduced sulphur, the subcuticular bacteria of *I. leukodermatus* store droplets of hydrophilic sulphur (polythionates, see Steudel 1989) and globules of polyhydroxy butyric acid (PHB) as reserve substances. In a well supplied worm, these together can make up 6 % of its whole body dry weight. The storage of these rich bacterial reserve substances obtained from the sulphidic milieu makes the worms usually appear shiny white. Only rarely, i.e. in the oxygenated surface horizons, are worms greyish-transparent. Hence, the position of the worms within the steep chemical gradients of the vertical sand column should have a marked influence on either the production or consumption of these storage substances which is evidenced by their appearance as "white" or "pale" worms (Giere et al. 1988 a). Consequently, experimental manipulation of the supply with reduced sulphur should influence not only the migratory behaviour, but also the appearance of the worms.

In this paper we describe the results of three different, but complementary approaches to the study of the ecological coupling between endosymbionts and host in *I. leukodermatus*. 1. The distributional ecology of the worms was determined by investigation of their vertical field distribution, 2. the distributional pattern and appearance ("white" versus "pale") of field-collected specimens were analysed following laboratory manipulations of sediment chemical gradients in order to study the effects of a) nutrient supply to the symbiotic bacteria, and b) variations in the position of the anoxic/oxic interface. 3. The nutritional role of the symbionts in the diet of the annelid host was investigated using biochemical markers for bacterial activity.

MATERIALS AND METHODS

Most field and experimental studies were done in the summer of 1989 in the laboratories of the Bermuda Aquarium, Museum and Zoo, close to the sample stations in Flatts Inlet, the narrow passage from the sea to Harrington Sound. We obtained fresh worms, original sand and seawater directly from the sampling site. All samples were taken by SCUBA divers from depths between 2 and 4 m. To supplement, data from vertical cores taken with essentially identical methods at the same location during former stays in Bermuda were included in the results and discussion.

Vertical cores from the sublittoral sediment in Flatts Inlet (Bermuda) were taken by SCUBA divers to study the microdistribution of the worms in relation to the pore water conditions (perspex corer of 5 cm internal diameter and 30 cm length). Cores were subdivided immediately after retrieval, and split into 2.5 or 3 cm fractions, the redox values were recorded and the worms quantitatively decanted and counted upon return to the laboratory.

The sulphide content in the pore water was measured in 10 vertical series (three in 1981, six in 1986 and one in 1989). For determination of dissolved sulphide in the natural sediment (pH 7.9–8.5), undisturbed pore water samples were collected by divers using small hypodermic syringes (Giere et al. 1982, Howes & Wakeham 1985). In 1989 spectrophotometric analysis (modified after Cline 1969) could not be done *in loco*, consequently the sulphide, precipitated by alkaline zinc acetate, was filtered through millipore filters (pore width 0.2 μm). These filters were kept on Millipore special filter jars in the refrigerator until return to the laboratory where the precipitate was dissolved for further photometric analysis by addition of 5.5 N HCl. Using this method the amount of sulphide in the samples might be slightly underestimated due to immediate oxidation during handling of the syringe, precipitation loss and bacterial degradation. The resulting data, therefore, represent conservative, yet in their overall range reliable values.

Porewater for analysis of the amino acids was sampled by a "pore water lance" (Giere et al. 1988 b).

Manipulation of the RPD depth: Migration experiments were designed to investigate the vertical distribution of worms allowing to set the RPD layer in a given horizon of a sand column. Perspex tubes ($\varnothing$ 2,6 cm, length 15 cm) were filled with reduced, gray or oxygenated, white sand, and a sharp redox-cline was set up at a given horizon. The bottom and top of each core was covered with nylon gauze (10–20 μm mesh size). After insertion of 20 active, undamaged worms into a chosen 3cm-layer, the cores were placed in a small glass tank which contained a reduced sediment layer in the depth and an oxygenated surface. The top of the tube rose above the sediment and aerated seawater was allowed to flow around it.

As a generalized indication for oxygenated or reduced conditions (Giere et al. 1988 b) in the experimental cores, redox values were recorded

in every 3cm-layer when fractionating the sediment core at the end of each experimental run (mostly after 72 h). By subsequent careful decantation of the 5 resulting subsamples (about 16 cm³ each) the new position of the worms was evaluated (retrieval rate mostly 90-100 %) and recorded with respect to the monitored redox value. A few cores from first experiments in 1986 were included in the evaluation. Regrettably, within the time available, not all experiments could be based on sufficient numbers of replicates for reliable statistical evaluation (see Results).

Experimental pale/white conversion of worms as a result of variations in nutrient supply of the symbiotic bacteria was performed in small experimental tubes (about 2 cm³) filled with the original sediment and water. After insertion of 5 worms, each box was capped with nylon gauze of 10-20 µm mesh size and implanted into the sulphidic or oxic sand layers of the same glass tanks used for the migration experiments. Average duration of each experiment was 72 hours before the worms were retrieved and their color inspected. Similar experiments performed during former stays on Bermuda were incorporated into the evaluation.

For measurement of stable isotope ratios (C and N), air-dried worms, sampled in October 1989 in Bermuda, were analyzed in Woods Hole (for detailed methods see Conway et al. 1989). Due to the limited amount of material, each determination was based on one pooled worm population (about 100 specimens); thus, the values obtained are preliminary.

Lipids were analysed chromatographically after conversion into fatty acid methyl esters following the methodology of Conway & McDowell Capuzzo (1990 b). The values given here are averages of 3 extractions of pooled worms.

The amino acid composition of the worms was measured after formation of phenylcarbamyl derivatives (modified after Yang & Sepulveda

1985). Determinations were based on 2 parallel subsamples of pooled, dried worms.

RESULTS

Vertical field distribution of chemical gradients and worms

a) relevant physiographical factors:

Except for the top 2.5 cm-layer the calcareous sand is fairly fine (Md in the range of 150 μm , lower and upper quartiles at 91 and 430 μm). The sorting coefficient of 2.1 characterizes it as "very poorly sorted" indicating the typical situation in calcareous sands with lots of fine detritus and shell debris clogging the interstices. The top layer, with its coarse shell particles (Md = 528) covering this fine material, prevents the sediment from being eroded, but apparently also reduces penetration of oxygenated water to a greater depth despite the strong tidal currents. This sedimentary situation is reflected by the gradients of redox potential and sulfide: Average Eh-values ranged from +250 mV in the uppermost subsample (3cm-layer) to approximately -150 mV with the discontinuity usually in the depth of 7-8 cm. However, rather than a sharp boundary, the change from oxic to reduced conditions was mostly gradual. This was paralleled by the transition zone from whitish to a greyish-dark coloration of the sediment due to formation of ironsulphides. It usually coincided well with the recorded RPD-threshold.

The layers with negative redox potential beyond about -50 mV smelled distinctly of H_2S . Values for sulphide in the pore water of the deeper layers showed large variations between samples depending probably on the local sediment structure and exposure to the water current. Their maximum values (in depths of 15-20 cm) were in the range of about 200 $\mu\text{mol S}^2$. In the layers preferred by the worm population (see below), the

sulphide content was far below these extreme values and usually had a range of 5 to 50 $\mu\text{mol S}^=$.

b) animal distribution:

In all vertical cores the populations of *Inanidrilus leukodermatus* centered around, or somewhat underneath, the RPD layer with a steep decrease towards the oxic subsurface layer and the sulphidic depth horizons. This is shown in Fig. 1, a combined evaluation of 5 vertical cores. Although taken from just one locality (approximately 3x2 m²), the density of worms per core was variable resulting in high standard deviations particularly around the RPD layer.

If worm numbers from all samples are compiled and calculated in a polynomial regression against either sediment depths (Fig. 2) or redox values respectively (Fig. 3), the result in both cases is a significant relation. The preferred depth is shown to be again between 5 and 7 cm and at redox values (Eh) between +50 and -50 mV, i.e. positioned symmetrically around the RPD threshold. Also the Spearman-Rank Test showed a clearly significant relationship between worm number per subsample and these parameters ($p=0.01$ for redox values, $p=0.001$ for depths).

c) Experimental variation in nutrient supply for the symbiotic bacteria:

If "white" worms with bacteria containing a rich supply of light-refractile reserve substances, were kept for 72 hours in oxygenated sediment, the colour of the worms changed gradually and became completely greyish-pale and rather transparent ($n=15$ replicates). The worms stayed fully active and showed no signs of sluggishness during this experimental period.

In controls, white worms from the same sample unit, kept for the same period of time in highly sulphidic sediment ($>300 \mu\text{mol S}^=$), stayed fully white. However, under these conditions a certain portion was always

found dead or sluggish, albeit still white. This result indicates that the worms, despite their high tolerance to anoxia and sulphide, are more adapted to short-term stays than to a permanent exposure to these conditions. In contrast, anoxia and sulphide did not seem to adversely affect the endosymbiotic bacteria, which maintained their supply of reserve substances, indicated by the persisting white colour of the worms.

When "pale" worms from oxygenated sediment with bacteria lacking reserve substances, were inserted into sulphidic sediment, they usually turned white after 72 h ($n=15$ replicates). However, in these experiments some specimens only became just whitish, not comparable to their normal shiny white appearance. Pale worms were much more delicate to handle and less resistant, so that some of the test animals died. In controls, pale worms, kept under permanently oxygenated conditions, always stayed pale, but over an experimental duration of 72 h the percentage of sluggish or dead worms increased to about 30 %. Interestingly, in some longer-term experiments we could change white animals into pales and, after a further 72-hours run in appropriate sulphidic conditions, reverse their appearance back into white.

These results, obtained in small "cages", were confirmed also in the tubes set up for migration experiments (see below). Here, the worms' colouration was often found to change as a result of the oxic/sulphidic character of the ambient sediment.

Correspondingly, under natural conditions (i.e. in vertical cores taken in the field) the appearance of the worms is not uniform. Most animals are clearly white, however, a small part of the population (about 5 %) is found pale. Considering the fact that the appearance of the animals evidently mirrors the supply with sulphide and oxygen, this field observation indicates vertical migrations along the steep chemical gradient system.

Migration experiments:

In this set of experiments we wanted to experimentally verify the assumption that worm migrations are dependent on the chemical regime.

a) Redox and sulphide gradients in the test tubes:

The redox potential in the reduced sand we used was about - 210 mV Eh, that of its oxygenated counterpart was recorded as + 230 mV Eh. Under the experimental conditions described above, redox potentials in the various layers of the artificial sand column stayed relatively stable throughout the duration of the experiment, sometimes with a slight upward drift of the RPD layer which usually clearly coincided with the characteristic colour change of the sand grains from whitish to gray. Sulphide content in the reduced sediment used for the experiments was around 300 $\mu\text{M S}^{2-}$ indicating complete anoxia. The development of sulphide concentrations in the experimental tubes with the RPD positioned at 13 cm depth was followed in a time course series of 6 parallel tubes over the duration of 96 hours. Between 24 and 72 h (the usual end point of the experiments) sulphide in the two bottom cm of the tubes remained relatively stable around a median concentration of 260 $\mu\text{M S}^{2-}$ with variations between 240 and 300 $\mu\text{M S}^{2-}$. This maintenance of the redox and sulphide regime throughout the time period of the experiments underlines the reliability of the experimental set-up.

b) Reaction of the worms to changes in redox conditions:

All experimental runs have been tested statistically for randomness of the central distributional trend (Friedman test). All samples clearly showed a non-random trend in their distribution. The various experimental runs (each lasting for about 72 h) can be grouped in four categories:

White worms:

- The RPD was established in about 13.5 cm depth, 1.5 cm above the bottom of the tube, with the worms inserted at the top (Fig. 4). After 3 days, almost 70 % of the worms were regularly found in the bottom layers around the RPD ($n = 10$ experimental runs, differences of mean values highly significant; $p=0.001$ for a non-random distribution).
- Setting the RPD in a median position (6-7 cm) and inserting the worms at the bottom resulted in an accumulation of worms in the middle of the tube (Fig. 5) with a more gentle decrease of worm numbers towards top and bottom ends ($n = 4$ runs, mean values of middle layers significantly different at 90 % level from those of top and bottom).
- A similar, but more pronounced distribution pattern became established, if the RPD was positioned close to the top end of the tube (in the 3cm horizon) with the worms again starting from the bottom. 90 % of the worms were found in the two upper fractions with a clear maximum in the moderately reduced, low sulphidic horizon close to the RPD (Fig. 6). Due to the low number of runs ($n = 2$), we could not calculate the statistical significance, however, the migration pattern is clear.

Pale worms (generated by leaving "whites" for about 24 to 48 h in oxygenated sediment):

- Again the RPD was established in about 13.5 cm depth, 1.5 cm above the bottom of the core, while the worms were inserted at the top (Fig. 7). After 3 days, 80 % of the worms were regularly found near the bottom around the RPD ($n = 5$ experimental runs, differences of mean values in bottom layers highly significant from those in 3 uppermost layers; $p=0.005$ for a non-random distribution).

These 4 groups of experiments suggest that it is the specific environmental condition at the RPD which attracts the worms. This is confirmed by the additional experimental runs in which the worms were inoculated directly into the redoxcline. The worms stayed here, for the duration of the experiment, exhibiting no migration. Correspondingly, in some tubes, where maintenance of the RPD-threshold was not kept constant over the experimental time period, and which, consequently, showed at the end of the experimental period a homogeneous, positive or a negative potential throughout, the worms were $\pm$ evenly distributed without any recognizable trend.

In experiments lasting shorter than about 60 h, the structuring impact of the RPD for the distributional pattern of the worms could not be evidenced. Here, only a migratory trend in the direction towards the RPD was discernible, but a marked maximum never developed over the short time period. In experiments lasting only 12 to 24 h, no clear pattern was evident even when a constant RPD layer was maintained.

Stable isotope ratios:

In the minute tubificid *Inanidrilus leukodermatus* the symbiotic bacteria are intimately integrated in the cuticle/epidermis complex and a separation of bacteria-containing and bacteria-free tissues is not possible. Thus, the total worm homogenate had to be measured. The resulting $\delta^{13}\text{C}$ value ($^{13}\text{C}:^{12}\text{C}$ ratio) was -26.0‰ ; the corresponding $\delta^{15}\text{N}$ ($^{14}\text{N}:^{15}\text{N}$ ratio) was -1.4‰ .

The lipid composition of *I. leukodermatus* tissue (Fig. 8) was dominated by the five fatty acids 16:0, 16:1 ω 7, 18:0, 18:1 ω 7 and 18:1 ω 9, which accounted for 77.9 % of the total fatty acid pool. *Cis*-vaccenic acid (18:1 ω 7) made up for 43.0 %, while the other dominating fatty acids,

palmitic acid (16:0) and stearic acid (18:0) accounted for 13.5 % and 9.3 % respectively.

Among amino acids, Glycine, Alanine, Valine and Leucine accounted for 56 % of total amino acids. Although in marine organisms these are generally the most common amino acids, it is noteworthy that in our symbiotic worms the whole set of "essential" amino acids was present (Fig. 9, Tab. 1), even those which usually cannot be synthesized by the eukaryotic host. They amounted to about 50 % of total. (Tryptophan has not been recorded here, but this amino acid often gets destroyed during acid hydrolysis.)

DISCUSSION

Under natural conditions, the redox chemocline is a complicated and dynamic "landscape" (Ott & Novak 1989) representing, in calcareous sands, a rather gradual transition zone from positive to negative values. Thus, for sulphur bacteria the area where microoxic and mildly sulphidic conditions overlap, is favourably wide. Movement towards this horizon of optimal factorial combinations and mobility within this network seems particularly important in these types of symbiotic associations as sulphur bacteria prefer changing gradients of sulphur compounds and oxygen (Jørgensen & Revsbech 1983, Jannasch, pers. communication). Here, even micro-electrodes are too insensitive to measure the ever fluctuating redox conditions in due resolution and detail. (Revsbech, personal communication). Thus, our Eh-recordings give only relative "background informations" about the general oxic conditions, and should not represent a basis for quantitative calculation of fluxes.

Thiobiotic animals adjust in different ways to the preferred conditions at the chemocline: Less mobile symbiotic animals like bivalves or the sessile pogonophorans may bridge over the range of reduced,

sulphidic and low oxic conditions by anatomical or physiological adaptations (ventilation currents by siphons, specific transport properties of blood, see Cavanaugh 1985, de Burgh 1986, Dando et al. 1986, Reid & Brand 1986). Small meiobenthic thiolbiota, however, can actively move along the steep chemical gradients around the RPD adjusting themselves to the optimal position in this temporally and locally much changing zone (Giere et al. 1982, 1984, Powell et al. 1983, Fox & Powell 1987, Meyers et al. 1987). Meyers et al. (1988) showed the prompt response of various meiobenthic thiolbiota to experimentally fluctuating water changes with subsequent changes in oxic and sulphidic gradients. Ott & Novak (1989) in preliminary experiments with thiolbiotic nematodes found a corresponding positive reaction to the conditions around the redox cline.

Our experimental studies provide direct evidence, to show that the depth of the RPD layer controls the distribution of thiolbiotic animals. In the field situation fluctuations in the depth distribution of the annelids are more related to the changing location of the oxic/anoxic interface of the sediment than to the actual depth *per se*.

The pronounced attractivity of the RPD layer for symbiotic worms in contrast to asymbiotic interstitial annelids raises the question as to which factor might act as a trigger directing the animals along a gradient. It is conceivable, that the complicated interdependence in the redox chemocline between pH- and CO₂-values (Böttcher & Strebel 1985, Gardner 1973, Gardner et al. 1988) could be an important aspect. Both, sensitivity for increasing alkalinity towards anoxic depths (Gardner 1973) and the general importance of pH effects on the annelid metabolism (Weber 1978) could be envisaged as relevant arguments.

Yet discrepancies remain between the clear preference of the worms for the redox chemocline shown in the laboratory experiments and their

field distribution. Why did we find some animals in vertical cores away from the RPD? In particular, why is there always some portion of the population in the completely anoxic deep layers with high concentrations of hydrogen sulphide (both in the field and the experiments)? This might be an "overshoot reaction" of the worms on their way to the deeper layers. However, there are indications that the two basic metabolic processes involved here, the uptake of reduced sulphur compounds and their oxidation, do not necessarily occur simultaneously. In the enclosure experiments reported above, pale worms inoculated into completely anoxic sand with very high concentrations of hydrogen sulphide not only survived for some days, but also turned completely white. This shows that the symbiotic bacteria managed to build up their sulfur reserves without access to oxygen.

The underlying metabolic processes in the symbiotic bacteria are still controversial (see Kelly 1989), and not to be discussed here, but one of the more frequent pathways in sulphur bacteria is the splitting of thiosulfate into sulphur and sulphite without the immediate need of oxygen. Hence, the very uptake of reduced sulphur compounds could, temporarily and partially, occur in complete anoxia in those layers with rich supply of reduced substances. This uptake will, of course, later be followed by the necessary binding of oxygen in order to obtain the energy needed. Although a certain amount of oxygen will be stored in the blood of the worms which have a well developed hemoglobin containing blood vessel system, the direct uptake of oxygen in the upper, microoxic sediment layers may be more important and would explain the preference of the worms for the RPD layer.

A temporal and spatial partitioning of sulphide and oxygen supply has also been suggested by Reid and Brand (1986) for the symbiotic bivalve *Saxidomus nutalli* and for meiofauna by Meyers et al. (1987) who suggested

that "most of daily metabolism in thiobiotic species must take place in an oxygen-free environment so that oxygen, if required, must be obtained by migratory behavior plus an oxygen storage system".

The vertical distribution of thiobiotic nematodes also indicates a separated uptake and metabolization of reduced substances. Ott & Novak (1989) showed a maximum around the RPD layer, however, they often found another aggregation in the anoxic depths (pers. communication). The fact, that in our field studies we never encountered a second maximum in these depths, apart from some "runaways" or "outsiders", is not necessarily controversial. It might relate to the different metabolism in Inanidrilus leukodermatus. This oligochaete species mainly metabolizes thiosulfate and, thus, is more closely linked to the main occurrence of this oxygen containing compound around or closely underneath the RPD layer - as opposed to the possibly sulphide consuming bacteria of the nematodes mentioned.

If a system exists to "guide" the worms to the optimal bacterial growth conditions, it is surprising that it takes about 3 days to yielding clear distributional patterns considering a) the length and motility of the worms (as observed in Petri dishes) and b) the fact that it is a high turnover bacterial metabolism which seems to underly the migrations. However, the chemical microgradients in the sediment column are not only directed vertically. Thus, if undisturbed, the animals may migrate only relatively little in a unidirectional distance. Moreover, these oligochaetes might also clump together for a while after insertion, an artifact found in studies on other positive thigmotactic annelids.

With pale worms, even longer times were needed to obtain clear results and scatter was higher than in white worms. We have to consider that these "stressed" worms (Giere et al. 1988 a) are not always in an

identical physiological situation. A "pale" appearance covers the wide span from specimens whose reserves had just been used up to those whose metabolism is critically reduced. It may also result from this stressed state that, in earlier experiments, pale worms showed a low CO₂-uptake capacity despite an ample supply with thiosulfate (Giere et al. 1988 a).

Another possible explanation involves the life history of the bacteria. The symbiotic sulphur bacteria, in contrast to their free-living counterparts, appear to be relatively long lived with slow division rates (Belkin et al 1986). Slow autotrophic growth was also described by Wood & Kelly (1989) for their bacteria cultures suggested to represent the first isolates of symbiotic sulphur bacteria. Uptake rates for CO₂ in L. leukodermatus had been measured in up to 6 hours (Giere et al. 1988 a). It is conceivable that the formation of reserve substances and their re-utilization proceeds in correspondingly slow processes, so that the worms' reaction to the lack of reduced sulphur is much retarded.

Recently, Riemann et al. (1990) pointed to another possible explanation for the relatively slow reaction of the worms: Under the chemical conditions prevailing around the RPD attractive chemical substances might become accumulated (in interstitial nematodes it was shown to be acetate) which would contribute to the microdistribution of these thiotrophic animals. It is conceivable that formation of - and/or detection of attractive organic substances would require some time and, thus, would be responsible for the relatively slow distributional reaction of the worms.

We have shown in earlier EM-studies that the worms receive dissolved metabolic products and particulate cell material from their symbiotic bacteria (Giere & Langheld 1987). Also, the high excretion rate of extracellular slime and fixed carbon measured in cultures of isolated Thiobacillus n. sp. from the symbiotic Thyasira flexuosa (Wood & Kelly

1989) indicates the possibility of an intensive nutritional supply for the animals through their bacteria. Substantial utilization of bacterial biomass and metabolic products by *I. leukodermatus* is corroborated by our stable isotope data.

The analysis of stable isotope values has become an important method in analyzing the trophic interrelationships between animals (Rounick & Winterbourn 1986, Peterson & Fry 1987), particularly where chemosynthetic bacteria are an important nutrient source and conventional nutritional studies impossible. Consequently, stable isotope ratios are of special relevance in studies on animals with symbiotic bacteria (Spiro et al. 1986, Southward 1987, Conway et al. 1989), since their food source is often largely based on chemosynthetic primary producers. In the case of the gutless oligochaetes, the only trophic pathway beside direct transfer from their endobacteria is transepidermal uptake of dissolved organics from the ambient porewater. This has been shown to be an important food source in annelids (Stephens 1975) including *I. leukodermatus* (Liebezeit et al. 1983, Giere et al. 1984). However, the position of the symbiotic bacteria as a 'mantle' around the animal's inner tissue suggests that a substantial part of this uptake is in fact due to the bacteria and only to a lesser extent due to direct incorporation by the animals.

The stable isotope ratios of *I. leukodermatus* enabled us to attempt quantify this delicate interaction for the first time. On the basis of microscopical studies of the worms (longitudinal and cross-sections, see Giere 1981), the endosymbionts appear to account for about 25 % of the symbiosis volume (Feller & Warwick 1988). This value even takes into account the scarcity of bacteria in the preclitellar body region. In order to calculate the contribution of the bacterial endosymbionts to the carbon budget of *I. leukodermatus*, a $\delta^{13}\text{C}$ of - 32 to 35 ‰ was postulated for the sulfur bacteria (Conway et al. 1989). This figure is rather conservative

considering the average values of -30 ‰ to -42 (-47 ‰) for sulfur-based chemoautotrophic symbioses given by Cary et al. (1989) and Brooks et al. (1987). Using a two-source mixing model (Conway et al. 1989), the animal tissue in the symbiosis of *I. leukodermatus* would then have a $\delta^{13}\text{C}$ of -23 to -24 ‰. These values are more negative than the isotope ratios of photosynthetically based carbon sources in benthic animals, which range between -15 and -21 ‰, or from dissolved inorganic carbon in sediment pore water (+18 to -20 ‰, Paull et al. 1985, Southward 1987). Hence, they suggest a dominant nutritive role for the chemosynthetic bacterial symbionts of these gutless worms.

Nitrogen stable isotope ratios cover a wide range in animals as the N-ratio is altered during trophic transfers, usually increasing the ^{15}N content each time by 3-5 ‰ (Minagawa & Wada 1984, Paull et al. 1985, Peterson & Fry 1987). The ratio recorded in *I. leukodermatus* is clearly lower than that found in tissues derived from photosynthetic-based food chains (+1 to +20, see Paull et al. 1985), and also lower than in organisms from the Galapagos and 21°N hydrothermal vents, including annelids (Rau 1985). However, in *I. leukodermatus* it is more positive than in sulfur bacteria or in the symbiotic bivalve *Solemya velum* (Conway et al. 1989). This range indicates that the organic matter of the worms is not likely to be derived from a phytoplankton-based food chain, but is largely dependent on unusually depleted N sources. In the absence of geothermal vents these values suggest that much of the nitrogen in the symbiosis may originate from bacterial nitrogen fixation or other bacterial N-metabolic processes (Rau 1985).

It is conceivable that the symbiosis has access to and assimilates a very large supply of bacteria-mediated ammonia or nitrate. Indeed, ammonia values of $980\ \mu\text{mol}\cdot\text{g}^{-1}$ dwt of animal tissue were measured. For interstitial water analyzed in cores from the sampling area, values in the

range of $100 \mu\text{mol}\cdot\text{dm}^{-3}$ with maxima $>700 \mu\text{mol}\cdot\text{dm}^{-3}$ were not unusual (Liebezeit, unpublished data). Felbeck et al. (1983) showed the presence of substantial nitrate reductase activity in *I. leukodermatus*. Interpreting these data one has to consider the fact that there are two forms of bacteria incorporated in this symbiosis (Giere & Langheld 1987). They differ in size, structure and possibly also physiological pathways. So far, only the larger have been shown to be involved in sulphur metabolism.

Amino acids: The interesting point in these analyses is the presence of the complete set of amino acids with the essential ones accounting for about 50 % of the total. Since animals are usually unable to synthesize the "essential" amino acids, and it is quite unlikely that *I. leukodermatus* would be a rare exception, they must come from its diet. Many chemoautotrophic bacteria, e.g. *Thiomicrospira crunogena*, can produce all the essential amino acids (Conway 1990). Also, Trytek & Allen (1980) demonstrated the synthesis of essential amino acids by the prokaryotic symbionts of the shipworm *Bankia setacea* (Bivalvia) and the inability of the animal host alone to synthesize them. Hence, it could be concluded that also *I. leukodermatus* derives its supply of these substances from its bacterial symbionts. Perhaps the unusually low nitrogen stable isotope fractionation, demonstrated above, is a good indication for the prokaryotic origin of many amino acids.

On the other hand, it cannot be excluded that the worms take up (parts of) their supply of amino acids directly from the high concentrations of amino acids dissolved in the ambient pore water. Compared to the seawater 3 cm above the sediment surface, the pore water of the sampling area was about 50 to 60 times enriched in free dissolved amino acids. In the 5 cm sediment-horizon (a depth of regular worm occurrence) concentrations in the range of several hundred $\mu\text{M}\cdot\text{l}^{-1}$ have been measured (analyses performed in 1981 by Liebezeit, unpublished data). Although the

analytical methods differed from those used here for the tissue analysis, the percentage relation of amino acids in the pore water of three sediment cores monitored can be compared (see Tab. 1). While almost half the amount is made up by Glutamic Acid, the complete set of amino acids could be evidenced in the pore water. However, essential amino acids accounted for only 15 % of the total. Particularly Valine and Leucine were present in the pore water in much lower relative concentrations (mostly < 1 % each) than in the animal tissues (Tab. 1).

Tentative uptake experiments of amino acids with *I. leukodermatus* showed preferred utilization of the acidic amino acids Glutamic Acid and Aspartic Acid with only low uptake rates of the neutral or alkaline acids Glycine, Serine, Arginine and Histidine. This possibly points to an autochthonous production by the bacteria proper rather than to an uptake from the ambient medium.

Uptake experiments with a pool of ^{14}C -labeled amino acids comparing normal animals with worms treated with antibiotics regularly showed a linear incorporation with a rate being about 4 times faster in untreated worms than in those having their bacteria inactivated. Also these results favour a predominantly bacterial uptake as compared to pathways through the animal tissues.

Lipids: Although based on just three extractions from one pooled worm sample, the overall percentage relation of lipids is probably representative, even though the samples could not be analyzed immediately nor kept frozen. The large amount of unsaturated fatty acids indicates that oxidation has not occurred. With the high ratio of one specific bacterial lipid (*c/s*-vaccenic acid) the spectrum of lipids underlines impressively the dominating metabolic role the prokaryotes play in the symbiosis with the anenteric annelid. This mono-unsaturated fatty acid is often the major end product of a bacterial pathway in the absence of oxygen, with 16:1w7

produced as a secondary product (Goldfine 1972, Fulco 1983). This could be interpreted as another indication for the regular occurrence of *L. leukodermatus* in completely anoxic layers where its bacteria not only can take up sulphide but also can drive an active anaerobic metabolism.

Similarly high amounts of *cis*-vaccenic acid have been found in cultures of free-living sulfur bacteria. In *Solemya velum*, the only animal-bacteria symbiosis where the lipid composition is published, the relative amounts of this bacterial lipid was much lower (Conway & McDowell Capuzzo 1990 a, b), but amounts were still remarkably high in comparison with other marine species. Thus, this lipid probably reflects utilization of bacterial lipid metabolites. *L. leukodermatus* like *Solemya velum* contains only a small amount of highly unsaturated fatty acids in contrast to most non-symbiotic marine animals (compare Conway & McDowell Capuzzo 1990 b). Although $\delta^{13}\text{C}$ ratios of fatty acids have not been measured, it can be concluded that in this mouthless worm the pattern of lipids is symbiont-based reflecting the strong metabolic influence of its endobacteria. Palmitic acid and stearic acid, the two other dominating amino acids in *L. leukodermatus* (23 % of total, see Fig. 9), are preferred substrates utilized in normal, aerobic pathways.

In this context, the rich amounts of the bacterial reserve substance polyhydroxy butyric acid (2 % of the total dwt of the worm, Giere et al. 1988 a) should be stressed.

CONCLUSIONS

Our earlier studies on the structure (Giere & Langheld 1987), physiology (Felbeck et al. 1983; Liebezeit et al. 1983), microbiology (Giere et al. 1988) and field ecology (Giere et al. 1982), all underlined more or less indirectly the relevance of the prokaryotic symbionts for the nutrition of the gutless *Janidrilus leukodermatus*. In this paper, for the first time

direct ecological experimentation have shown that the bacterial metabolic needs, which depend on a particular physico-chemical regime, dominate the system and, thus, regulate the microdistribution of their annelid hosts. These results contribute to the often neglected ecological aspects of our understanding of animal/sulphur-bacteria symbioses. In addition to these ecological results, we corroborated our conclusions through the use of bacterial biochemical "biomarkers". These clearly show the extent to which the worms depend on bacterial chemoautotrophy as their trophic source. Simplified and very roughly summarized, the worms resemble bacteria more than animals.

On the other hand, the migration experiments underline also the beneficial role of the annelid hosts for the prokaryotes in this obligate and mutualistic symbiosis. The bacteria are transported by the worms in a spatially complex, temporally variable gradient system of oxygen and sulphur compounds to the appropriate milieu. This permanent adjustment enables them to maintain an effectively high metabolic level. If this migratory behaviour of mobile thiobios over a range of changing oxic and sulphidic gradients (including extended stays in layers of complete anoxia) can be generalized (Meyers et al. 1988, Ott & Novak 1989), it may answer most of the controversies about a meiobenthic thiobios which arose from the limited ecological and physiological understanding of animal life in sulphide-based ecosystems at that time (Reise & Ax 1979, Boaden 1980, Powell & Bright 1981).

The tight metabolic, ecological and structural coupling between bacteria and animal host shown in our studies on *I. leukodermatus*, represents an evolutionary potential apparently powerful and advantageous enough to allow for the complete loss of the digestive and excretory organs. Regarding the high number of gutless phalloporine tubificids (approximately 100 mostly undescribed species; Erséus, personal

communication), probably all in symbiosis with endobacteria, this trend seems more than just an isolated evolutionary event. It renders these oligochaetes favourable objects for further studies on the evolution of animal/bacteria symbioses.

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Fig. 1. Vertical pattern of redox potential (left) and distribution of *Inanidrilus leukodermatus* (right) in the sampling area; compilation of 5 parallel cores

Fig. 2. Polynomial regression curve of sediment depth *versus* worm distribution in the field indicating preferred occurrence around 5 cm depth; compilation of 5 parallel cores

Fig. 3. Polynomial regression curve of sediment redox conditions *versus* worm distribution in the field indicating preferred occurrence around the redox discontinuity layer; compilation of 5 parallel cores

Fig. 4. Migration experiments with redox discontinuity (RPD) set in a bottom position of the tube and (white) worms inserted at the top: aggregation in deeper layers

Fig. 5. Migration experiments with RPD set in middle position of the tube and (white) worms inserted at bottom: aggregation in middle layers

Fig. 6. Migration experiments with RPD set underneath the surface of the tube and (white) worms inserted at bottom: aggregation in top layers

Fig. 7. Migration experiments with RPD set in a bottom position of the tube and (pale) worms inserted at top: aggregation in deeper layers

Fig. 8. Lipid composition of *Inanidrillus leukodermatus* calculated as percentage of fatty acids in total pool. Note dominance of bacteria-derived 18:1ω7 (*cis*-vaccenic acid)

Fig. 9. Amino acid composition of *Inanidrillus leukodermatus* calculated as percentage of total pool. Note presence of all essential amino acids. (for methodological reasons Tryptophan not measured)

Amino Acid	Worm Tissue		Pore Water, 5cm depth
	[μ M .g-1 dry wt.]	% of Total	% of Total
Aspartic Acid	144,09	3,9	10,6
Glutamic Acid	321,84	8,8	46,1
β -Glutaric Acid			2,5
Hydroxy-Proline	14,98	0,4	
Asparagine "	5,3	0,1	5,2
Serine *	54,26	1,5	1,6
Glutamine "	0	0	13,2
Glycine	640,37	17,5	2,3
Histidine	43,29	1,2	1,1
Taur./Argin./Tyros.	149,45	4,1	1,9
Threonine *	135,67	3,7	
Alanine	559,75	15,3	6,8
β -Alanine			
Proline	40,56	1,1	
Valine/Methionine	474,51	12,9	2,8
Cystine/Cysteine	21,47	0,6	
Isoleucine	289,8	7,9	0,8
Leucine	395,79	10,8	0,8
Phenylalanine	186,29	5,1	1,4
Tryptophan *	0	0	
Lysine	193,3	5,3	0
Others			0,9
Total	3670,72	100	100

Table 1. Amino acid composition: tissue homogenate of *Inonidrilus leukodermatus* in contrast to pore water from sediment in sampling area (obtained with different analytical methods).

* Yields of these amino acids are reduced during acid hydrolysis;

" these amino acids are largely converted to aspartic and glutamic acid.

APPENDIX III

UPTAKE OF RADIOLABELLED PHYTOPLANKTON BY THE
SOLEMYA VELUM SYMBIOSIS.

Renee White, Noellette Conway, and
Judith McDowell Capuzzo

AI.1 Abstract

The ability of *Solemya velum* to ingest ^{14}C -labelled *Isochrysis galbaena* algal cells was examined in the presence and absence of a reduced sulfur source. Uptake of algal cells by *S. velum* was compared with uptake by the symbiont-free bivalve, *Mya arenaria*. The radioactivity of the experimental seawater was reduced in all the experimental vials, with counts for the vials containing *M. arenaria* significantly lower than those of the blank vials and the vials containing *S. velum* samples. The maximum possible number of algal cells ingested by *S. velum* was almost two orders of magnitude lower than the maximum number ingested by *M. arenaria*. The maximum possible number of algal cells ingested could provide for <1% of the oxidative requirements of *S. velum*. The results clearly show that ingestion of phytoplankton is unlikely to contribute greatly to the nutritional needs of the *S. velum* symbiosis, although small amounts of phytoplankton may be ingested and may be important in the provision of essential nutrients.

AIII.2 Introduction

Throughout this dissertation, the question of nutrition in animal-bacteria symbioses has been addressed using biochemical markers which can be measured in preserved animals. The results of these experiments all indicate a vital role for the endosymbionts and a minimal role of exogenous phytoplankton-derived organic matter in the nutrition of *S. velum*. However, in order to demonstrate this directly, physiological feeding studies with live animals were required, as *S. velum* does have a small but apparently functional gut, and algal cells can sometimes be seen in the digestive system.

Experiments to determine the ability of *S. velum* to ingest algal-derived nutrients are also important in view of the small amounts of PUFAs found in this species. If these are not produced by the symbiosis *de novo*, they must be derived from marine phytoplankton. During the summer of 1989 some preliminary feeding experiments with *S. velum* and *M. arenaria* were carried out in order to determine whether ingestion of phytoplankton cells could contribute substantially to the nutritional requirements of the *S. velum* symbiosis.

AIII.3 Materials and methods.

Isochrysis galbaena cells were grown in F/2 medium spiked with $10 \mu\text{Ci L}^{-1} \text{Na}^{14}\text{CO}_3$. Once the cell suspensions were turbid, algal cells were centrifuged out of suspension in a bench top centrifuge and cell viability and cell concentrations were determined under the microscope. A known concentration of radiolabelled algal cells were added to Scintiverse scintillation fluid and disintegrations per minute (dpm) counted in a United Technologies Packard Minaxi β Tri-carb 4000 scintillation counter in order to determine dpm per algal cell at the start of the experiment.

Specimens of *Solemya velum* and *Mya arenaria* were collected from sediments at Little Buttermilk Bay, MA, and added to large

centrifuge tubes containing sediment and 25 ml of 0.2 μ m filtered seawater, to provide sufficient oxygen for 12 h (with $\leq$ 25% depletion of available oxygen). Thiosulfate was added to provide a reduced sulfur source in some of the vials as follows.

Table AIII.1 Experimental setup used in radiolabelling experiment

Treatment	# of <i>S. velum</i> tested	# of <i>M. arenaria</i> tested	Control vials (no animal)
(I) 0.5 mM Na ₂ SO ₄ , 2 hour exposure to algae(pulse)	4	2	2
(II) 0.5 mM Na ₂ SO ₄ , 10 hour exposure to algae(pulse)	4	2	2
(III) No Na ₂ SO ₄ , 2 hour exposure to algae (pulse)	4	2	2
(IV) No Na ₂ SO ₄ , 10 hour exposure to algae (pulse)	4	2	2

Animals were added to the experimental vials and allowed to equilibrate for 2-3 h then equal aliquots of ¹⁴C-labelled algal suspension were added to each vial to result in final algal concentrations and disintegrations per minute (dpm) of 2.64 x10⁴ cells ml⁻¹ and 5989 dpm/ml respectively in each treatment vial. After the appropriate exposure time (2 or 10 hours, as outlined above) animals were removed from the experimental vials, washed briefly in distilled water and added to similar vials containing no algae where they were left for 24h (chase period). Water was replaced in these vials after 12 h to prevent oxygen limitation. The

experimental water from the pulse period was spun in a table top centrifuge to pellet out any remaining algae. These algae and known aliquots of water from the pulse vials (water¹) were added to Scintiverse scintillation fluid and dpm measured. Counts from the algal fraction could be used to determine the algal depletion that had occurred, while water¹ fractions indicate the amount of radiolabel that is in the dissolved carbon pool either as a result of cell lysis or leakage from algal cells. The radiolabel incorporated into the sediment fraction could not be determined.

After the 24h chase period both sets of water samples from the chase vials of each experimental animal were combined (water²). Aliquots of this water fraction were added to scintiverse scintillation cocktail and radioactivity determined. Shells were removed from each animal and the soft parts were rinsed briefly with 0.01N HCL solution to remove radiolabelled organic material adhering to the outside of the animal. Each animal was divided into foot and body (all remaining soft parts), rinsed again in 0.01N HCL, dried briefly on a paper towel and added to scintillation vials. Tissue weight was determined then the animal tissue was roughly homogenized with a razor. Protosol or scintigest, strongly basic tissue dissolving agents, was added to each scintillation vial, and the tissue was kept in a ventilated 60°C oven until all the tissues had dissolved. After neutralization of the dissolving agents with glacial acetic acid, 30% hydrogen peroxide solution was added to bleach the dissolved tissue. The radioactivity of all the tissue fractions was determined after the addition of scintiverse scintillation cocktail.

Disintegrations per minute were calculated using a program that corrects for chemiluminescence and quenching. Blanks of the entire procedure (all the chemicals with no tissues added) were also run to determine background counts. Background counts were subtracted from the dpm values obtained for each tissue.

AIII.4 Results and Discussion

The results for the animals in each experimental treatment and the control vials are outlined in table AIII.2 which will be referred to throughout the discussion.

Table AIII.2 results of 14 -C radiolabelled algae uptake studies.

<i>Solemya velum</i>				
	+ 0.5mM Thiosulfate		No Thiosulfate	
	2 h pulse	10 pulse	2 h pulse	10 pulse
Foot dpm/mg wet wt.	4	5	3	4
Body dpm/mg wet wt.	2	6	2	3
Total dpm/mg wet wt.	49	115	41	51
Final Algae dpm/ml	1107	537	1518	480
Water 1 dpm/ml	1226	1090	797	591
Water 2 dpm/ml	10	9	29	19
Max. possible # cells per mg wet.wt.*	11	26	9	12
Max possible # of cells ingested* per animal	3077	5517	1877	2477

<i>Mya arenaria</i>				
	+ 0.5mM Thiosulfate		No Thiosulfate	
	2 h pulse	10 pulse	2 hour pulse	10 pulse
Foot dpm/mg wet wt.	158	289	156	142
Body dpm/mg wet wt.	229	138	110	184
Total dpm/mg wet wt.	4385	2923	2156	3110
Final Algae dpm/ml	131	129	146	143
Water 1 dpm/ml	442	578	255	715
Water 2 dpm/ml	94	64	98	79
Max. possible # cells per mg wet.wt.*	995	623	489	706
Max possible # of cells ingested* per animal	177265	123866	128257	104027

Blank				
	+ 0.5mM Thiosulfate		No Thiosulfate	
	2 hour pulse	10 hour pulse	2 hour pulse	10 hour pulse
Initial algae dpm/ml	5989	5989	5989	5989
Final algae dpm/ml	1102	571	1547	372
Water 1 dpm/ml	948	851	992	950
% of cells lost to sediment	82	91	74	94

*Calculated by assuming that any radioactivity present was due to the ingestion of algal cells.

AIII.3 1. Effect of sulfate treatment on tissue radioactivity.

S. velum showed a trend towards lower cell uptake on the absence of sulfate during both pulse periods (Table AIII.2, Figures AIII.1, AIII.2). *M. arenaria* also seemed to take in less cells when no sulfate was present! Because of the relatively small sample sizes and individual specimen variability it is not possible to determine whether these are true phenomena.

AIII.3.2 Differences in phytoplankton uptake between the two pulse periods.

In *S. velum*, the amount of radiolabel incorporated increased over time (Table AIII.2, Figures AIII.1, AIII.2) although not in a linear fashion; at best, the dpm/mg tissue increased by a factor of two although the second pulse treatment lasted five times as long as the first. In *M. arenaria* there was also only a small increase in the amount of cells ingested over time. In the *M. arenaria* vials it is likely that after only 2 h. the concentrations of cells available had dropped below a limiting threshold. After 2 h, the dpm/ml in both the blank vials and the *S. velum* vials corresponds to about 4800 cells per ml, at these concentrations the chances of an animal encountering a cell will be reduced. In the *M. arenaria* vials, after just 2 h cell concentrations had fallen to only 570 cell per ml (Table AIII.3). This is probably below the threshold for cell uptake. The experimental vials contained sediment and a considerable proportion of the suspended cells probably settled to the sediment. This is shown in the blank vials where over 90% of the algal cells ended up in the sediments at the end of just a 2h period. Uptake by *S. velum* appeared to be so low, that cell limitation probably did not exert a large effect; however, in *M. arenaria*, the cell availability almost certainly fell below a specific threshold of abundance. In this context it is interesting to note that *Mytilus edulis* stopped taking in algal cells once cell concentrations fell to about 1000 cells/ml in similar experiments recently conducted (Page *et al.*, 1990).

AI.3.3 Differences in phytoplankton uptake between species.

Differences between the maximum possible numbers of cells taken up by the two species of bivalves were significant. In *S. velum*, 9-26 cells were taken up by the whole animal per mg wet weight of tissue while the values for *M. arenaria* were 489-995 cells per mg wet weight. (Table AIII.2, Figures AIII.1, AIII.2). This is readily apparent when the decrease in algal cells over time is plotted (Table AII.3, Figure AIII.3). Although an extremely large number of cells were lost to the sediment, the cell depletion in the *M. arenaria* vials was significantly greater than in the *S. velum* vials, in fact the cell depletion in the *S. velum* vials differed little from the blank controls (Figure AIII.3). These differences are also apparent when the water fractions are examined. Water¹ represents the dissolved radioactivity in the ambient water at the end of each pulse period and is probably the result of algal leakage or lysed cells. In the *M. arenaria* vials water¹ dpm values are lower than in the *S. velum* vials suggesting that either less cells were present to leak or lyse, or that *M. arenaria* was more effective at removing dissolved radioactive carbon compounds. In fact, water¹ dpm/ml values were very similar between the *S. velum* vials and the blank vials (Table AIII.2). The water² fractions, which represent loss of radiolabelled carbon that was incorporated by the animal during the pulse, either by respiration, excretion, or mucous production, had higher dpm/ml values in the *M. arenaria* vials than in the *S. velum* vials.

We can set limits on the maximum contribution the phytoplankton cells could make to the respiratory requirements of the *S. velum* symbiosis by converting the number of cells ingested to the amount of carbon ingested, and comparing this with the respiration rates of *S. velum*. In order to provide a range of values for the contribution of the algae to the nutrition of *S. velum*, we used the biochemical composition of the majority of algal species studied in the comprehensive paper of Parsons *et al.* (1961). It is probably safe to assume that *I. galbaena* falls within this range and these calculations allow us to estimate the potential contribution of larger and smaller algal species not examined in our study.

These calculations are as follows:

Monochyrisis lutheri

Dunaliella salina

Number of cells/mg dry wt.

(Parsons et al., 1961)

$$\Rightarrow 2.9 \times 10^7 \text{ cells} = 1 \text{ mg}$$

$$7.6 \times 10^6 \text{ cells} = 1 \text{ mg}$$

(Of the 11 species of algae examined in the paper of Parsons *et al.* (1961), 9 fell within this range, one species had more cells per mg and one species had less. So this range seems to be fairly representative of a wide variety of algae).

$$\Rightarrow 1 \text{ cell} = 3.45 \times 10^{-8} \text{ mg}$$

$$1 \text{ cell} = 1.32 \times 10^{-7} \text{ mg}$$

Carbon content of cells

(Parsons et al., 1961)

44.6%

45.4%

$\Rightarrow$ Carbon content

$$1.55 \times 10^{-8} \text{ mg C/cell}$$

$$5.9 \times 10^{-8} \text{ mg C/cell}$$

This gives us a reasonable range of carbon values for marine phytoplankton.

The O_2 consumption rates of *S. velum* calculated by Cavanaugh (1985) are:

$$= 0.5 \text{ to } 1.5 \mu\text{mol } O_2/\text{h}/100 \text{ mg}$$

$$\Rightarrow 0.005 - 0.015 \mu\text{mol } O_2/\text{h}/\text{mg}$$

If we assume a respiratory quotient of 1, and 100% assimilation efficiency

$$\Rightarrow 0.005 - 0.015 \mu\text{mol C/h}/\text{mg} \text{ required by } S. \text{ velum.}$$

From Table AIII.2, it is apparent that *S. velum* took up at most 11 cells during the 2 h pulse and 26 cells during the 10 h pulse. In order to ensure that we are determining maximum intake rates, hourly rates were calculated from the 2 h pulse period yielding the highest uptake:

⇒ 6 cells take in per hour per mg by *S. velum*.

⇒ range of values for the amount of carbon ingested =

If algae = <i>Monochrysis lutheri</i>	<i>Dunaliella salina</i>
9.3×10^{-8} mgC/mg animal/h	3.54×10^{-7} mgC/mg animal/h
⇒ 7.8×10^{-9} mmol C/mg animal/h	2.95×10^{-8} mmol C/mg animal/h
⇒ 7.8×10^{-6} μ mol C/mg animal/h	2.95×10^{-5} μ mol C/mg animal/h

= Range of maximal contributions to the oxidative requirements of *S. velum*;

0.05 to 0.2 % if respiration rates = $0.015 \mu\text{mol O}_2/\text{h}/\text{mg}$

0.16 to 0.6% if respiration rates = $0.005 \mu\text{mol O}_2/\text{h}/\text{mg}$

Thus, it would appear that marine phytoplankton are unlikely to contribute greatly to the energetic requirements of *S. velum*.

Figure AIII.1 Comparison of the radiolabel incorporated by a) *Solemya velum* and b) *Mya arenaria*.

Figure AIII.2. Comparison of the maximum possible number of cells ingested by a) *Solemya velum* and b) *Mya arenaria* during the two hour pulse period.

Table AIII.3 Decrease in algal cells in suspension during the experimental period.

	Blank		<i>S. velum</i>		<i>M. arenaria</i>	
	+ S	No S	+ S	No S	+ S	No S
Start	26400	26400	26400	26400	26400	26400
2hours	4854	6813	4875	6686	577	644
10 hours	2517	1640	2366	2115	568	629

Figure AIII.3 Decrease in algal cells in suspension during the experimental period

The ability of an undescribed seep mussel to clear, ingest and assimilate both bacteria (*Vibrio pelagicus*, *Escherichia coli*) and phytoplankton (*Dunaliella tertiolecta*) has recently been investigated and compared to uptake by *Mytilus edulis* (Page *et al.*, 1990). In that study it was found that the seep mussel cleared bacteria and algal cells more slowly than *M. edulis*, with the amount of algal cells ingested about 3.4×10^5 cells per hour for mussels with an average wet weight of 2.6 g. This is about 130 cells/mg/h which is about an order of magnitude greater than the uptake we found for *S. velum*. However, the cell uptake found for *M. edulis* was about 1923 cells/mg/h calculated from results of Page *et al.* 1990). These are similar to the values we found for *M. arenaria*; the lower values found for *M. arenaria* are probably due to the fact that we are calculating hourly rates from the 2 h pulse period, and it is likely that cell concentrations were already highly reduced after just one hour, and during the second hour cell limitation probably influenced ingestion rates.

AIII.5 Summary

We conclude from these preliminary data that *S. velum* appears to be able to ingest small numbers of algal cells. Thus, although the algae are unlikely to provide the animals with a significant proportion of its nutritional requirements, the algae could be vital in the provision of small amounts of essential nutrients. When these data and the data of Gallagher (unpub data, see chapter 5) are considered it would appear that *S. velum* is capable of supplementing its diet of endosymbionts with small amounts of exogenous organic matter either through direct digestion of marine algae, or via epithelial DOM uptake processes.

AIII.6 Suggestions for future research

One problem still exists with the interpretation of the data presented in this thesis. The possibility that *S. velum* may be utilizing external chemoautotrophic bacteria has not been ruled out.

External "farming" of bacteria are unlikely to contribute greatly to the dietary needs of *S. velum* for a number of reasons.

- (1) *The digestive system of S. velum is extremely small and it is unlikely that the small gut would be able to process enough bacteria to provide the animal with large amounts of nutrients.*
- (2) *The feeding palps of S. velum are also reduced, consequently the animal would be unable to take in sediment for processing as is normally found in protobranchs. Thus, the animal could only rely on free living bacteria in the water column.*
- (3) *If the animal was ingesting sufficient quantities of bacteria from the porewaters of its burrow, it is probable that significant amounts of phytoplankton nutrients would also be ingested, particularly when the phytoplankton $\delta^{13}\text{C}$ signature of the sediments where S. velum is found are considered. The stable isotope ratios and lipid composition of S. velum (Chapters 3 and 4) do not suggest that this is occurring.*

Thus, it is unlikely that the bacterial compounds found in *S. velum* are externally derived. To demonstrate this directly, however, it is necessary to carry out similar experiments to the ones described here using radiolabelled bacteria. Results of experiments of this type have been reported by Page et al. (1990). In addition, it would be informative to repeat the experiments described here *without* the addition of sediment to the vials. We were attempting to mimic the animals natural environment; however, none of the animals burrowed, and the sediments only served as a sink for settling algae. If the animals are incubated without sediment, the experimental vials should be placed on gently shaking tables to ensure even distribution of algae/bacteria. Determining the algal/bacterial cell depletion over time will also allow the ratio of cells filtered: radiolabel incorporated to be calculated, and assimilation efficiencies to be calculated. In addition, a greater

number of pulse times should be used, including pulse times as little as one hour. If possible, faeces and psuedofaeces should also be collected and radioactivity determined.

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APPENDIX IV

**ELECTRON-IMPACT MASS SPECTRA OF THE STEROLS OF
SOLEMYA VELUM (I-II) AND *MYA ARENARIA* (III-XIV),
SHOWN AS THEIR ACETATE DERIVATIVE**

III

26,27-Dinoregosta-5,22-dien-3 β -ol acetateC₃₀H₄₈O₂

M=412

Biographical note

The author was born at an early age in Dublin, Ireland. She received her bachelors degree in Natural Sciences from Trinity College, Dublin, Ireland in 1984 and entered the WHOI/MIT Joint Program in Oceanography in the summer of 1985.

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THE AMAZING SPIDER-MAN

By Stan Lee and Larry Lieber