This is to certify that the

dissertation entitled

The Biosynthesis of
Dimethylsulfoniopropionate
(DMSP) in Marine Algae

presented by

Wayne Anthony Hicks

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Douglas Gage
Major professor
per W.L. Smith

Date August 23, 2002

O-12771

THE BIOSYNTHESIS OF DIMETHYLSULFONIOPROPIONATE (DMSP) IN MARINE DINOFLAGELLATES

By

Wayne Anthony Hicks

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

2002

ABSTRACT

THE BIOSYNTHESIS OF DIMETHYLSULFONIPROPIONATE (DMSP) IN MARINE DINOFLAGELLATES.

By

Wayne Anthony Hicks

Dimethylsulsoniopropionate (DMSP) is a member of a class of compounds known as "compatible osmolytes". These compounds accumulate to high cytoplasmic concentration in response to water stress without incurring any damaging effects to cellular structures, such as proteins. DMSP is produced by a few land plants in diverse plant families. It is also produced and accumulated by seven different classes of marine algae. DMSP has a number of biological functions. It helps to regulate osmotic pressure, it serves as a cryoprotectant, and it also functions as a defense compound as a precursor to acrylic acid.

DMSP is a precursor to acrylic acid through the action of the enzyme DMSP lyase, produced by marine bacteria and some marine algae. The lyase cleaves DMSP into DMS and acrylic acid. DMS produced from DMSP is a major fraction of the total amount of sulfur in the global sulfur cycle. Marine sources contribute up to 40 metric tons/ year of DMS to the environment according to one source. DMS in the atmosphere forms cloud condensation nuclei, and thereby contribute to cloud albedo and the cooling of the earth's surface. DMS is oxidized in the atmosphere to sulfuric acid and precipitates as acid rain.

Some land plants also synthesize DMSP. Previous research has shown that they synthesize DMSP from methionine via the enzymatic sequence of methylation, deamination/decarboxylation, and oxidation. Research is described in this thesis that describes the synthesis of DMSP in marine algae. The pathway in marine algae has no steps in common with that of the higher plant pathway. Algae also synthesize DMSP from methionine, however they do so through the enzymatic sequence of transamination, reduction, methylation and oxidative decarboxylation. This research has led to the identification of a novel biological compound, 4-dimethylsulfoniohydroxybutyrate (DMSHB) which may also function as a compatible osmolyte. Dinoflagellates are the largest producers of DMSP among the algal classes that synthesize DMSP. Previous research on DMSP synthesis in the dinoflagellate *Crypthecodinium cohnii* suggests that decarboxylation is the first step. This pathway was tested and results indicate that the DMSP biosynthetic pathway in dinoflagellates is consistent with other marine algae that proceed with transamination as the first step of the pathway. The effect of nitrogen on increasing DMSP production by dinoflagellates is also described.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AdoHcy	Adenosyl homocysteine
Ala	alanine
Asn	asparagine
Asp	aspartate
BADH	Betaine aldehyde dehydrogenase
DDH	3-dimethylsulfoniopropionaldehyde dehydrogenase
DMS	dimethyl sulfide
DMSHB	4-dimethylsulfonio-2-hydroxybutyrate
DMSKB	4-dimethylsulfonio-2-ketobutyrate
DMSP	3-dimethylsulfoniopropionate
DMSP-ald	3-dimethylsulfoniopropionaldehyde
DMSP-amine	3-dimethylsufoniopropylamine
FABMS	Fast Atom Bombardment Mass Spectrometry
GCMS	Gas Chromatography-Mass Spectrometry
gfw	grams fresh weight
Gln	glutamine
Glu	glutamate
HMT	Homocysteine methyltransferase
K_m	Michaelis-Menten constant
MCW	methanol:chloroform:water
MDH	Malate dehydrogenase
Met	methionine
MMT	S-methyl methionine S-methyltransferase
MTA	methylthio adenosine
MTHB	4-methylthio-2-hydroxybutyrate
MTOB	4-methylthio-2-oxobutyrate
MTP	methylthiopropionate
MTP-amine	methylthiopropylamine
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
PLP	pyridoxal phosphate
QAC	quatenary ammonium compound
SAM	S-adenosyl methionine (S-AdoMet)
SIM	selected ion monitoring
SMM	S-methylmethionine
TBDMS	t-butlydimethylsilyl
TLC	thin layer chromatography
TLE	thin layer electrophoresis
TMSCN	trimethylsilylcyanide
TSC	tertiary sulfonium compound
V_{max}	maximum velocity of a reaction

CHAPTER 1

LITERATURE REVIEW

Introduction

Dimethylsulfoniopropionate (DMSP) is a member of a class of compounds known as biologically compatible osmolytes that are accumulated by halophytic or drought tolerant plants and some microorganisms. This class of compounds includes zwitterionic tertiary sulfonium compounds (TSCs), and quaternary ammonium compounds (QACs), as well as polyols such as glycerol, and mannitol. Amino acids and amino acid derivatives are also known to provide osmoprotective effects, particularly proline, and proline betaine, a QAC, which have been shown to accumulate in some osmotically stressed plants (37). Some members of this class of compounds are known to accumulate to cytoplasmic concentrations of up to 1.4 M (36) without disrupting cellular structures. Similar cytoplasmic concentrations of chaotropic salts would be deleterious to protein, and membrane structure. High internal concentrations of compatible solutes reduce osmotic stress in dry or saline conditions. DMSP and other biologically compatible osmolytes therefore serve a very important role in plant adaptation to the environment. Their accumulation to such high levels regulate osmotic pressure in the presence of high external salt concentrations, and may also protect some organisms from freezing damage at low temperatures (14, 53, 92).

The role DMSP plays in biological systems is important in understanding how salt tolerant plants regulate their internal environment; however DMSP also has important environmental implications as a major source of atmospheric dimethyl sulfide (DMS). DMSP is a precursor of dimethylsulfide, which is released to the atmosphere from ocean and coastal waters (1, 2, 8,11). DMS is produced from catabolism of DMSP through the action of a DMSP lyase found in some marine bacteria and marine algae (89). The lyase produces DMS and acrylate from DMSP (11, 13).

$$\text{(A)}\quad \underset{\text{DMSP}}{(CH_3)_2S^+CH_2CH_2COOH} \rightarrow \underset{\text{DMS}}{(CH_3)_2S} + \underset{\text{Acrylate}}{CH_2{=}CHCOOH}$$

The estimate of biogenic sulfur emission is thought to be in the range of 32 to 103Tg/yr, which is equivalent to about half of the atmospheric sulfur from anthropogenic sources (1, 78, 43). The percentage of biogenic sulfur attributable to DMS is about 45% (76). DMS has been implicated as a nucleation center for aerosol and cloud formation and also in acid precipitation by its oxidation to sulfuric acid (2,). Its role as a cloud condensation nucleus directly impacts climate regulation patterns (Fig.1) (2,11). The ecological role of DMSP as a major precursor of DMS has provided an impetus to uncover the biosynthetic mechanism by which it is produced.

Biological Distribution of Osmolytes

Betaines, small nitrogen containing compounds in which the nitrogen atom is fully methylated, occur in a wide range of higher plants, and even in mammalian kidney (50). The most widely studied and distributed member of this class of QACs is glycine betaine (4, 5, 7, 15, 37). Glycine betaine has also been found to occur in trace amounts in some plants, which do not accumulate this compound to osmotically significant quantities. Glycine betaine is thought to have evolved early in plant evolution and the class angiospermeae seem to retain glycine betaine production as a primitive evolutionary characteristic (21). In some members of the genus *Limonium* of the Plumbaginacae (35), and *Wollostonia biflora* (Compositae)(68), the osmoregulatory functions of glycine betaine have been replaced or supplemented by other QACs or TSCs. *Wollastonia* is among the few flowering plants that accumulate dimethylsulfoniopropionate.

DMSP occurs in seven classes of marine algae, but is found in only a few unrelated land plant families. It has been isolated in *Spartina alterniflora* (Poaceae), an intertidal grass species (51, 78), sugarcane (Gramineae) (65), and as mentioned above, *Wollastonia biflora* (Asteraceae) (36, 79).

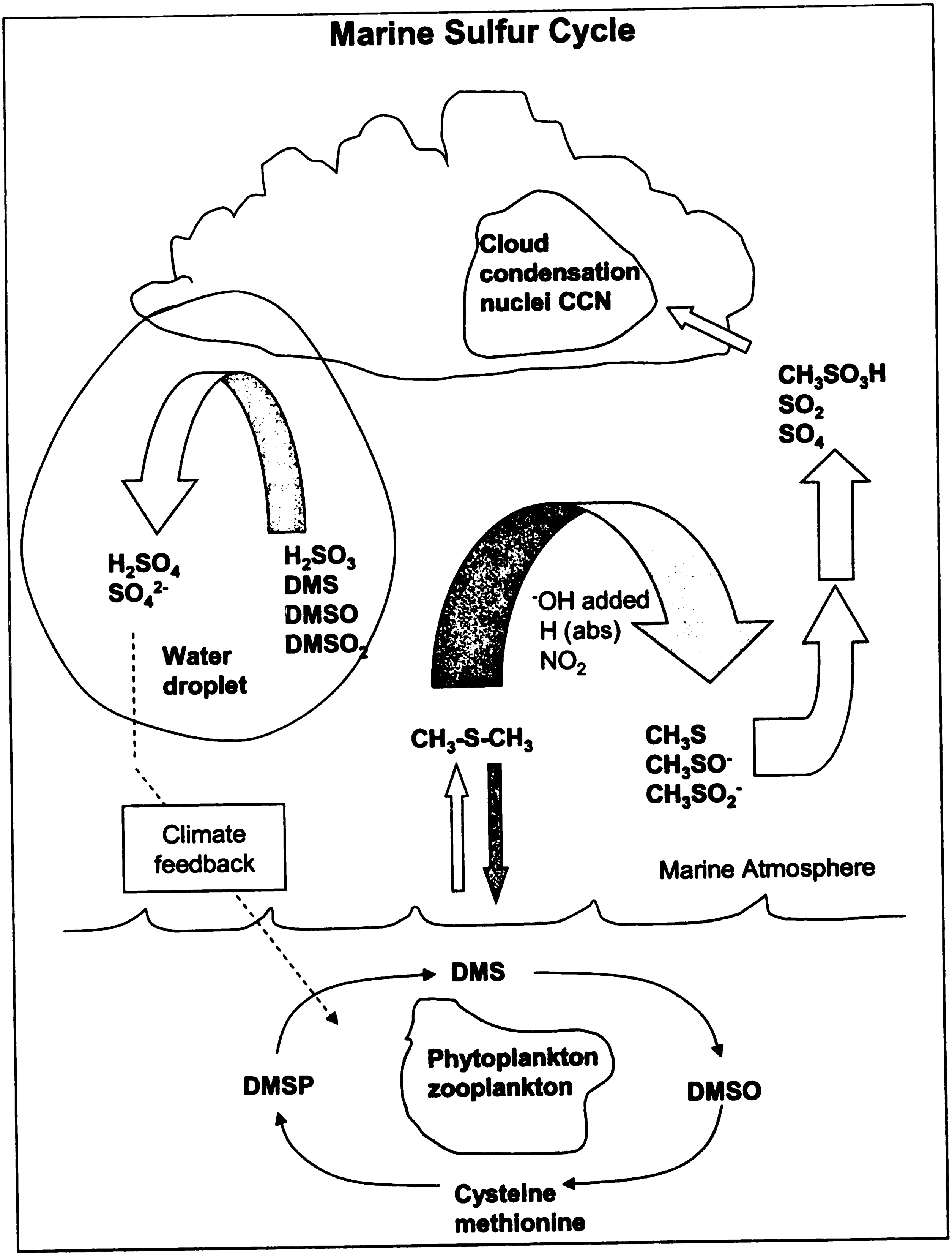


Figure 1) The role of Marine phytoplankton in the Sulfur Cycle

Comparison of DMSP Biosynthetic Pathways in Higher Plants and Marine Algae

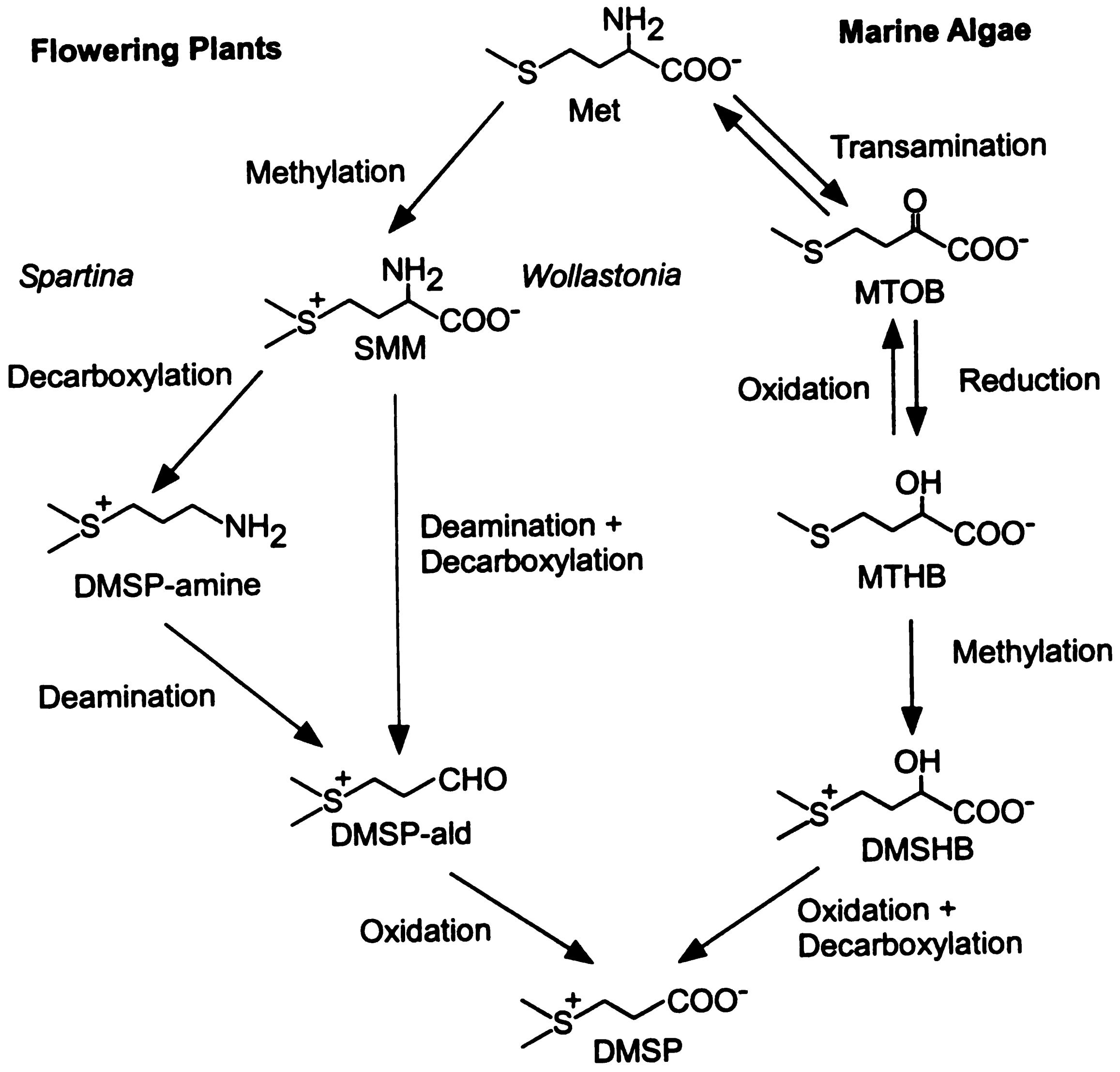


Figure 2)Side by side view of known DMSP biosynthetic pathways in higher plants and marine algae

DMSP Biosynthetic Pathway in *Crypthecohdinium cohnii*

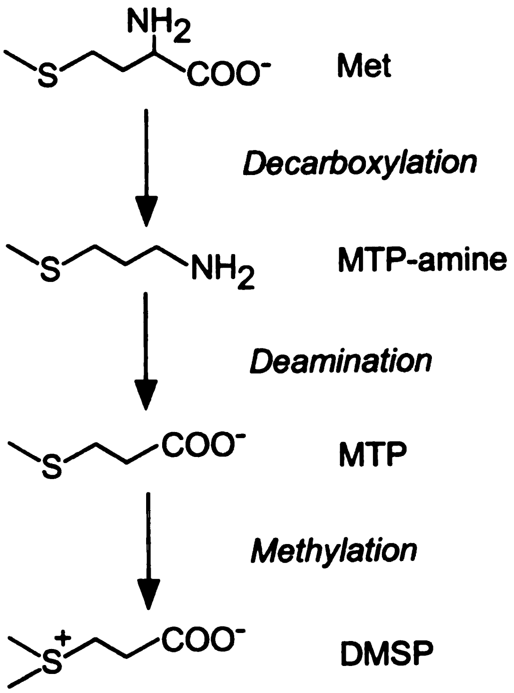


Figure 3) DMSP biosynthetic pathway proposed by Uchida et al. for the dinoflagellate *C. cohnii* (86)

Biological Function of Osmolytes

Compatible osmolytes serve a variety of biological functions. Their role in adaptation to water stress has been extensively demonstrated (6, 9,14, 15,16, 32, 35, 37). This includes changes in temperature, salinity, water availability, and nitrogen levels. The term "compatible osmolytes" differentiates between those molecules which lower water potential and maintain turgor pressure without perturbing effects on other biological molecules, and those, such as inorganic salts, which effect a perturbation of the system.

There are two ideas, not mutually exclusive, that are commonly invoked to explain the mechanism by which compatible osmolytes function. Osmolytes may stabilize protein structure by direct interaction with proteins or ligands, or through solvent-solute interactions (6, 92). Timasheff offered that glycerol was able to stabilize protein structure by re-ordering water structure in such a way that enhances the structure of the solvent layer of the protein (24, 25). The other theory is that they function in much the same way as the "stabilizing ions" of the Hofmeister series. It is worth noting that the highly methylated nature of betaines, such as glycine betaine, and trimethylamine N-oxide, closely resemble the non-organic salt ammonium sulfate (Fig. 4).

Ammonium occupies a position as a stabilizing cation in the Hoffmeister series, in which ions are ranked according to their affinity for protein denaturation, or their ability to promote a "salting out" effect. The structure of methylamines is very similar to that of quaternary ammonium ions. Yancey and

Somero suggest it is this similarity that is responsible for the stabilizing effect of betaines on protein structure (92). Indeed, they showed that the effects of compatible and non-compatible osmolytes were algebraically additive. This phenomenon has been taken advantage of in nature as some species of urea accumulating vertebrates (e.g. *Xenopus laevis*) maintain a 2:1 ratio of methylamines: urea. This strategy allows them to adapt to osmotic stress while offsetting the protein denaturation effects through the inclusion of methylamines (7, 50, 92)

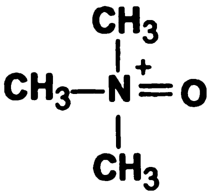
Figure 4) **A**) Ions of the Hofmeister series grouped as to whether they tend to stabilize protein structure, “salting in”, or destabilize protein structure, “salting out”. **B**) Some selected molecules that are similar to the ions of the Hofmeister series in their effect on protein structure, and their electronic structure.

A

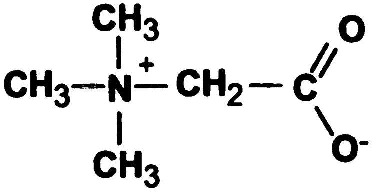
	Stabilizing (salting - out)				Destabilizing (salting - in)						
Anions	F^-	PO_4^{3-}	SO_4^{2-}	CH_3COO^-	Cl^-	Br^-	I^-	CNS^-			
Cations	$(CH_3)_4N^+$	$(CH_3)_2NH_2^+$	NH_4^+		K^+	Na^+	Ca^+	Li^+	Mg_2^+	Ca_2^+	Ba^{2+}

B

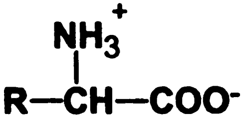
Nonperturbing or stabilizing



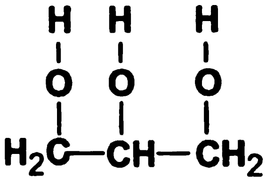
Trimethyamine N-oxide



Betaine

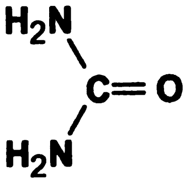


Amino acid

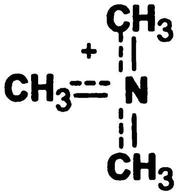


Glycerol

Perturbing



Urea



Guanidinium

DMSP is a sulfonium compound, the structure of which closely resembles that of the QACs. It has been shown to function in the same capacity as that of the QACs (4, 5,14). In some plants, such as *Spartina alterniflora*, and *Wollastonia biflora*, DMSP and glycine betaine are both present. Some research suggests that DMSP serves as a constitutive osmoticum is such systems, while glycine betaine functions as a quick response compound whose concentration is adjusted in response to changing conditions of salinity (9).

Colmer and Higashi authored a paper (9) in which they looked at the effects of salinity, and nitrogen availability on the concentration of sugars, glycine betaine, proline, and DMSP in *Spartina alterniflora*. They found elevated levels of glycine betaine when plants were exposed to high concentrations of NaCl, and nitrogen was provided at a concentration greater than 0.5 M. DMSP showed decreased levels in response to the high NaCl, high nitrogen growth regimen. However, when nitrogen levels were reduced below 0.5 M DMSP accumulated to higher levels vs. control plants. The accumulation of glycine betaine was sharply reduced compared to that seen for high salt and nitrogen > 0.5 M. In marine environments where nitrogen levels are more limiting, DMSP plays more of a primary role in osmotic adjustment.

Grone and Kirst conducted several studies in which DMSP levels were monitored in response to a variety of environmental stressors (31, 32). In a 1992 paper they studied the effects of nitrogen availability, and the intracellular methionine pool size on the DMSP content of *Tetraselmis subcordiformis* (32). (As will be discussed in detail below, earlier studies by Greene in 1962 established that DMSP was produced from methionine) (30). Cultures of *Tetraselmis* grown in nitrogen deficient media showed a two-phase increase in DMSP production. In the first 24 h. DMSP content/cell was increased by ~30%. After 14 days DMSP content increased almost three-fold. This increase is DMSP was accompanied by a decrease in total protein where cultures grown in nitrogen deficient media showed a decrease from 52 pg/cell to 20 pg/cell. The onset of protein reduction occurred within the first 48 h of culture in nitrogen deficient media. When exogenous methionine was provided to cultures growing in a complete medium a 3-fold increase in DMSP/cell was observed. Cultures were also subjected to hyperosmotic shock, protease inhibitors were added, and DMSP content was assayed. Control cultures, which were subjected to hyperosmotic shock without the addition of protease inhibitors, underwent an initial period in which DMSP content /cell was significantly reduced. This was followed by a period of synthesis in which intracellular DMSP recovered to its initial values. The cultures with added proteases did not recover to starting values after the initial decline. This indicated that cells were able to synthesize DMSP as a result of the increase of the free methionine pool resulting from protein degradation.

DMSP's suggested role as a cryoprotectant is naturally demonstrated as polar macroalgae have a significantly higher DMSP content than their counterparts from temperate or tropical regions. Chlorophyceaen species collected from polar regions have an average DMSP content of

40 – 70 mmol kg^{-1} fresh weight, whereas species from temperate waters contain 25 – 30 mmol kg^{-1} fresh weight (43, 44). Several species of green macroalgae collected from an Antarctic habitat were cultivated for one year at temperatures of 0°C and 10°C. Those cultivated at 0°C showed a 2 – 5.5-fold increase in intracellular concentrations of DMSP. The increase in DMSP production seems to be linked to its ability to stabilize enzyme structure at reduced temperatures.

Malate dehydrogenase (MDH) was assayed at 30°C and at –2°C in the presence of increasing amounts of DMSP. Enzyme activity measured at 0°C was stimulated to a level of 165% of the control in the presence of DMSP concentrations up to 300 mM; at 30°C just the opposite was found, increasing DMSP concentration up to 300 mM lowered enzyme activity by up to 35%. This cryoprotective response was not seen with the addition of proline or sucrose (45).

The ability of DMSP to promote enzyme activity in the presence of high salt concentrations was tested using four enzymes isolated from *Tetraselmis subcordiformis* (31), glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, MDH, and a proteolytic enzyme. Of the four enzymes tested only MDH showed a pronounced sensitivity to salt concentration. When MDH was assayed in the presence of saturating substrate concentration,100 mM NaCl produced a 35% decrease in activity, whereas the addition of the chloride salt of DMSP (DMSP-CL) at a concentration of 100 mM did not have such an effect (31). 200 mM NaCl, and 200 mM DMSP-Cl produced similar levels of inhibition. Under substrate limiting conditions near the K_m value for MDH, DMSP proved much less inhibitory than NaCl. MDH activity in the presence of NaCl or

DMSP-Cl showed a decrease in V_{max} and an increase in K_m as is characteristic of mixed inhibition. DMSP-Cl, alone, lowered V_{max} by an additional 7%.

There is a 2.5 fold increase in the rate of malate synthesis to oxaloacetate synthesis under hypersaline conditions when DMSP is not present (31). The K_m of MDH for oxaloacetate in 100 mM Na Cl was four-fold higher versus a two-fold increase in the presence of DMSP-Cl (31). Furthermore under hypersaline conditions when the oxaloacetate hydrating activity of MDH is assayed with no DMSP present; malate acts as an uncompetetive inhibitor by lowering both V_{max} and K_m. At low oxaloacetate concentration malate even acts as an activator rather than an inhibitor (31). When DMSP is present K_m and V_{max} are both decreased, but the enzyme is still responsive to inhibition by malate. Increased substrate levels can overcome inhibition of enzyme activity by NaCL. Overall, the most significant effect of NaCl within a biochemical pathway, maybe its effect on turnover balance. In this case, turnover balance is referred to as the rate of malate formation to oxaloacetate formation (31). In the presence of high NaCl, when oxaloacetate levels are low, malate becomes an activator rather than an inhibitor. In the presence of DMSP, malate continues to act as an inhibitor and the MDH turnover balance is maintained closer to that of control values. The effect of DMSP in this system would be to maintain regulation of the pathway in the presence of hypersaline conditions by preventing malate buildup (31).

The correlation of DMSP production with photosynthetic organisms suggests a link (32). The effect of daylength, and light intensity was tested for its effect on DMSP production (43). Five separate benthic species of Antarctic

Chlorophyceae were grown under differing lighting regimens of low, moderate and high intensity, as well as long and short day lengths (18:6 light/dark cycle, or 6:18 light/dark cycle). The concentration of DMSP increased linearly with increased light intensity, and with longer daylength. Three of the five species tested also showed significant reduction of DMSP concentration when cultured in darkness, although one showed such a reduction only after 170 days. This may have been due to senescence of the culture. The link between DMSP biosynthesis and photosynthetic processes may be due to the requirements of DMSP biosynthetic enzymes for the reductants NADPH and ATP (37).

Acrylate produced from DMSP via DMSP lyase has been shown to be an effective chemical defense agent against herbivore grazing (88, 89). Microzooplankton, which are the primary herbivores of marine phytoplankton, have been shown to avoid DMSP accumulating species when offered a choice of prey. DMSP-lyase is produced by DMSP accumulating marine algae, but is kept physically separate from internal DMSP. This is demonstrated in that exponentially growing cultures produce little DMS, however stationary cultures containing senescent cells produce significant amount of DMS (88, 89). When DMSP containing phytoplankton are ingested by herbivores DMS emission is increased as a result of the mixing of DMSP-lyase and DMSP in the predator gut (88, 89).

DMSP Biosynthesis in Higher Plants

The biosynthesis of DMSP in plants is a complex story. There are at least two distinct pathways for synthesizing DMSP in higher plants. (Fig.2). In marine algae one biosynthetic pathway has been established for the classes Bacillariaphyceae, Chlorophyceae, Prasionophyceae, and Prymnesiophyceae (Fig. 2) and a second has been postulated for Crypthecohdinium cohnii, a heterotrophic dinoflagellate (Fig. 3). This means the pathway has evolved independently at least three times. There are no other examples of such biosynthetic diversity for a single naturally occurring compound. The biological relevance of DMSP must be very significant, possibly because, unlike QACs it is an effective compatible osmolyte that does not tie up significant amounts of a limiting nutrient, nitrogen. It's role as a defense compound subsequent to catabolism by DMSP-lyase may add to its biological value.

Prior to the work done in *Wollastonia biflora*, nothing was known of the intermediates between the putative precursor methionine and DMSP. The necessary steps to convert methionine to DMSP involve deamination, methylation, decarboxylation and oxidation, however the sequence of these steps was not known (Fig. 5). Hanson et. al. (36)used a strategy of radioisotope labeling with [U-^{14}C]Met in feeding studies. He also conducted feeding studies with ^{14}C labeled putative precursors (36) S-methylmethionine (SMM) and methylthiopropionate (MTP) to identify SMM as an intermediate in DMSP biosynthesis. This work was complemented with cold trapping studies, stable isotope labeling, and mass spectrometry to produce several lines of evidence, which unequivocally demonstrated that methylation of methionine to produce SMM was the first step in the DMSP biosynthetic pathway. While SMM is ubiquitous in higher plants as part of the SMM cycle (62), in vivo double labeling of the two methyl groups showed that SMM was directly in the DMSP pathway and not first converted back to methionine.

In pulse chase experiments with [U-^{14}C]Met ,SMM acquired and lost label in a manner that is kinetically consistent with its role as an intermediate. When [^{14}C]SMM was provided; DMSP efficiently acquired label.

Biosynthesis of DMSP in Higher Plants

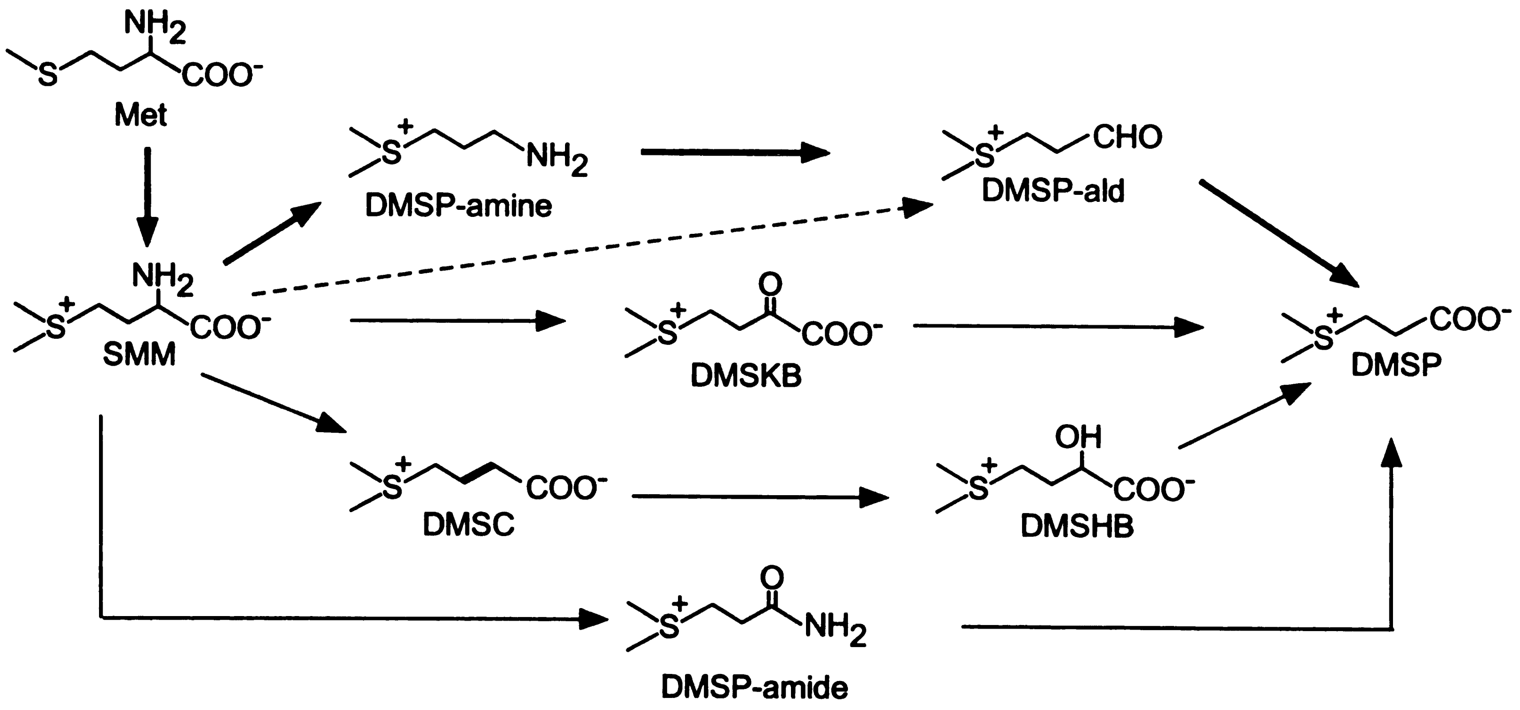


Figure 5) Different possible routes for synthesis of DMSP the heavy arrows indicate the pathway defined in *Spartina alterniflora*. The dashed arrow indicate the unusual transamination in *Wollastonia biflora*.

When unlabeled SMM was used as a cold trapping pool in combination with [U-^{14}C]Met flux of ^{14}C label from [U-^{14}C]Met into DMSP was diminished. The evidence from these experiments was consistent with SMM as an intermediate, but interpretation was complicated by the SMM cycle (Fig.6).

The SMM cycle, as described by Mudd and Datko (61, 62) showed that Met and SMM are interconnected in the species *Lemna paucicostata* through what was described as a futile cycling between the two compounds (Fig.6). In this system of two interdependent, and opposing reactions; SMM is synthesized from methionine and S-adenosylmethionine (S-AdoMet) to yield SMM and S-adenosylhomocysteine (AdoHcy). This irreversible reaction is catalyzed by S-methylmethionine-methyltransferase. The opposing reaction, catalyzed by homocysteine S-methyltransferase, utilizes SMM as a methyl donor to convert homocysteine to Met.

The presence of the SMM cycle provides for the possibility that SMM acquires label from [U-14C]Met not because it is an intermediate in DMSP biosynthesis, but because it is connected to Met through the SMM cycle. To test for this possibility a dose of racemic SMM, in which the methyl groups were differentially labeled with $^{13}CH_3$ and C^2H_3, was provided to leaf discs of *W. biflora* (36). Analyses by FABMS showed that 93 – 96% of the DMSP came from the doubly labeled SMM as evidenced by the DMSP ion at m/z 139, which indicates a four mass unit increase above that of unlabeled DMSP MH$^+$ ion. If SMM did give rise to DMSP indirectly through the SMM cycle, the methyl carbons would have been randomized. Met synthesized from homocysteine and SMM would have a mixture of methyl groups labeled with $^{13}CH_3$, $^{13}CH_3$ -, C^2H_3, C^2H_3 -, $^{13}CH_3$, C^2H_3 in a 1:2:1 ratio. This would have produced mass shifts of M+2, M+4 and M+6 respectively.

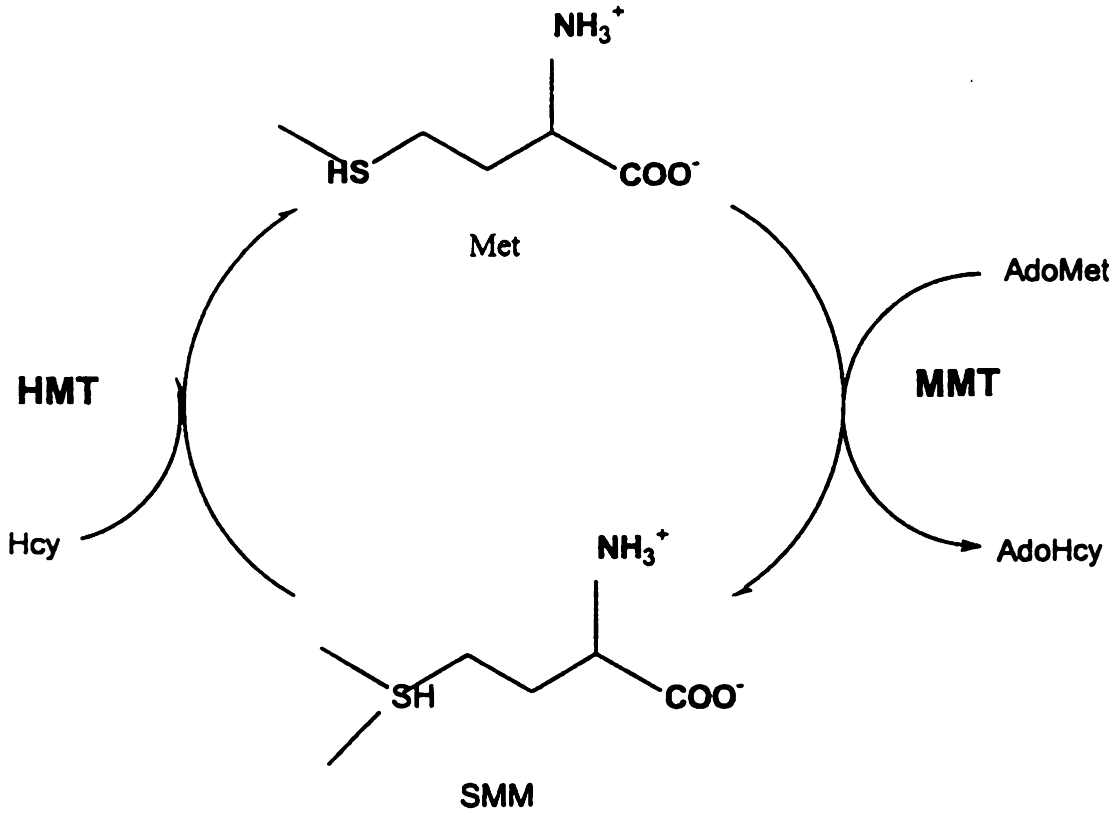


Figure 6) The SMM Cycle: MMT (methionine methyltransferase) uses Met and AdoMet to form SMM and homocysteine. HMT (homocysteine methyltransferase) catlyzes the opposing reaction to form Met from homcysteine and SMM.

Hanson and co-workers used similar methods to demonstrate that 3-dimethylsulfoniopropionaldehyde (DMSP-ald) was the next intermediate in the DMSP pathway (42). [^{35}S]SMM was given to Wollastonia leaf discs and DMSP-ald incorporated and lost label in parallel with the decline in specific activity of the SMM pool. In addition unlabeled DMSP-ald was supplied as a trapping pool and shown to be effective in decreasing flux of label from [^{35}S]SMM into DMSP. The methylation of Met to form SMM, and oxidation of DMSP-ald to DMSP can be explained by "well established" biochemical reactions

The conversion of SMM to DMSP-ald requires two steps, transamination, or oxidative deamination, and decarboxylation. DMSP-amine is a stable species and would be a likely intermediate resulting from decarboxylation of SMM. Hanson et al. never isolated a free intermediate. The possible transamination product 4-Dimethylsulfonio-2-ketobutyrate (DMSKB) is an unstable species, which spontaneously undergoes β, γ-elimination to yield DMS, and vinylglyoxalate (10). The absence of DMSP-amine in the labeling studies and the inherent instability of DMSKB led Hanson and co-workers to propose that a unique multi-enzyme complex catalyzes the SMM → DMSP-ald conversion in which there are no free intermediates (68).

The studies in *Wollastonia* were extended to *Spartina alterniflora* (42). Both plants were known to be DMSP accumulators, however they are phylogenetically distant. In *Spartina*, SMM and DMSP also acquired label when a pulse of [^{35}S]Met was given. Unlike *Wollastonia* labeled DMSP-amine, was detected. As discussed above DMSP-amine is the stable decarboxylated product of SMM. When [^{35}S]SMM was supplied DMSP-amine, acquired and lost label in response to the depletion of radiolabeled SMM from the SMM pool. In studies which used [^{35}S]DMSP-amine as a precursor the DMSP-ald became labeled in a manner consistent with its role as a downstream intermediate in the DMSP pathway. The labeling experiment with [^{35}S]DMSP-amine provided one additional surprise. Regardless of whether a large or small dose of precursor was supplied; about 20% was metabolized to DMSP. This indicated a high capacity for the metabolism of DMSP-amine. A metabolic model developed to fit the data (42) suggests there are large storage pools of DMSP-amine and SMM, and small metabolically active pools with a low flux rate between them compared to rate of DMSP synthesis. Exogenously supplied DMSP-amine, therefore first enters the storage pool prior to being metabolized to DMSP. The evidence garnered from this experiment indicated that the DMSP synthetic pathway in *Spartina* was distinct from that seen in *Wollastonia* sharing several steps, but with a different enzyme yielding DMSP amine as a free intermediate.

Enzymes of the DMSP Biosynthetic pathway in Higher Plants

The S-adenosyl methionine (SAM) dependent methionine-S-methyltransferase (MMT) was purified and characterized from *W. biflora* (51). This enzyme catalyzes the formation of SMM, which is the first committed intermediate in DMSP biosynthesis in higher plants. MMT appears to be a novel enzyme. It is a homotetramer of 110 KDa subunits. Most O and N-methyltransferases are typically monomers or dimers with subunits in the range of 20 – 45 KDa in size (21). Mammalian small molecule methyltransferases involved in DNA modification are typically ~250 –300 residues in length (21). The AdoMet binding domain for MMT is contained in its first 100 residues which suggests it may contain a novel methyltransferase domain. The 750 residue C-terminus of the protein shows some similarity to starch and glycogen phosphorylases. The highest region of similarity of MMT is to the pyridoxal 5'phosphate (PLP) domain of glucan phosphorylases. No other known methyl transferase is PLP-dependent. In phosphorylase this domain includes the lysine to which PLP is bound as a Schiff base; however, in MMT this lysine is absent. If PLP is present in MMT, it must be bound in a different position. Spectroscopic studies reveal a small absorption peak at 330 nm and fluorescence at 550 nm, similar to that of PLP-bound phosphorylase. It is important to emphasize that the meanings of the unusual features of MMT have yet to be determined. Kinetic analyses of MMT indicate that it has an ordered Bi Bi mechanism in which

AdoMet is the first substrate to bind and AdoHomocysteine (AdoHCy) is the last product released.

The second step of the DMSP anabolic pathway in *Wollastonia* converts SMM to DMSP-ald. This is a two step process requiring deamination/transamination, and decarboxylation. The unstable nature of the 2-keto acid intermediate DMSKB suggests two possibilities (10). One possibility is that DMSKB only exists as a tightly bound intermediate for an unusual enzyme capable of carrying out both required steps. The second possibility is that the two enzymes operate as a multi functional complex in which the unstable 2-keto acid intermediate is immediately channeled from one active site to the other. Both possibilities represent unusual biochemical reactions. Stable isotope labeling and GCMS experiments were carried out using ^{15}N labeled Met to help determine the enzyme mechanism (69). Amino acid pools were analyzed by GCMS to ascertain the nature of the amino acceptor. MS analyses showed the glu pool to be more highly labeled than gln or asn. This indicated a transamination mechanism rather than oxidative-deamination, which would generate free $^{15}NH_3$. It would be expected that free $^{15}NH_3$ would subsequently be assimilated into the amide nitrogen of glutamine pools via the GS-GOGAT (glutamine synthase-glutamate dehydrogenase, glutamate aminotransferase) pathway.

There is not much information on the SMM → DMSP-amine conversion or the DMSP-amine → DMSP-ald potential conversion. The final step in which DMSP-ald is converted to DMSP is common to both pathways and some initial characterization has been done. The oxidation of DMSP-ald to produce DMSP has been shown to occur via 3-dimethylsulfoniopropionaldehyde dehydrogenase (DDH) (83). This enzyme prefers NAD to NADP and is localized to chloroplast stroma. It has a low K_m for DMSP-ald of 1.5 μM, which is consistent with the calculated small pool sizes of available substrate. DDH follows Michaelian kinetics, and is subject to substrate inhibition. Other small aldehydes are also capable of acting as inhibitors, including betaine aldehyde at concentrations below 0.4 mM. In fact, this dehydrogenase is likely related to the dehydrogenase involve in glycine betaine biosynthesis. Antiserum to betaine aldehyde dehydrogenase (BADH) neutralizes DDH activity (83). DMSP-ald is also a superior substrate for BADH than is betaine aldehyde. This strongly suggests structural similarity between the two enzymes (21).

Early work on Algal Biosynthesis of DMSP

In 1962 Ronald Greene published the first paper on the synthesis of DMSP in marine algae (30). His study used *Ulva lactuca*, a macro-algae of the class Chlorophyceae, to establish that DMSP was synthesized from methionine. In several rounds of labeling Greene provided cultures of algae with [^{35}S]Met, [^{14}C1]Met, and [^{14}C2]Met and analyzed the alkaline hydrolyzed products of DMSP (DMS and acrylate) for their incorporation of label from methionine. This work established that the carbon skeleton of DMSP originated from methionine, which underwent methylation, deamination, decarboxylation, and oxidation to form the penultimate product. Greene also used [^{14}CH$_3$]Met to show that the methyl donor for the formation of DMSP was also methionine. In an attempt to identify some of the intermediates in the pathway he used [^{35}S]SMM in the same type of feeding experiments and found no labeling of DMSP resulting from SMM.

Uchida et al, has done previous research on DMSP biosynthesis in dinoflagellates. The work of Uchida et al. was done using (86) *Crypthecodinium cohnii*, a heterotrophic dinoflagellate. (86) This is an interesting case, because biosynthesis of DMSP has typically been linked to photosynthesis (17, 37, 43, 48). Uchida et al. also used [^{35}S]Met in feeding studies to determine intermediates in the pathway; however, they did very long term labeling (1 week). The feeding studies were done in the dark, whereas others have always been done under illumination. The authors suggest a different pathway for synthesis of DMSP in *C. cohnii*, in which the first step is decarboxylation (Fig. 3).

Their scheme is based on research which investigated the ability of putative intermediates to effect a diminution in flux of the ^{35}S label from [^{35}S]Met into DMSP. The results showed that 4-methyl-2-oxobutyrate (MTOB), which would be the product if the first step in the pathway were deamination of Met, and SMM were both ineffective as trapping pools. In contrast, methylthiopropionate (MTP) was a very effective as a trapping pool. This compound could be formed by several routes, but they extrapolated that decarboxylation was the first step in the DMSP pathway based on the purification of a methionine decarboxylase from *C. cohnii.* Methionine decarboxylase has previously been shown to be present in algae and plants that do not accumulate DMSP (66). Without direct evidence Uchida et al. proposed that methylthiopropylamine (MTP-amine) is the first intermediate in the DMSP pathway, because it is the product of methionine decarboxylation. The research by Uchida and co-workers while provocative doesn't make a convincing case for MTP and MTP-amine as intermediates. There were no direct feeding studies reported that showed these compounds exhibited the type of labeling kinetics that would be expected of an intermediate. The presence of a decarboxylase that uses methionine as a substrate doesn't argue convincingly either, as amino acid decarboxylases are widespread (72, 86).

Nakamura and colleagues have done some interesting work with the autotrophic dinoflagellate *Linglodinium polyedra*. They sought to establish a link between the biosynthesis of DMSP, and two other sulfonium compounds, gonyol, and gonyauline (Fig. 7). Nakamura used a combination of stable isotope labeling and NMR in his investigation. He fed [methyl-$^{13}C_1$]Met and [methyl-$^{13}C_1$]SMM and determined that the methyl groups in gonyauline come from Met (63).

DMSP Biosynthesis in *L. polyedra*

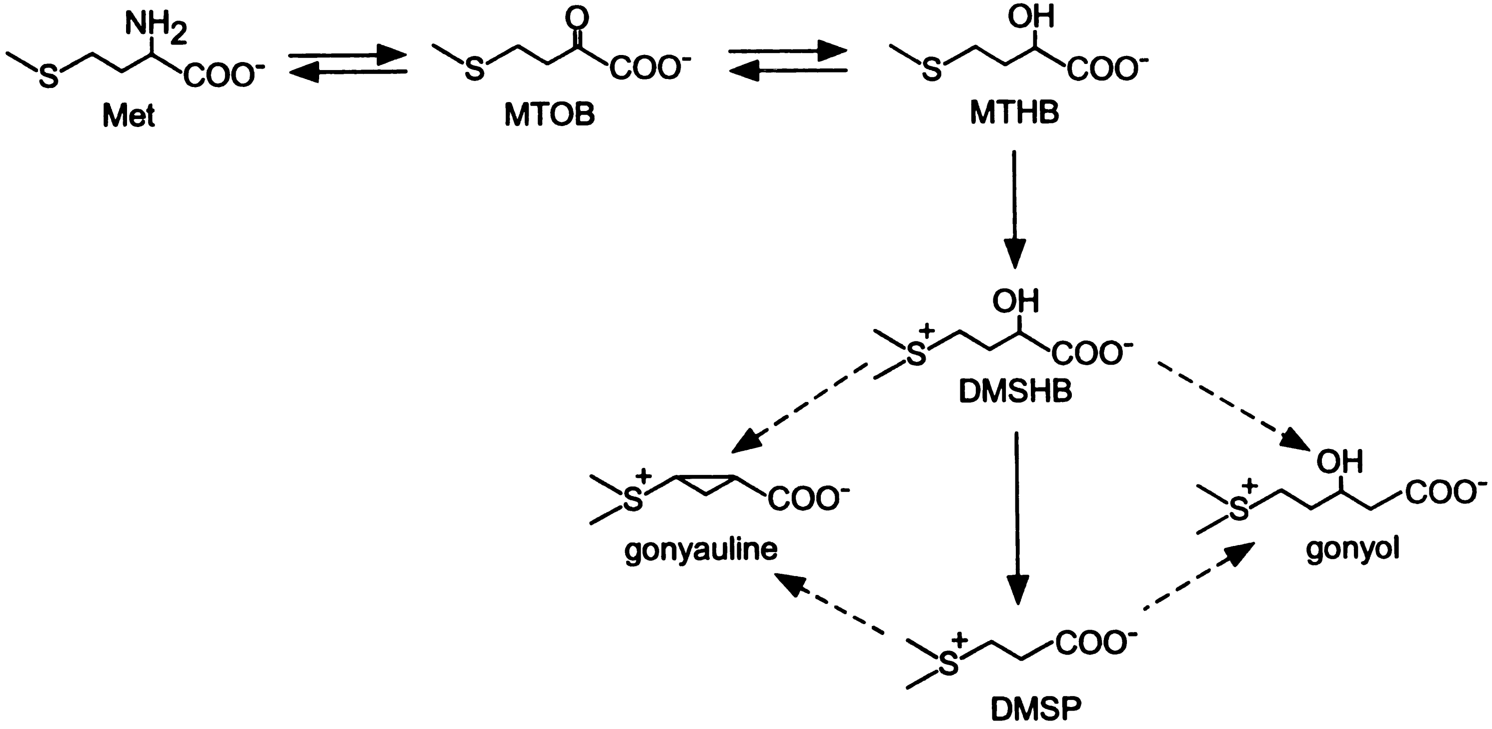


Figure 7) Known biosynthetic pathway in marine algae showing possible connection to synthesis of gonyauline and gonyol.

Using [1-^{13}C]Met they found no ^{13}C in gonyauline. He also fed [U-^{13}C]DMSP and found that the C1-C3 positions and the methyl groups were incorporated into gonyauline. In gonyol however only the methyl groups and the C3-C5 positions labeled. This suggested gonyauline was synthesized from Met, but the carbon atom, which occupies the C1 position, was different in both gonyauline and gonyol when labeled Met was used as a precursor. Nakamura et al. proposed that the C1 and C2 atoms of gonyol come from acetate. This was supported by feeding studies with [1,2-^{13}C]AcOH, which showed that ^{13}C was incorporated into the C1 and C2 positions of gonyol. They concluded that gonyol and gonyauline were synthesized from DMSP, however the C1 of gonyauline originates from CO_2 and the C1 and C2 positions of gonyol are derived from acetate. Their statement that the C1 position of gonyauline comes from CO_2 is based on the increase of ^{13}C incorporation at the C1 position when $NaH^{13}CO_3$ was added to the medium. This increase was only seen when the organisms were cultured in the light, not in the dark. This led them to the conclusion that the incorporation of CO_2 was photosynthesis dependent. The conclusions drawn from the work of Nakamura et al. require additional confirmation as the long term nature (7days) of his experiments allows for the possibility of scrambling of carbon atoms from the supplied precursors into other intermediates.

Research Objectives

The work described in the following chapters is directed toward elucidating the DMSP biosynthetic pathway in marine algae. This will be done using a strategy of isotope labeling with radioactive and stable isotopes, thin layer chromatography, Thin layer electrophoresis, mass spectrometry, and autoradiography in a similar means to that used in defining DMSP biosynthesis in higher plants. This work was first done in species of four diverse classes of marine algae, *Enteromorpha intestinalis* (Chorophyceae) *Tetraselmis subcordiformis* (Prasinophyceae) *Melosira nummuloides* (Bacillariaphyceae) and *Emiliana huxleyi* (Prymnesiophyceae) and then extended to dinoflagellates because they accumulate more DMSP and produce more DMS than marine algae of other classes. *Heterocapsa triquetra* is used as a model organism for dinoflagellates, but DMSP biosynthesis in *Crypthecodinium cohnii*, and *Linglodinium polyedra* is also investigated because of the unusual features highlighted by previous research done in these organisms.

CHAPTER II

A NEW ROUTE FOR THE SYNTHESIS OF DIMETHYLSULFONIOPROPIONATE IN MARINE ALGAE

Acknowledgement

The work contained in this chapter was previously published in the journal *Nature* as Gage, D. A., Rhodes, D., Nolte, K. D., Hicks, W. A. Leustek, T., Cooper, A. J. L., and Hanson, A. D., (1997) A new route for synthesis of dimethylsulfoniopropionate in marine algae. Nature, 387, 891-894

Introduction

As discussed in Chapter 1 the biosynthesis of DMSP has been established in higher plants. Flowering plants synthesize DMSP through the sequential methylation, decarboxylation, transamination, and oxidation of methionine. There are two variants of the pathway found in *Spartina* and *Wollastonia*. Algae also synthesize DMSP from methionine, but until recently none of the intermediate steps were known, only that the carbon skeleton and sulfur of DMSP were incorporated intact into DMSP (31).

DMSP synthesis in marine algae has now been shown to share no common steps with the pathway in higher plants. There were some early studies on the synthesis of DMSP in the dinoflagellate *Linglodinium polyedra* by Nakamura et al, (63). They used radiolabeling methods to show that the sulfur, C2, and C3 atoms of methionine are incorporated into DMSP. This was consistent with Greene's result in *Ulva lactuca* that DMSP is derived from methionine.

Uchida et al also conducted some early experiments in the dinoflagellates. They used the heterotrophic species *Crypthecodinium cohnii*. The evidence they obtained suggesting the distinct pathway Met → MTP-amine → MTP → DMSP was based on indirect evidence. The conversion of Met to MTP-amine, which is the first step of their pathway, was based on the purification of a methionine decarboxylase from *C. cohnii*. Methionine decarboxylases are however present in many other species which do not produce DMSP (72, 77, 87).

At the time I joined the lab experiments were underway to define the route for biosynthesis of DMSP in *Enteromorpha intestinalis*, a macroalgae of the class Chlorophyceae, *Melosira nummuloides*, a diatom, *Tetraselmis*, a Prasinophyte, and *Emiliana Huxleyi*, a prymnesiophyte. Methods of in vivo isotope labeling were combined with chromatography, electrophoresis, and mass spectrometry to elucidate the biosynthetic pathway of DMSP in these diverse marine algae. From this work the pathway has now been shown to synthesize DMSP from the sequential transamination, reduction, methylation, and oxidative - decarboxylation of methionine to form DMSP. This pathway includes the biosynthesis of the novel sulfonium compound 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB). The intermediate DMSHB has also been demonstrated to serve many of the same biological functions as DMSP. This project was a collaborative effort between the laboratories of Andrew Hanson, Univ. of Florida, Tom Leustek, Rutgers Univ. Arthur Cooper, Cornell Univ. David Rhodes, Purdue University, and Doug Gage. A collaborative paper was published in the journal, Nature (20) that described the pathway elucidation and included my analytical studies presented below.

My specific role, described herein, was quantitative and qualitative analysis of metabolites isolated from stable isotope feeding studies. The objective of my work was to define the enzymatic mechanisms involved in two of the biosynthetic steps: the deamination of methionine and the decarboxylation/oxidation of DMSHB. New analytical methods were developed as a part of this project.

Materials and Methods

Materials. Andrew Hanson, Florida State University, provided DMSHB that was used as a standard for mass spectrometric measurements, zwitterionic fractions isolated from *Enteromorpha* that contained DMSHB and DMSP, and DMSHB and DMSP that was labeled with stable isotopes. David Rhodes, Purdue University, provided amino acid samples that were labeled with ^{15}N and isolated from *Enteromorpha*. All other chemicals and reagents were purchased from commercial sources.

Derivatization of sulfonium compounds. The sulfonium compounds isolated from the zwitterionic fractions of *Enteromorpha intestinalis* were derivatized to their tert-butyl dimethylsilyl (TBDMS) esters for analysis by GC-MS. The zwitterionic fractions of algal lysates were lyophilized to complete dryness. The dried sample was then further dried overnight in a vacuum dessicator in the presence of P_2O_5 in order to remove all traces of water. A derivatization cocktail was prepared by mixing 20 μL N-(tert–butyldimethylsilyl)-N-methyltrifluoroacetamide with 2 μl of 10% (TMSCN) in chloroform, 4ul 10% trimethylpyridine In chloroform, and 2 μL 10% dimethoxypropane in chloroform. The above reactants were mixed in the above order. 25 μL of the above derivatization cocktail was added to the dried sample. The sample and cocktail were incubated for 2 h at 60°C.

Gas Chromatography-Mass Spectrometry of TBDMS derivatives of sulfonium compounds. Mass spectrometric data was obtained using a JEOL AX-505H double focusing mass spectrometer coupled to a Hewlett-Packard

5890J gas chromatograph. Gas chromatography was acquired using a 30 m DB1 column (O.25mm ID and 0.25μm film coating). The column was procured from J&W Scientific. Direct (splitless) injection was used. Helium gas flow was approximately 20 ml/min. GC conditions were as follows: Injector temperature 260°C, initial temperature 100°C, initial time 2 min, rate 20°C/min, final temperature 310 °C, final time 0 min MS conditions were as follows: separator temperature 285 °C, ion source temperature 200°C, electron energy 30 eV, scan rate 45-500 a.m.u. at 0.8 scans/sec. DMSHB was derivatized as its *t*-butyldimethylsilyl ester/ether and analyzed by GC-MS with selected ion monitoring after on-column nucleophile assisted *S*-demethylation. Authentic DMSHB was used to calibrate the selected ion monitoring parameters. The diagnostic fragment ion clusters at mass/charge ratio (m/z) 321, due to loss of a *t*-butyl radical, was monitored in the SIM mode with peak centering at 8'26", which corresponds to the appropriate retention time. The SIM parameters for ^{13}C labeled DMSHB were programmed to monitor ions from 321.2 m/z - 327.2 m/z in increments of 1, with peak centering at 8'25".

Derivatization of ^{15}N labeled amino acids. 100 mg batches of *Enteromorpha intestinalis* were incubated for 1, 2, and 4h with 1 μmole of [^{15}N]ammonium chloride of with 5 μmole [^{15}N]Met in 2.5 ml of seawater. These were extracted using a standard methanol-chloroform-water extraction procedure. I analyzed samples of the neutral amino acid fraction from [^{15}N]Met feeding studies that were provided by David Rhodes. The samples were derivatized for analyses by mass spectrometry using 60 μl of a 60:30:10 mixture of water-isobutanol-pyridine, 10 μl of isobutylchloroformate and 60 μl of chloroform.

Gas chromatography-mass spectrometric analyses of Glutamine and Asparagine. GC-MS data was acquired as described above except that the GC conditions were as follows: 280°C, initial temperature 100°C, initial time 2 min, rate 10°C/min, final temperature 300°C. MS conditions were as follows: separator temperature 300°C, ion source temperature 200°C, electron energy 30 eV, scan rate 0.8 scans/sec over a mass range of 45-500 a.m.u.

Results

In order to determine the biosynthetic intermediates in the marine algae *Enteromorpha intestinalis*, large scale labeling studies were carried out in the laboratory of Andrew Hanson at the University of Florida with the assistance of Doug Gage, Michigan State University, David Rhodes, Purdue University, Thomas Leustek, Rutgers University, and Arthur Cooper, Cornell University. Andrew Hanson coordinated the project, and oversaw the labeling studies with all of the algae except those done with *Tetraselmis*, which were conducted by David Rhodes. David Rhodes also developed a computer model to analyze the kinetics of metabolite interconversion in the DMSP pathway. Isolation and mass spectrometric analyses of acidic, and basic amino acid fractions from [^{15}N]Met and $^{15}NH_4OH$ fed *Enteromorpha* was also done by David Rhodes. I performed GC-MS analyses of DMSHB samples and analyzed FAB-MS spectra of DMSP isolated from [^{13}C]Met fed *Enteromorpha*. I also performed GC-MS analyses of neutral amino acid fractions isolated from *Enteromorpha* that had been fed [^{15}N]Met or $^{15}NH_4OH$.

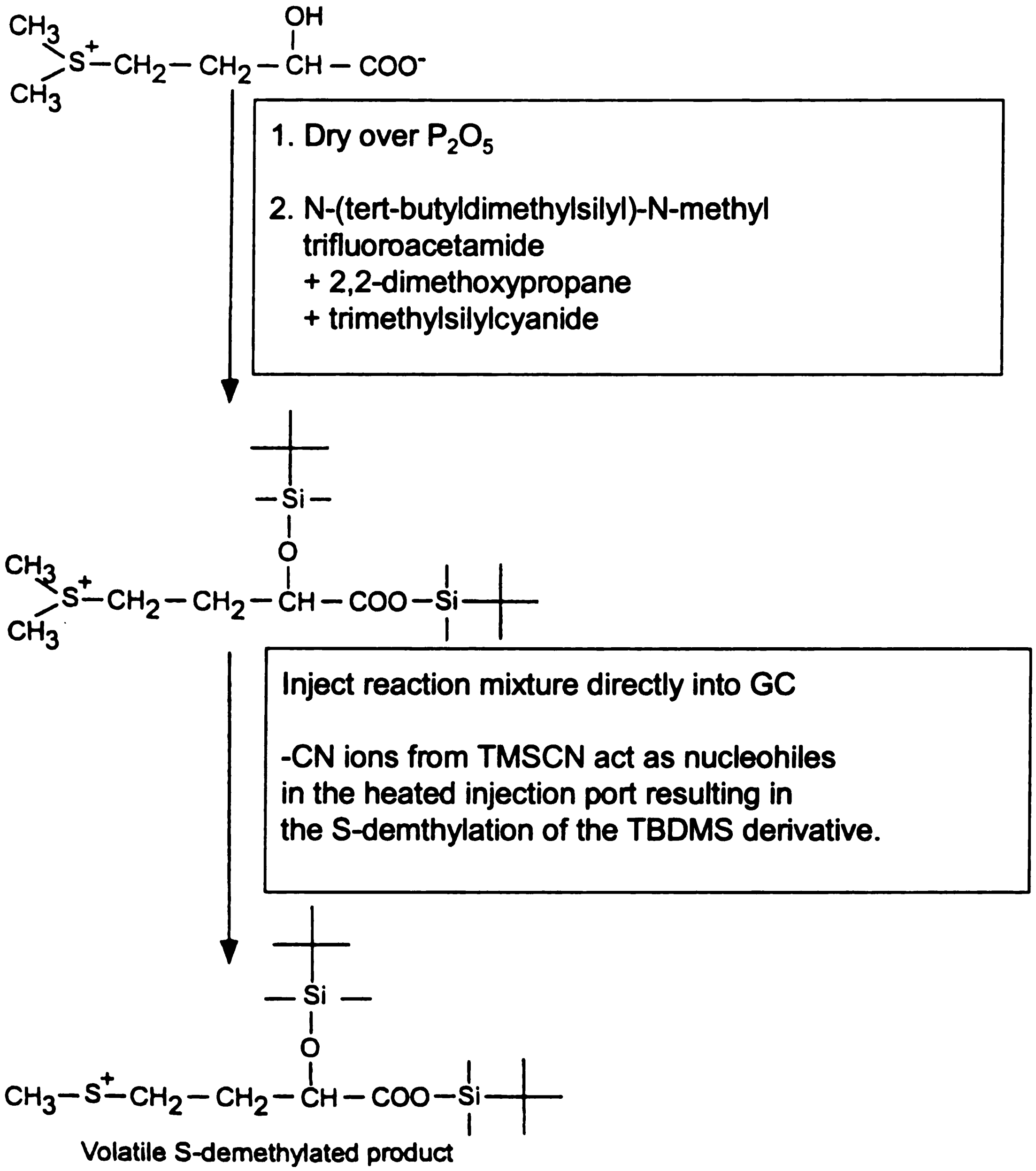
100 mg fronds of algae were incubated with a 500 pmol dose of [^{35}S]Met. The metabolic fates of the Met were followed using thin layer chromatography (TLC) and thin layer electrophoresis (TLE). The major fates of Met were incorporation into protein and conversion to DMSP. Two compounds labeled in a manner that is consistent with the expected kinetics for an intermediate. These two compounds were 4-methylthio-2-hydroxybutyrate (MTHB) and its

S-methylated product dimethylsulfonio-2-hydroxybutyrate (DMSHB). The most likely route from Met to MTHB is via the relatively unstable α-keto acid , 4-methylthio-2-oxobutyrate (MTOB), which can undergo spontaneous decarboxylation to form methylthiopropionate (MTP). An alternative gentle extraction method was employed to quantify MTOB. MTOB was also converted to a stable derivative, MTHB, with sodium borohydride. Comparisons of reduced and unreduced samples were used to estimate MTOB pool size. Both methods showed that MTOB acquired and lost ^{35}S label in parallel with MTHB. MTP that was produced as a result of the spontaneous decarboxylation of MTOB could also be estimated based on the difference between reduced and unreduced samples of MTOB. Analysis of MTP levels by this procedure shows that a significant amount, but not all of the MTP which labels in the [^{35}S]Met labeling experiment is derived from MTOB (personal communication from Andrew Hanson).

Compounds other than MTOB, MTHB, and DMSHB which were considered likely intermediates based on other studies of DMSP biosynthesis, or because of known biological reactions were also monitored for uptake of label from [^{35}S]Met. Two of the known intermediates in higher plant DMSP biosynthesis, S-methylmethionine (SMM) and 3-dimethylsulfoniopropionaldehyde (DMSP-ald) (36, 42) showed little or no uptake of label. MTP-amine, which was proposed to be an intermediate of DMSP biosynthesis in dinoflagellates by Uchida et al (86), slowly accumulated label as a minor end product. MTP, . another compound from the pathway suggested by Uchida et al., labeled in a variable manner, from undetectable to a level commensurate with that of MTOB. This was shown in repeated experiments with different lots of *E. intestinalis*. The data from MTP can be explained based on a catabolic route involving oxidative decarboxylation of MTOB to MTP, which occurs in other plants and algae that do not accumulate DMSP (12, 58, 66). This same pathway is also present in animals and varies with nutritional status.

The labeling data acquired from [^{35}S]Met labeling is consistent with the pathway Met → MTOB → MTHB → DMSHB → DMSP. This pathway was further supported when ^{35}S labeled versions of the putative intermediates, SMM, MTHB and DMSHB were supplied. Very little SMM was metabolized. Taken together with the data from the [^{35}S]Met labeling studies SMM does not appear to be an intermediate in the algal pathway. MTHB was mainly converted to Met, which was then converted to protein, and a small amount was also found in DMSP. These data are consistent with the conversion of Met → MTHB through a freely reversible transamination reaction. DMSHB was metabolized to DMSP. The identification of intermediates in the ^{35}S labeling studies was based on co-migration with standards during TLC and TLE. In order to further support the data; experiments using stable isotope labeling were conducted. *E. intestinalis* was supplied [U-^{13}C]Met in atmospheres containing $^{16}O_2$ or $^{18}O_2$. The latter was used to investigate the enzymatic reactions involved, since incorporation of ^{18}O into DMSP would provide evidence if an oxygenase was involved in the formation of DMSP from DMSHB (20). The zwitterionic fractions were isolated and these samples were submitted to me for analyses by GC-MS, and fast atom bombardment mass spectrometry (FABMS). These analyses were done as a means to verify the presence of DMSHB, which is a novel biological compound, and to test if an oxygenase reaction is involved in its conversion to DMSP.

Analysis of the sulfonium compound, DMSHB, required the development of a new technique, because this metabolite is found in low levels and is difficult to completely separate from other compounds. DMSHB was converted to its TBDMS-derivative and analyzed by GCMS in order to take advantage of the separation capability and sensitivity of the method. Trimethylsilylcyanide (TMSCN) was included in the derivatization mixture as a source of CN^- ions, which promoted the S-demethylation of DMSHB (Fig. 8). As salts, sulfonium compounds would not normally be amenable to analysis by GCMS because of their fixed charge and polar nature. This novel approach for analysis of sulfonium compounds was developed from a similar approach used to analyze acylcarnitines (39). The derivatization and demethylation procedure greatly enhances the volatility of sulfonium compounds, and makes their analyses by GCMS possible (19, 39). I used single ion monitoring (SIM) for the GCMS analyses. This was shown to be very sensitive with a 10 pmol detection limit (Table I). The S-demethylated DMSHB-TBDMS derivative had a retention time of 8 min 26 s. The M-57 peak at m/z 321 (loss of a t-butyl group) was diagnostic for the presence of DMSHB.



1. Dry over P_2O_5

2. N-(tert-butyldimethylsilyl)-N-methyl trifluoroacetamide
   + 2,2-dimethoxypropane
   + trimethylsilylcyanide

Inject reaction mixture directly into GC

-CN ions from TMSCN act as nucleohiles in the heated injection port resulting in the S-demthylation of the TBDMS derivative.

Figure 8) Method for the dervatization of DMSHB for analysis by GCMS

Detection Limit for DMSHB		
Sample	Retention time	Peak area
800 pmol DMSHB + 1 – 2 μmol DMSP	8 min. 27 s	20.142
200 pmol DMSHB + 1 – 2 μmol DMSP	8 min. 27 s	1.0691
40 pmol DMSHB + 1 – 2 μmol DMSP	8 min. 27 s	0.2252
20 pmol DMSHB	8 min. 26 s	7.8909
10 pmol DMSHB	8 min. 25 s.	0.7460
10 pmol DMSHB *	8 min. 25 s	6.2817

Table I) GCMS data acquired in the SIM mode for the S-demethylated TBDMS esters of DMSHB.

(Note: The first three entries in the table are samples from ion-exchange purified extracts of *Enteromorpha* that was spiked with the 20, 5, and 1 nmols of DMSHB respectively. The amount of DMSHB entered in the table is the amount injected onto the GC column assuming 100% recovery from the ion exchange columns. The last entry in the table (*) was taken with the collection slit opened to 250 this increased the signal by a factor of 8.37.)

The *E. intestinalis* samples that had been supplied with [U-^{13}C]Met in chambers with atmospheres containing $^{16}O_2$ or $^{18}O_2$, showed a significant peaks at m/z 326, which correspond to the M+5 peak of the M-57 peak of the S-demethylated DMSHB-TBDMS ion (Fig.9). Because the analyte is S-demethylated, the sixth carbon is not directly detected. Nevertheless, we can infer that the entire carbon skeleton of DMSHB is derived from Met, which is consistent with Greene's earlier result in *Ulva lactua* (30). Met is the likely methyl donor in the methylation of MTHB to form DMSHB (via S-AdoMet). There was no indication of ^{18}O incorporation into DMSHB, which suggests that oxygens in the hydroxyl and carboxyl groups are not derived from molecular oxygen

Because DMSP is an abundant metabolite, a less sensitive MS technique, fast atom bombardment mass spectrometry could be used for its analysis. Derivatization is not required for this technique. FABMS of DMSP samples from the [U-^{13}C]Met feeding study done in an $^{16}O_2$ atmosphere showed significant M+4 and M+5 peaks (Fig. 9). The M+4 peak is likely the result of the [$^{13}C_4$]homocysteine moiety. Homocysteine is recycled back to Met after it has been used in methylation reactions as AdoMet, with the resulting formation and release of AdoHcy. GCMS and FABMS (Fig.10) confirmed the presence of the recycled Met. Modeling studies indicated the [$^{13}C_4$]DMSP/[$^{13}C_5$]DMSP ratio agreed with expected values for the recycling of [$^{13}C_4$]homocysteine into DMSP. GCMS data of DMSHB formed from [U-^{13}C]Met in an ^{18}O atmosphere show no significant increase in the M+2 peak indicating the α-hydroxyl group does not originate from O_2. The FABMS spectrum of DMSP resulting from *E. intestinalis* treated with [U-^{13}C]Met /^{18}O shows strong signals at m/z 141 (^{13}C4,^{18}O) and 142 (^{13}C5, ^{18}O) indicating that 30-40% of the DMSP formed became labeled with ^{18}O.

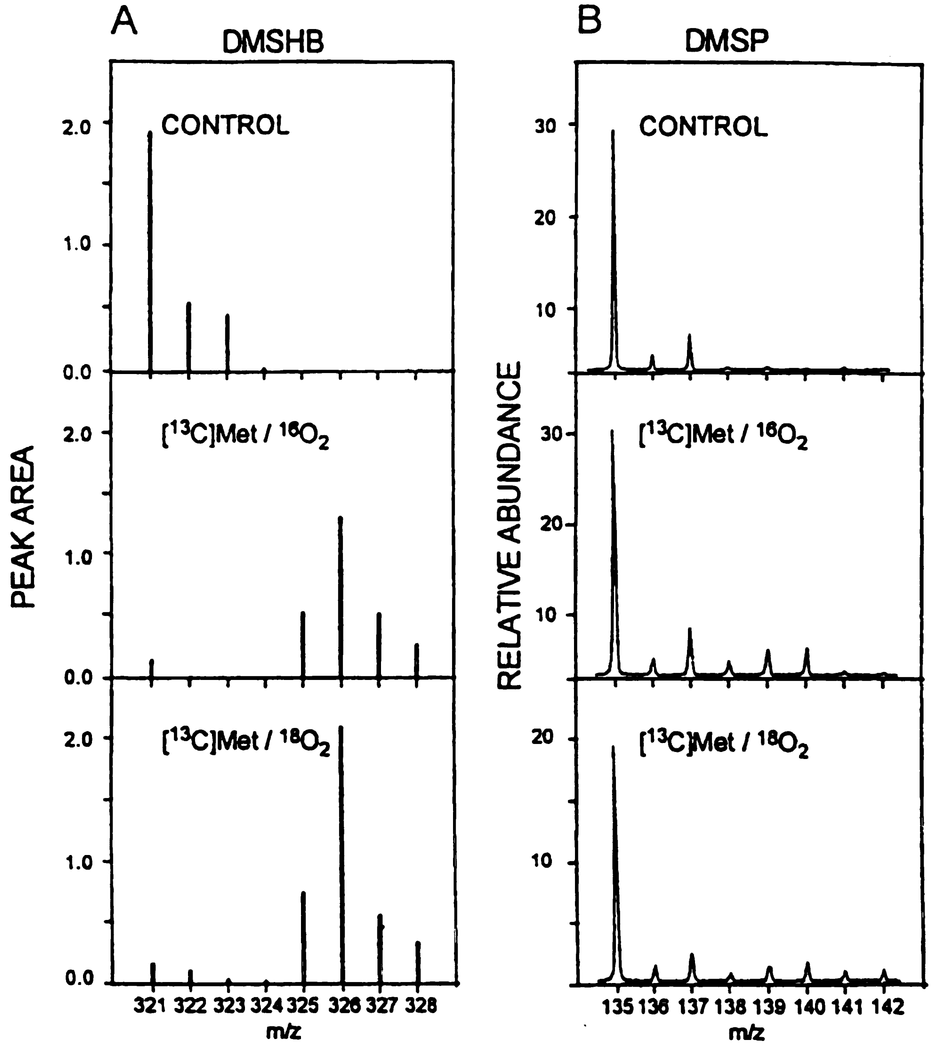


Figure 9) Mass Spectral evidence for DMSHB and for its conversion to DMSP by an oxygenase reaction. E. intestinalis fronds (100 mg fresh weight) were incubated for 24 h with L-[U-^{13}C]Met (5 µmol) in a flask initially containing 21% $^{16}O_2$ or $^{18}O_2$ in N_2; controls received no Met and no $^{18}O_2$.
A) SIM analysis of DMSHB. The peak areas in endogenous DMSHB was replaced by $^{13}C_4$-and $^{1}3C_5$-labelled species the S-demethylatedderivatives of [^{13}-C_4]- originate from O_2.
B) FABMS analysis of DMSP. In the [^{13}C]Met/$^{16}O_2$ treatment, the peaks at m/z 141 and 142 ([$^{13}C_4$, ^{18}O]-and [$^{13}C_5$, ^{18}O]DMSP) show that 30-40% of the ^{13}C-labelled molecules contain an ^{18}O atom.

Photosynthetically generated $^{16}O_2$ likely lowered this percentage. The information gathered from the ^{13}C and ^{18}O labeling data indicate that an oxygenase reaction is involved in the oxidative decarboxylation of DMSHB to DMSP.

Fate of Methionine Nitrogen in DMSP Biosynthesis

Oxidative deamination or transamination are both plausible mechanisms for the deamination of Met to form MTOB. Stable isotope labeling was used to differentiate between the two mechanisms. If MTOB were formed via oxidative deamination, free$^{15}NH_3$ from the α-$^{15}NH_3$ of [^{15}N]Met would have been generated. Incorporation of free $^{15}NH_3$ into the amide nitrogen of Gln through the action of glutamine synthase would then be expected. In the case of transamination the α-$^{15}NH_3$ of methionine would have been transferred directly to α-ketoglutarate, oxaloacetate, or pyruvate. This would result in the formation of ^{15}N labeled Glu, Asp and Ala respectively (69). 5 μmol of [^{15}N]Met was exogenously supplied to 100 mg of *E. intestinalis* and GCMS was used to examine the pattern of labeling in the amino acid pool. Glu (7.0% abundance at 2 hrs), Asp, and Ala acquired ^{15}N rapidly (Table II). This is consistent with a transamination mechanism.

The neutral amino acid fraction was isolated, and I converted them to their *N*-butylcarbonyl isobutyl esters prior to analyses by GCMS (Fig. 11)(40, 88).

nmol 15N amino acid/gfw (rounded to the nearest nmol)							
amio acid	T = 0 h	Met 1 h	Met 2 h	Met 4 h	NH4+ 1 h	NH4+ 2 h	NH4+ 4 h
Ala	0	13	11	20	137	150	84
Met	n.d.	4,374	5,815	8,965	n.d.	n.d.	n.d.
Asn	0	1	2	7	29	61	68
Gln	0	15	13	25	702	812	612
Asp	0	6	11	12	26	41	49
Glu	0	51	77	119	283	383	374

Table II) 80 mg fresh weight samples of *E. intestinalis* were fed 5 μmol [^{15}N]Met or 1 μmol $^{15}NH_4$. The amino acids were isolated and derivatized to their N(O,S)-heptafluorobutyryl isobutyl esters for analyses by GCMS. Quantitation was based on ^{15}N abundance and predetermined pool sizes.
(Personal communication from David Rhodes, Purdue University)

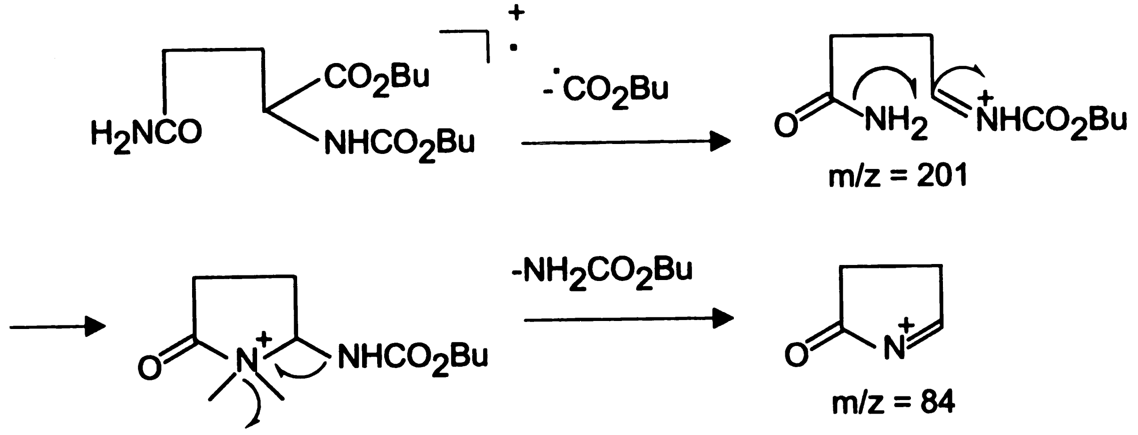


Figure 11) Fragmentation pattern of isobutylchloroformate isobutylester of Gln

The abundance of the M+1 peak of the ion containing the amide nitrogen at m/z 84, for Gln, and m/z 69, for Asn, was compared to the value for the naturally occurring isotopes in order to determine isotopic enrichment. The isotopic enrichment was less than 1% at 2 h. The low level of the isotopic enrichment in the Gln amide nitrogen is also consistent with transamination of Met, but not with oxidative deamination (Fig.12). A mechanism involving oxidative deamination would have resulted in the labeling of the amide nitrogen of Gln via the operation of glutamine synthetase. The presence of glutamine synthetase in *E. intestinalis* was verified by supplying the algae with a 1 μmole/100 mg dose of $^{15}NH_4^+$ (Fig.13). The amide nitrogen of Gln showed a 42% increase in abundance at 2h.

Several lines of evidence therefore indicated that the first step of DMSP biosynthesis in *E. intestinalis* is transamination, as opposed to oxidative deamination. The above stable isotope labeling data are consistent with transamination, but not oxidative deamination. The conversion of [^{35}S]MTHB to Met also indicates the reversibility of the Met to MTOB step, which is also consistent with transamination.

Discussion

Taken collectively the data above establish a pathway for the synthesis of DMSP in marine algae for the first time. The findings were extended to other species of marine algae, which are known to be large producers of DMSP.

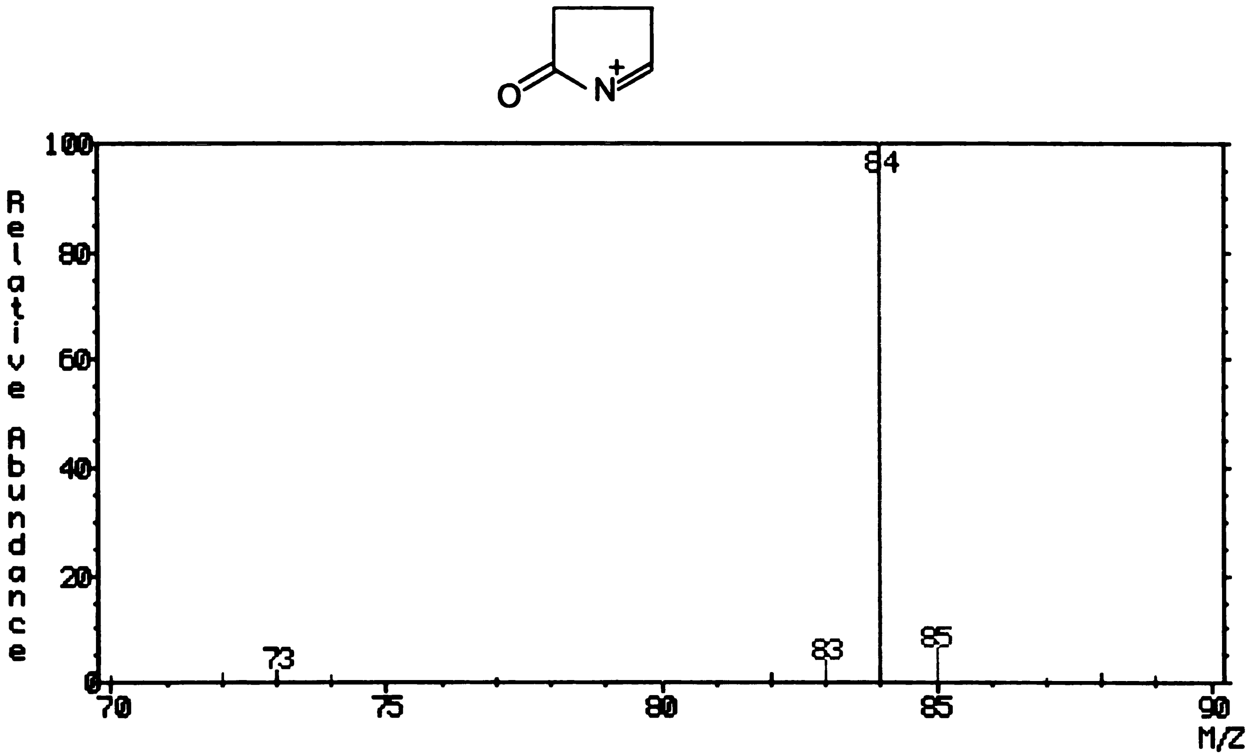


Figure 12) GCMS spectrum of amide N containing fragment ion of Gln. 2 hour sample from [^{15}N]Met fed *E. intestinalis*

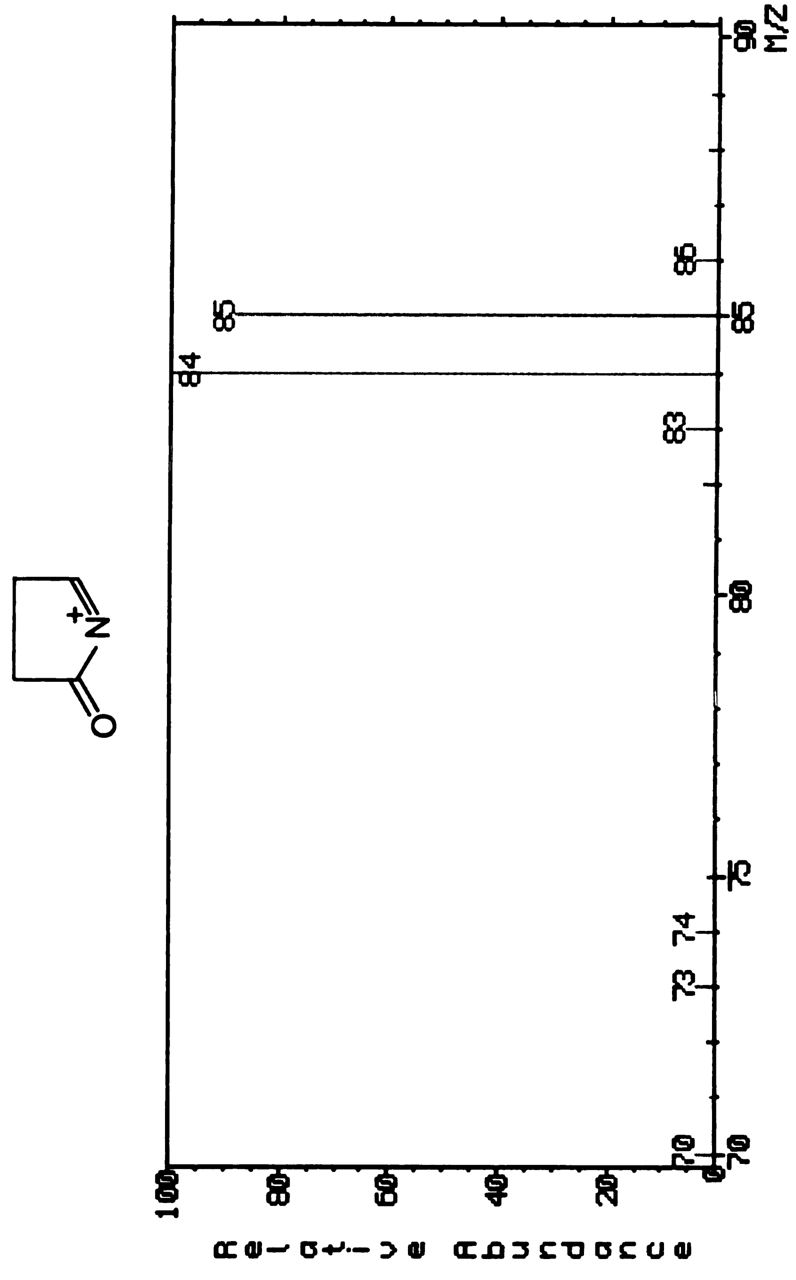


Figure 13) GCMS spectrum on amide N containing fragment ion of Gln. 2 hour sample from $^{15}NH_4$ supplied *E. intestinalis*

Emiliana huxleyi (Prymnesiophyceae) and *Melosira nummuloides* (Bacillariophyceae) both contained small pools of DMSHB, which acquired and lost label in parallel to a dose of supplied [^{35}S]Met. Both also metabolized a dose of [^{35}S]DMSHB to [^{35}S]DMSP. It is very likely that they share the same pathway.

This newly established pathway for the biosynthesis of DMSP shares no common steps with the pathway in higher plants. This indicates that the DMSP pathway has evolved independently at least twice. The pathway proposed by Uchida et al. for the heterotrophic dinoflagellate, *C. cohnii*, presents the intriguing possibility for yet another biological means for the synthesis of the same molecule.

The transamination step at the beginning of the algal pathway for the first time provides a possible explanation for the observation of increased DMSP synthesis in response to nitrogen limitation. Mobilization of the nitrogen in α-amino groups via amino acids provides a supply for the synthesis of other nitrogenous metabolites. This may explain the 50% reduction in protein content of Tetraselmis cultured in nitrogen reduced medium seen by Grone and Kirst (32). Methionine transport and its subsequent deamination would provide a ready supply of reduced nitrogen. The resulting α-keto acid , MTOB, can then be shunted through the DMSP synthetic pathway.

DMSHB was also identified as a novel sulfonium compound, and a possible substrate for DMSP lyase. Some preliminary evidence in feeding studies indicates significant catabolism of DMSHB supplied to marine algae. It is therefore another possible contributor to DMS emissions. Understanding of the synthesis of DMSP in marine algae and its regulatory mechanism should contribute to an improved insight to biogenic sulfur emissions and its impact on our environment.

Some preliminary isolation and characterization of the first three enzymes in the DMSP biosynthetic pathway has been done (80). The methionine aminotransferase at the head of the DMSP pathway has been shown to be non-enantioselective with respect to substrate methionine. It has a preference for MTOB or 2-oxoglutarate as the amino acceptor and for L-Glu as the amino donor for the reverse reaction. All algae would be expected to show some methionine aminotransferase activity as a constituent of the Met salvage pathway (12). In *E. intestinalis* this activity was 30-100 times higher than in non-DMSP accumulating species (80).

The reductase that catalyzes the MTOB → MTHB conversion is NADH linked, and appears to prefer NADH to NADPH. It also appears to be enantiospecific in its conversion of MTOB to MTHB, producing exclusively the D-form of MTHB. The MTOB reductase is also a speculative point of connection in linking DMSP synthesis to photosynthesis, because of its need for NADH, whose levels are photosynthesis dependent.

The formation of the sulfonium compound DMSHB in *E. intestinalis* is an AdoMet dependent methyltransferase. Studies conducted in the Hanson laboratory show it has a preference for D-MTHB (80). It has not been shown to have any MTP methylating activity. This enzyme also appears to be a novel methyltransferase whose presence is exclusive to DMSP accumulating algae.

The enzyme data are intriguing and could be diagnostic for characterizing some of the DMSP producing classes of marine algae whose pathways have not yet been rigorously characterized.

The unique nature of the sulfonium compound DMSHB, and its formative enzyme, MTHB methyltransferase could be used as a single point of data to define the DMSP pathway in other species. The heterotrophic dinoflagellate, *Crypthecodinium cohnii*, could provide a good point of comparison. The presence, or lack of DMSHB could indicate whether the pathway defined for *E. intestinalis* is operative, or as a heterotrophic species whether it has evolved another means for DMSP synthesis.

Future Directions

The newly identified pathway presented in this chapter represents a significant step in our understanding of the biosynthetic origin of DMSP. The identification of the novel sulfonium compound DMSHB provides for the possibility that it may have osmoregulatory properties. Comparison of its osmoregulatory properties, if any, with DMSP will provide another variable, which must be considered when investigating osmotic adjustment in DMSP accumulating organisms. Furthermore, what are the phylogenetic ramifications of its discovery? If DMSHB is an effective osmolyte, the question must be asked if there are organisms that have evolved a mechanism to produce DMSHB and not DMSP. We must also consider DMSHB as a new possible source of DMS emissions to the atmosphere.

The methyltransferase that catalyzes the formation of SMM from Met in the DMSP biosynthetic pathway of higher plants has been shown to have some unique characteristics among methyltransferases (41). The MTHB methyltransferase in the marine algal pathway offers an interesting target for study, as it catalyzes the formation of a similar sulfonium product. The data herein establishes the pathway in four of the seven classes of marine algae that produce DMSP (Chlorophyceae, Prasinophyceae, Bacillariaphyceae, and Prymnesiophyceae). The dinoflagellates however, as a class accumulate more DMSP and produce more DMS than any of the other classes (48). The possibility of another distinct pathway for the synthesis of DMSP in *C. cohnii* is presented in the paper by Uchida et al. These are some of the compelling reasons to extend the research tools developed in the elucidation of DMSP synthesis in *E. intestinalis*, to DMSP biosynthesis in dinoflagellates. This will resolve what is now an outstanding biochemical question. Do dinoflagellates have their own unique pathway for the biosynthesis of DMSP?

Chapter III

BIOSYNTHESIS OF DMSP IN THE MARINE DINOFLAGELLATE, *HETEROCAPSA TRIQUETRA*.

Introduction

Dinoflagellates have long generated scientific interest as the culprit in producing "red tides". These massive algal blooms, which kill large numbers of fish every year, are produced by a variety of species. Some of the neurotoxins produced by dinoflagellates, such as the neurotoxic shellfish poison, saxitoxin, are far more potent than curare (76). Dinoflagellates produce the well-known paralytic shellfish poisoning (PSP). PSP is accumulated by mollusk through filtering, and accumulates in mollusk tissue. The accumulation of these toxins can render many mollusks used as food sources inedible at particular times of the year when dinoflagellate populations are high. In areas where dinoflagellate populations are consistently high shellfish can never be eaten.

Dinoflagellates are the largest producers of DMSP among the seven classes of marine algae that accumulate DMSP. DMSP concentrations among these organisms show interspecies variation. Some of these species which produce large amounts of DMSP are also red tide organisms. *Linglodinium polyedra* (Fig. 14) (formerly known as *Gonyaulax polyedra*) is a red tide organism that produces ciguatoxins. Exposure to ciguatoxin is seldom fatal, but their effects can be long term and severe (76). There are even anecdotes of fishermen becoming ill simply by breathing the air in the vicinity of large algal blooms. Understanding the physiology of dinoflagellates is therefore very important both from an economic and public health standpoint.

The marine algal synthetic pathway for DMSP is completely different from that of higher plants (Chapter II). This points to independent evolution of the pathway in the two different phyla.

DMSP biosynthesis is usually linked to photosynthesis as its production has been shown to be light dependent (43). As discussed in Chapter 1 the marine dinoflagellate *Crypthecodinium cohnii* is a heterotrophic dinoflagellate that produces DMSP in a non-light dependent manner. In a paper by Uchida et al. a synthetic route for DMSP was proposed, which is distinct from the pathway in higher plants or other marine algae. The evidence for this pathway however, was somewhat equivocal. Dinoflagellates therefore present the intriguing possibility of a third or even fourth route for the synthesis of DMSP.

Linglodinium polyedra is a photosynthetic dinoflagellate that produces two sulfonium compounds, whose structures are very similar to DMSP. Nakamura et al. (63) showed that the synthesis of these two compounds, gonyol and gonyauline, were linked to DMSP.

Heterocapsa triquetra was selected as a model organism for the study of DMSP biosynthesis in dinoflagellates, because it is known to accumulate DMSP and it grows well in laboratory conditions. There is the added benefit that *H. triquetra*, unlike *L. polyedra*, is not known to produce any sulfonium compounds other than DMSP.

This study utilizes a strategy of labeling with radioactive and stable isotopes to establish the pathway of DMSP biosynthesis in *Heterocapsa triquetra*, a photosynthetic dinoflagellate.

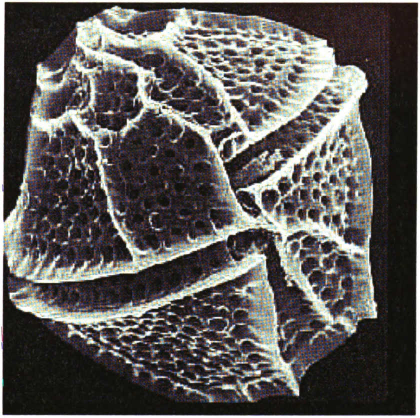


Figure 14) Linglodinium polyedra is an armored dinoflagellate. This particular example has lost its flagella. Normally there would be one flagella within the transverse furrow, which is the deep groove encircling the cell, and one flagella in the longitudinal furrow, which is the groove dividing the cell into right and left halves. The armored appearance is composed of a polysaccharide and microfibrils. It is actually interior to the cell and is covered by a cell membrane.

Materials and Methods

Chemicals. Racemic MTHB was purchased from Sigma and was recrystallized from aqueous ethanol. SMM-Cl (SMM chloride salt), MTP, DMSP-Cl (DMSP chloride salt) and MTP- amine were purchased from TCI America. MTOB was purchased from Sigma.

Radiochemicals. L-[^{35}S]SMM was synthesized by treating L-[^{35}S]Met with 250 mM methanol in 6 M HCl at 110°C for 4 hrs. L-[^{35}S]DMSHB was synthesized using a modification of the method of Zappia & Schenk) (93) by treating 7 nmol L-[^{35}S]SMM with 9 nmol H_2SO_4 in 60 μl H_2O and 5 μl glacial acetic acid. 10 μl of $NaNO_2$ was added dropwise to the reaction vial kept on ice. The reaction was incubated at 22°C for 3 hrs. The product was isolated by ion exchange chromatography using 2 columns arranged in tandem. The first column was 2 ml of mixed bed resin (2:1 AG1 $^{-}$OH, and BR70 H^{+}). The second was an AG50-X8 (H^{+}) column. The sample was applied to the mixed bed column and washed with 2 ml of H_2O. The product was eluted from the second column with 5 ml of 2.5 N HCl. The column eluate was lyophilized to dryness, and resuspended in double distilled (dd) H_2O to give the desired final concentration. Product purity was ascertained by co-migration with standard during TLE according to the protocol for TLE of sulfonium compounds in the following section. [^{35}S]Met 43.5 TBq/nmol (NEG-009A) was purchased from NEN Life Science products. It was treated with 4% thioglycollic acid at 95°C for 4 h to reduce Met sulfoxide. Basic impurities were removed by treating with AG50 (NH_4^+) resin.

Acidic impurities were removed with AG1 (COO^-) resin. Racemic MTHB was purchased from Sigma and was recrystallized from aqueous ethanol.

Growth conditions for Heterocapsa triquetra. H. triquetra was obtained from The Center for Culture of Marine Phytoplankton (CCMP449) and cultured axenically in L1 medium at 18°C and a 14:10 light :dark regime with a photon fluence of 105 μE m^{-2} s^{-1}. A daughter culture was made by diluting an aliquot of the stock culture into reduced nitrogen L1 medium in a 1:3 ratio. This was passaged on the fourth day of culturing by removing 1/3 of the total volume and replacing with an equal volume of the reduced nitrogen medium. The final nitrogen concentration after the first passage was 0.26 mM. Cell populations were calculated using a hemacytometer with a 0.1 mm cell depth.

Labeling with [^{35}S]Met. Two labeling experiments were done. Labeling of cultures grown in reduced nitrogen medium were used on the seventh day. Nine 12 ml subcultures were prepared from the reduced nitrogen culture. The nine subcultures corresponded to harvest time points of 0, 5, 10, 30, 60, 120, 240 and 1200 min. Cells were used in exponential growth phase at a population density of 3.85 x 10^4 cells/ ml. Cultures were inoculated with 19 μCi (0.4 nmol [^{35}S]Met) and incubated on a 2D rocker at a low to moderate speed. Labeling experiments with cells grown in a complete L1 medium were done with four 12 ml subcultures made from a stock in exponential growth at a cell density of 4.86 x 10^4 cells /ml. Cultures were inoculated with 8 μCi (0.4 nmol [^{35}S]Met). Lighting and temperature conditions were identical to those used in culturing. One culture was used as a 0 time point (control). We centrifuged the culture in order to pellet the cells. The supernatant was removed and a dose of [^{35}S]Met was added that was equivalent to that added to the other cultures. An aliquot was removed and assayed by scintillation counting. This was done to provide a baseline that could be used to monitor uptake of radioactivity by the other cultures. The amount of radioactivity remaining in the supernatant was assayed, and the percent uptake was calculated using the value obtained from the control culture as a baseline value.

Extraction of metabolites. Algal cultures were gently centrifuged after labeling at 4300 rpm using an S4180 rotor in a Beckman GS15R centrifuge. The pellets were frozen in liquid nitrogen. 1.0 μmol of MTP, MTOB, MTHB, SMM, MTP-amine, DMSP, and DMSHB were added as carriers. I.0 ml of 0.1 N HCl was added. The pellet was vortexed and centrifuged at 4300 rpm. The lysate was fractionated into acidic, basic, and zwitterionic components using a series of three columns AG1 (-OH), Bio-Rex 70 (H+), and AG50-X8 arranged in tandem. Cell lysates were washed through the three columns in series. Columns were eluted individually to isolate acidic, basic, and zwitterionic fractions.

Column eluates were lyophilized, resuspended in 300 μl of 60% methanol and lyophilized in a vacuum concentrator. The residues were suspended in a minimal volume of water for application to TLC plates.

Isolation of metabolites. MTP, MTOB, and MTHB and Met were isolated using a 1.5 ml column packed with AG1 – X8 ($^{-}$OH) 200 – 400 mesh resin. Cell lysates were applied in a 1 ml volume. The column was washed with 15 ml dd H_2O, and eluted with 5.0 ml 2.5 N HCl. SMM and MTP-amine were isolated by passing the entire wash fraction of the AG1 column through a 1 ml column packed with Bio-Rex 70 ($^+$H) 100 – 200 mesh resin. The column was washed with 15 ml of dd H_2O and eluted with 15 ml of 1.0 N HCl.

DMSHB and DMSP were isolated by passing the entire wash fraction from the Bio-Rex 70 column through a 1.0ml column packed with AG50W-X8 (H^+) 200 – 400 mesh resin. The column was washed with 5.0 ml of dd H2O and eluted with 5.0 ml of 2.5 N HCl.

TLE of cellular metabolites. ^{35}S labeled metabolites were identified by co-migration with authentic standards during thin layer electrophoresis.

TLE was done using a Hunter thin layer peptide mapping system. Organic acids, amino acids, and bases were separated by TLE in a 1:1:38 mixture of pyridine/acetic acid/H_20 for 20 min. at 1.8 kV. Sulfonium compounds were separated in 1.5 N formic acid at 1.8 kV for 20 min.

Identification of metabolites. Metabolites isolated from column fractions were spotted onto TLC plates in individual lanes. Authentic standards of expected metabolites within the column fractions were spotted in adjacent lanes. After TLE an autoradiogram was prepared. The radiolabeled bands were localized, excised from the plate, and counted by scintillation. The lane containing the authentic standards was then stained with a reagent specific to the type of metabolites isolated from the column fractions. Dragendorff's reagent was used to react with and positively identify sulfonium compounds, and QACs (DMSHB and DMSP) (20, 36) isolated from the AG50-X8 column. Ninhydrin was used to identify basic compounds (SMM, MTP amine) from the Bio-Rex 70 column (20, 36). Iodoplatinate was used for organic acids from the AG1 column (KTMB, MTP, MTHB) (20, 36). The mobilities of the standards could then be compared to the bands from the autoradiogram, and the individual metabolites subsequently identified.

Identification of DMSHB. TLE of the sulfonium compounds from AG50-X8 fractions produced an intense band corresponding to DMSP, that obscured the zone of DMSHB migration. The zone corresponding to DMSHB was scraped from the plate and eluted using 3 x 300 ml of 80% methanol. The samples were lyophilized and reapplied to TLC plates for separation by TLE.

Base destruction of DMSP. Half of the ^{35}S labeled sulfonium fractions were reserved. To these fractions 0.5 μmol and 0.5 μmol of DMSHB were added. The samples were then treated with 17% NaOH. The tubes were left open to air in a hood for evolution of [^{35}S]DMS. The samples were neutralized using 1.7 vols (relative to NaOH) of 2.5 N HCl. The samples were diluted to 0.5 ml and applied to a two column series consisting of a 1 ml mixed bed (2:1 AG1 $^{-}$OH and Bio-Rex 70 H^{+}) column, and a 0.5 ml AG50-X8 (H+) column respectively. Both columns were washed through with 2 ml of H_2O, and the AG50 column was washed with an additional 5 ml of H_2O. The AG50 column was eluted with 5 ml of 2.5 N HCl. The eluate was lyophilized, suspended in 300 ml of 60% methanol, and lyophilized again in a vacuum concentrator. The residues were resuspended in 5 μl of H2O and applied to a 0.1 mm glass backed cellulose plate for TLE.

Quantification of radioactivity. The amount of radioactivity recovered from columns was assayed by adding a small aliquot to 5 ml of Ready Gel scintillation fluid and assaying by scintillation counting. Radioactivity recovered from plates was calculated by scraping radioactive bands, and adding the scrapings to 2 ml of H_2O. This was mixed with 2 ml of Ready Gel scintillation fluid and assayed by scintillation counting.

Autoradiography. Subsequent to separation by TLE, the electrophoretograms were enclosed in an X-ray film cassette with one sheet of X-ray film. Exposure times varied according to the amount of radioactivity present on the TLE plates.

Quantitation of radioactivity in protein and lipids. ^{35}S incorporation into protein and lipid fractions was assayed by a modification of the method described by Mudd and Datko (1986). Extracted pellets were washed with 1 ml of 0.1 N HCl, vortexed and centrifuged. The wash was discarded. The pellets were then extracted with 2 x 1 ml of methanol:chloroform:water (12:5:1). The pellet was vortexed and centrifuged at 4300 rpm using an S 4180 rotor in a Beckman GS15R centrifuge. The supernatants were combined and phases broken by the addition of 0.5 ml $CHCl_3$ and 0.75 ml H_2O. The pellet (containing the lipid free protein fraction) was suspended in 1 ml of H_2O and vortexed. A 100 ml aliquot was removed and added to a 2 ml of H_2O. 2 ml of Ready gel scintillation fluid was added and the mixture was vortexed. The mixture was assayed by scintillation. Efficiency correction was carried out by adding a known amount of [^{35}S]Met and calculating an efficiency factor for each sample. ^{35}S incorporation into lipids was assayed by taking 200 µl of the organic phase and adding it to 4 ml of Ready gel scintillation fluid. The mixture was counted using a scintillation counter.

Cold trapping studies. A six-day-old culture of *H. triquetra* was subdivided into seven 12 ml subcultures. Three were used as controls. One of these three was used as a 0 time point. This culture was centrifuged to pellet the cells. 8 μCi of [^{35}S]Met was added to the supernatant. A 0.5 ml aliquot was added to 5 ml of Ready Gel scintillation fluid and assayed. The other six were used pairwise to provide a 30 min. time point and a 4 h time point. The control cultures had only 8 μCi [^{35}S]Met added. The other four cultures had either MTP or MTHB added 1 min. prior to the addition of an 8 μCi dose of [^{35}S]Met.

Results

A small dose of [^{35}S]Met was supplied to 12 ml cultures of axenic, exponentially growing cultures of *H. triquetra*. The fate of the ^{35}S label was followed into downstream metabolites, and into end products. The major fate of [^{35}S]Met was incorporation into protein. DMSP also accumulated significant amounts of ^{35}S. Several compounds, which have been identified or suggested as intermediates in other organisms that accumulate DMSP, also acquired label.

In agreement with studies done on DMSP biosynthesis in the chlorophyte alga *Enteromorpha intestinalis*, 4-methylthio-2-oxobutyrate (MTOB), 4-methylthio-2-hydroxybutyrate (MTHB), and 4-dimethylsulfonio-2-hydroxybutyrate all acquired label. MTOB and MTHB labeled heavily at early time points, and levels diminished as cellular pools of free [^{35}S]Met were depleted. This pattern of labeling is consistent with what would be expected of an early intermediate in the pathway. DMSHB acquired label at a constant rate in parallel with DMSP. This may indicate that there are storage pools of DMSHB, which accumulated label slowly in conjunction with the increase of intracellular DMSP levels. MTP also labeled quite heavily, and paralleled the pattern seen in MTOB.

S-methylmethionine (SMM), the first intermediate in the plant DMSP biosynthetic pathway, was not detected. We also did not detect the presence of 3-methylthiopropylamine (MTP-amine). Uchida et al. suggested MTP-amine was a metabolite of the DMSP pathway in the heterotrophic dinoflagellate *Crypthecodinium cohnii*, although no direct evidence for its presence was provided. (86).

The data from the [^{35}S]Met labeling studies favor the pathway Met → MTOB → MTHB → DMSHB → DMSP previously established in *E. intestinalis* (20). The pathway proposed by Uchida et al. is not operative in this dinoflagellate, because we failed to find any MTP-amine. However, the labeling pattern of MTP makes it difficult to rule this out as a potential intermediate. The data could be interpreted to support the pathway Met → MTOB → MTP → DMSP. In this scenario either MTP or MTHB would be a substrate for the methyltransferase, which catalyzes the formation of the sulfonium product DMSP, in the case of the former, and DMSHB in the case of the latter. I therefore complemented the [^{35}S]Met labeling experiment with a cold trapping experiment. Either MTP or MTHB was provided as a cold trap in conjunction with [^{35}S]Met to test for diminution of ^{35}S flux from [^{35}S]Met into the sulfonium products of the two possible pathways.

Feeding Experiments with [^{35}S]Methionine

A culture of *Heterocapsa triquetra* was grown in reduced nitrogen L1 medium for a week. This was done to maximize uptake of tracer Met, and production of DMSP. Met uptake was increased two-fold in the reduced nitrogen medium, as compared to similar feeding experiments in unmodified L1 medium (Fig. 15). Experiments with another dinoflagellate, *C. cohnii*, indicated that DMSP production was increased two-fold on a per molecule methionine basis. The combined effect was a five-fold increase in DMSP production at 2h (See Chapter 4.) This is consistent with the frequently observed inverse correlation between nitrogen availability and DMSP production (9, 32, 49). The mother culture was subdivided into eight 12 ml cultures. Each culture was inoculated with 19 μCi of [^{35}S]Met. Each of the eight cultures was harvested at eight different time points of 0, 5, 10, 30, 60, 120, 240, and 1200 minutes. Cells from the harvested cultures were disrupted and the soluble components fractionated using a series of 3 ion exchange columns. The column eluate was separated by TLE, and an autoradiogram was made to evaluate the components that incorporated ^{35}S.

MTOB, MTHB and MTP

The autoradiogram of the TLE plate from the organic acid containing AG1 fraction showed bands corresponding to MTOB, MTHB, MTP and Met. There was an unknown band that appears at 120, and 240 minutes and migrates intermediate to MTHB and MTOB (Fig. 16). When the bands were excised from the TLE plate, carryover from this band skewed the quantitation for MTOB, and MTHB upwards.

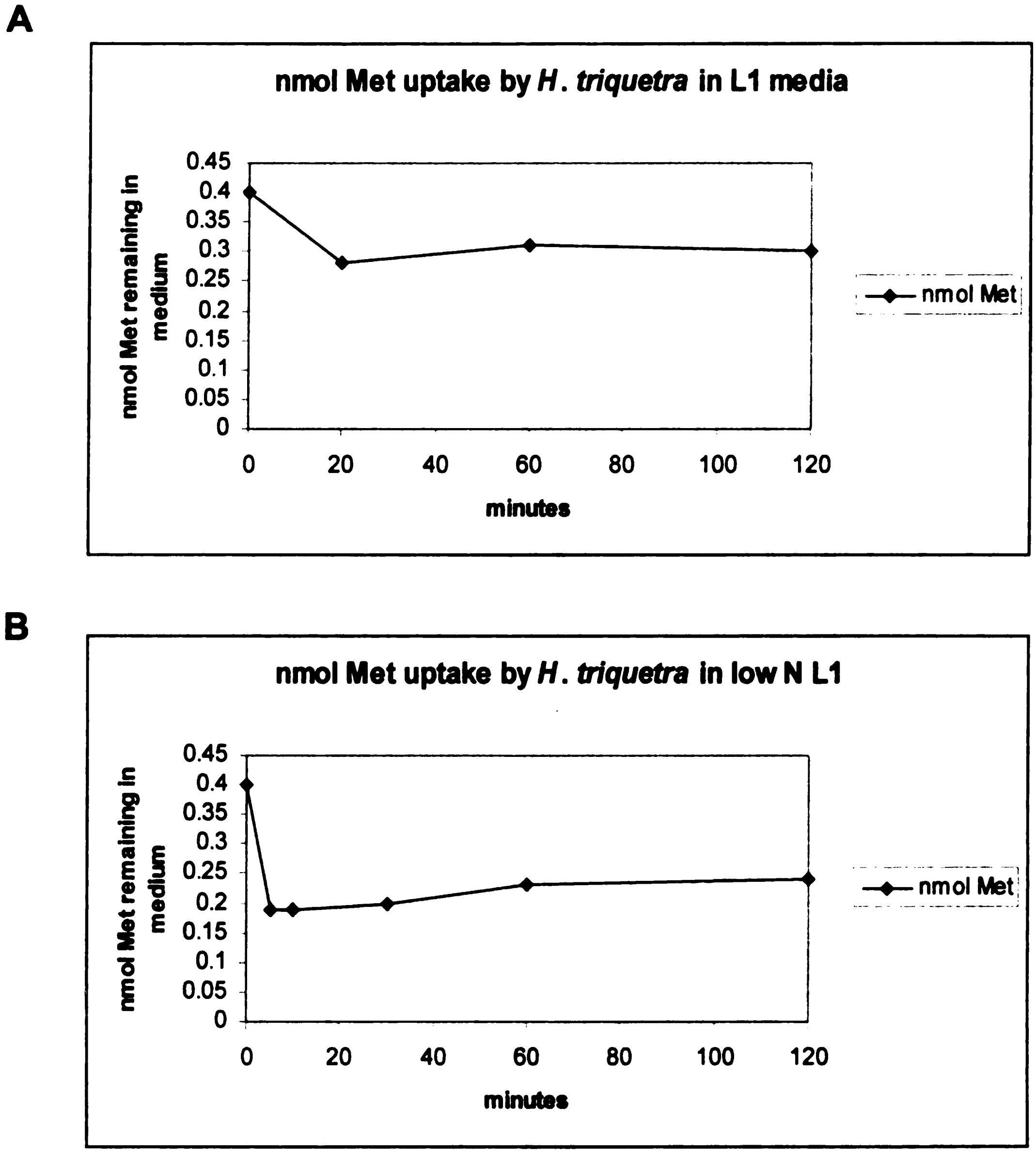


Figure 15) Graphs depict the nmol of Met remaining in the supernatant after cells were provided with exogenous methionine and centrifuged. A) Uptake of met by cells cultured in L1 media. B) Uptake of met by cells grown in L1 where nitrogen concentration was reduced ten-fold.

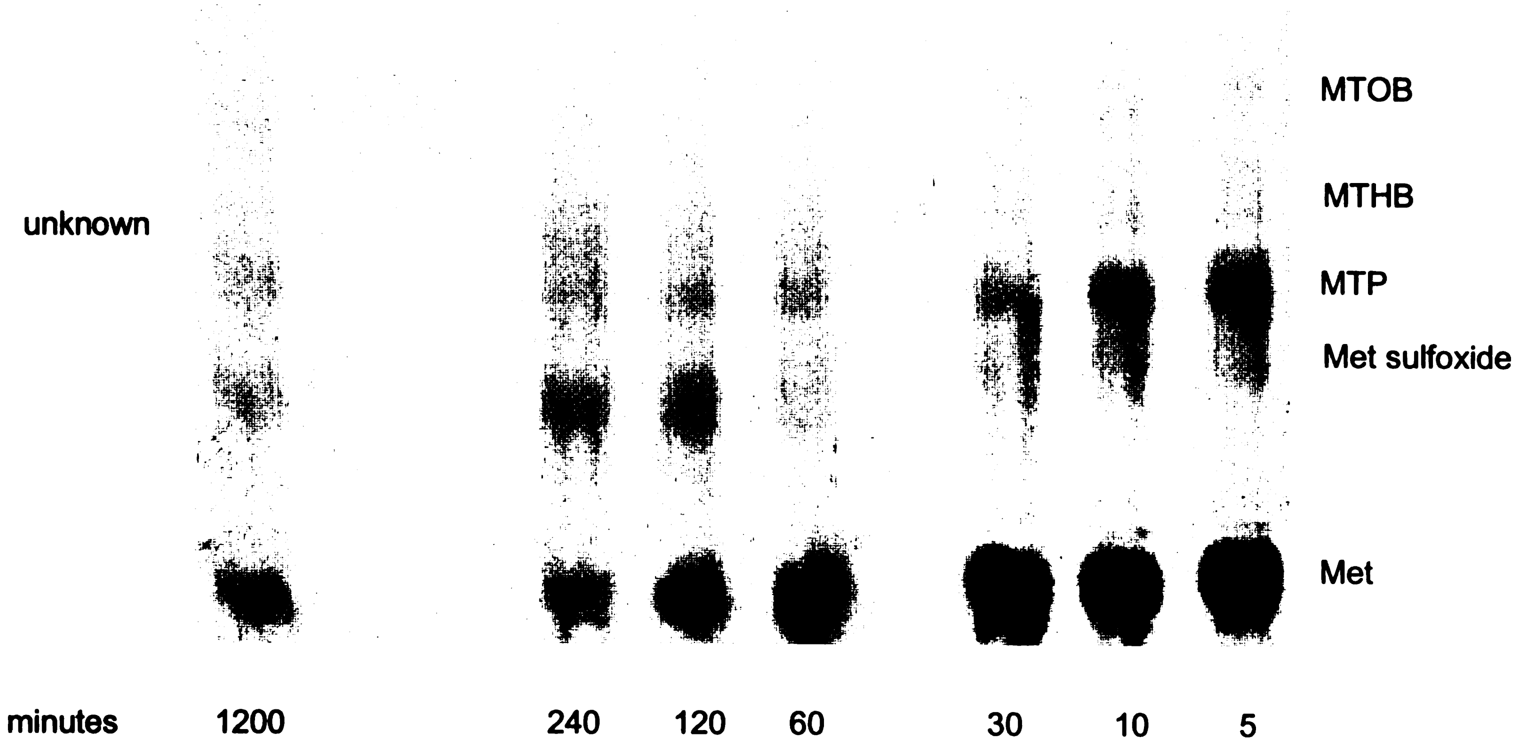


Figure 16) Organic acids from *Heterocapsa triquetra* extracted in 0.1 N HCl.

I, therefore carried out another [^{35}S]Met feeding experiment and this time the organic acids were extracted from the acidified cell lysate using ether. The ether extracted fraction, and the acidic fraction were analyzed by TLE. The ether extract contained MTOB, but not the unknown band (Fig.17). This showed that radioactivity incorporated into MTOB was drastically reduced after 60 min.

MTOB is somewhat unstable and can undergo spontaneous decarboxylation to form MTP. This was thought to be the source for some of the MTP formed in the experiments. The ether extraction was also done to improve recovery of MTOB and provide a more accurate assessment of MTOB and MTP levels. The autoradiogram of the ether extracted fraction still showed a band corresponding to MTP, however its labeling pattern was more variable, increasing then decreasing intermittently.

MTOB is the most likely intermediate between Met and MTHB, which increases its likelihood as a pathway intermediate (12, 20, 28). The increased flux through the DMSP pathway under nitrogen limiting conditions is also consistent with a transaminase reaction at the head of the pathway.

MTHB is a stable compound and its labeling kinetics are quite consistent with that of a pathway intermediate (Fig.18). It is also a natural precursor to DMSHB via methylation. It should be noted that in representative samples of DMSP accumulating algae no MTP methylating activity has been found previously (80). A methyltransferase, which catalyzes the formation of DMSHB from D-MTHB has been identified in *E. intestinalis* (80).

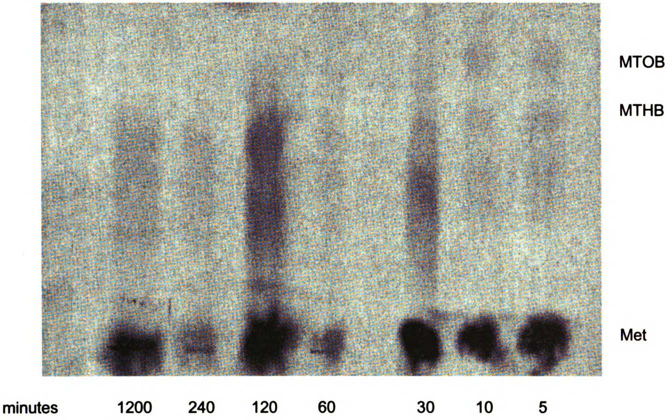


Figure 17) Organic acids extracted from *Heterocapsa triquetra* in ether. This shows disappearance of MTOB after 30 minutes.

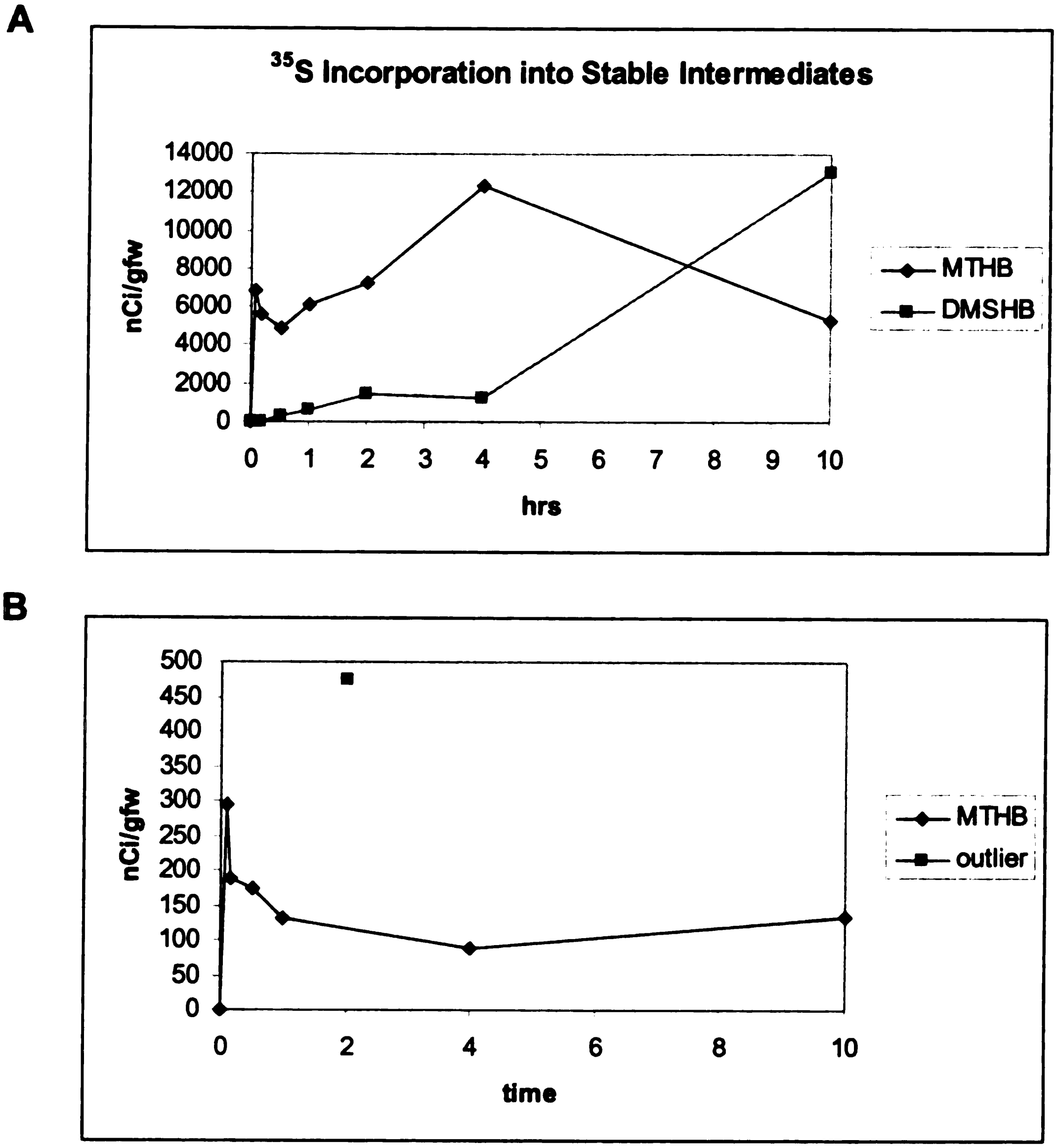


Figure 18) Graph showing incorporation of ^{35}S into selected metabolites. A) Quantitation of MTHB and DMSHB from acid extracted algae. This shows the skewing of MTHB quantities from 60 min onward due to presence of a contaminating band B) MTHB from ether fraction of acid extracted algae. There is still an increase at 120 minutes (plotted as a single point) but later values show a general downward trend.

DMSHB and DMSP

DMSHB accumulated label over the entire time period, as did DMSP (Fig.19). This was a somewhat surprising result as intracellular free Met levels showed a steady decrease in radioactivity. A large source of [^{35}S]Met between the 240 min and 1200 min was likely protein bound met, which continued to increase up to the 240 min. time point, but showed a sharp decrease at 1200 min (Fig. 19). Grone and Kirst have previously shown that an increase in DMSP synthesis is accompanied by as much as a 50% decrease in protein content/cell for *Tetraselmis* grown in nitrogen deficient media.

DMSP and DMSHB migrate very closely together during TLE. Autoradiography of one half of sulfonium containing fraction showed a strong tailing from the DMSP band that extended through the zone of migration for DMSHB. The zone corresponding to DMSHB was scraped, eluted and a new TLE, and autoradiogram was made, which showed a small band corresponding to DMSHB (Fig.20). The other half of the sulfonium fraction was treated with NaOH as another means to confirm the identity of DMSHB. DMSP is base labile, and undergoes α',β-elimination to yield DMS and acrylate, but DMSHB is stable to base treatment (12). Once DMSP was eliminated from the sample, TLE showed a strong band corresponding to DMSHB. Subsequent to scraping and quantification of the radioactive bands from the TLE plate the plate was stained with Dragendorff's reagent. There were no Dragendorff's positive bands in the sample lanes with electrophoretic mobility identical to DMSP. If DMSP had survived the base treatment there would have been a positive staining band just above the zone of migration for DMSHB. A single band was found in the sample lanes that had an electrophoretic mobility identical to that of authentic DMSHB added to adjacent lanes.

Heterocapsa did not take up [^{35}S]DMSHB. We were therefore unable to confirm its role as the direct precursor through direct feeding studies.

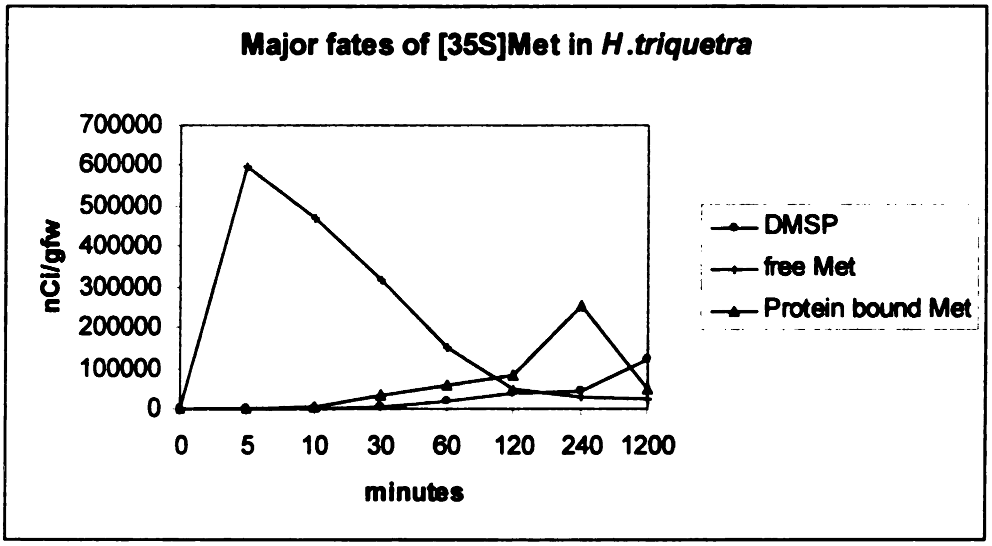


Figure19) Graph showing the major fates of a 0.4 nmol dose of [^{35}S]Met. The disparity between disappearance of intracellular free Met and products is likely due to production of DMS.

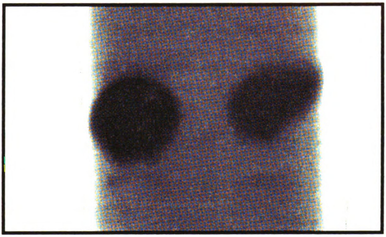


Figure 20) Autoradiogram of TLE showing the appearance of DMSHB bands after scraping lower zone of DMSP band from previous TLE, and eluting. DMSHB is the very faint band beneath the heavier DMSP spot.

Cold trapping studies with MTHB and MTP

The data from the [^{35}S]Met labeling experiments in which MTOB, MTHB and DMSHB all acquired label seems to confirm the pathway previously reported for other classes of marine algae (20). In order to further confirm this conclusion. I carried out cold trapping experiments to determine the ability of a pool of exogenously supplied MTHB, and MTP to diminish flux of label from [^{35}S]Met into the downstream sulfonium products DMSHB and DMSP (Table III). The experiment was done for two time points 0.5 h and 4 h. Six day old cultures were presented with 0.8 nmol of MTHB, or MTP 1 min. prior to the addition of a 0.4 nmol dose (0.8 μCi) of [^{35}S]Met. We monitored cellular uptake of radioactivity; and activity recovered in DMSP bands from TLE plates

Uptake of methionine was inhibited by 4.0% at 0.5 hrs. and 6.0% at 4 hrs for the culture with added MTHB In the culture with added MTP uptake was only slightly decreased with 5.0% and 7.0% respectively. The amount of radioactivity recovered from the sulfonium fraction in the MTHB treated sample was 62% of the control at 0.5 h and 59% at 4h. MTP was slightly less effective containing 61% and 75% respectively.

The effectiveness of MTP as a cold trap would seem to suggest that it both MTP and MTHB may be intermediates.

Sample	**Time (min)**	**[^{35}S]Met uptake (dpm)**	**[^{35}S] in sulfonium cmpds.**
Control	30	218,867	2,525
Control	240	272,113	28,799
MTHB	30	189,905	2,169
MTHB	240	234,112	17,597
MTP	30	182,647	2,002
MTP	240	237,897	20,651

TableIII) A single study showing the effect of a 1.0 nmol dose of MTHB or MTHB on the incorporation of [35S] from Met → DMSP and DMSHB.

Discussion

The data from the [^{35}S]Met labeling experiments are consistent with the pathway, Met → MTOB → MTHB → DMSHB → DMSP. They are not consistent with the pathway proposed by Ishida of Met → MTP-amine → MTP → DMSP. This is due primarily to the lack of [^{35}S]MTP-amine in the radiotracer studies. The labeling of MTP can be explained by oxidative decarboxylation of MTOB. It is likely that MTHB 's effectiveness as a cold trap is the result of its conversion to methionine. This is to be predicted based on the sequence of enzymatic steps in the known algal pathway. In this pathway both the transamination of Met to form MTOB, and the reduction of MTOB to form MTHB are reversible. It was also found that [^{35}S]MTHB was mostly converted to Met in *E. intestinalis*, although some was converted to DMSP (20). It is worth noting that the reductase responsible for the MTOB → MTHB conversion exclusively synthesizes D-MTHB. This means that the methyltransferase that converts DMSHB to DMSP is may also prefer the D-enantiomer. This was also shown in *E. intestinalis.* Most of the L-MTHB provided was likely converted to Met with only a small amount continuing through the pathway to DMSP. This small amount of conversion to DMSP may explain why MTHB is more effective as a cold trap than MTP.

The labeling pattern of MTP and its effectiveness as a cold trap are consistent with a role as an intermediate. MTP could be formed via the oxidation of MTP or MTHB and methylated to form DMSP in a parallel pathway. MTP is most likely not an intermediate. It is more likely that the efficacy of MTP as a cold trap is due to recycling back to Met via methylthioadenosine (MTA), or a reduction in Met transamination via an inhibition mechanism. No MTP methylating activity has been demonstrated in algal extracts of DMSP producers. If a parallel pathway is operative it may be unique to dinoflagellates.

The labeling pattern of DMSHB is a somewhat surprising result. It would be expected that its position as the immediate precursor of DMSP would produce a pattern opposite to that seen in the radiotracer experiments. One would expect higher levels at early time points with a progressive diminution of label at the later stages of the time course. One possible explanation of this pattern is a storage pool of DMSHB exists in slow equilibrium with a small metabolically active pool, which carries a high flux. This would produce the observed labeling pattern as the storage pool slowly becomes labeled over the time course of the experiment, while the bulk of the ^{35}S flux passes through the metabolically active pool into DMSP (20). This would be expected to produce a rapid accumulation of label in DMSP while DMSHB incorporates label at a much slower rate. A computer model developed to fit the labeling data for the DMSP synthetic pathway may support this explanation (20).

The data in this chapter establishes a pathway for DMSP biosynthesis in dinoflagellates for the first time. This suggests that the pathway is strongly conserved among marine algae. Dinoflagellates represent another class of marine algae for which the DMSP pathway has been defined. The novel compound DMSHB has been found in four other DMSP accumulating algae, *Tetraselmis subcordiformis* (Prasinophyceae) *Melosira nummuloides* (Bacillariophyceae) *Enteromorpha intestinalis* (Chlorophyceae) and *Emiliana* *huxleyi* (Prymnesiophyceae) and may itself be diagnostic for this pathway, as its presence has not been reported in any non-DMSP producers. Neither has it been reported in any DMSP producing organisms, which do not share this pathway.

It is important to point out that our data do not support the DMSP pathway proposed by Uchida et. al. Their model was developed for a heterotrophic species, while ours is for a photosynthetic species. *Linglodinium polyedra* is another dinoflagellate species in which some preliminary work has been done on DMSP biosynthesis. Nakamura confirmed Greene's result that DMSP was synthesized using methionine as a substrate. He also suggested that two other sulfonium compounds, gonyol and gonyauline, were synthesized from DMSP. It is likely that *L. polyedra* and *H. triquetra* share a common pathway for DMSP biosynthesis. The compound DMSHB provides an alternative substrate to DMSP however for the synthesis of gonyol, and gonyauline.

The DMSP story in dinoflagellates should be completed by extending the methods used in this study to *C. cohnii* and *L. polyedra*. Experiments to address this question are presented in Chapter IV.

Chapter IV

THE BIOSYNTHESIS OF DIMETHYLSUFONIOPROPIONATE IN TWO MARINE DINOFLAGELLATES: *CRYPTHECOHDINIUM COHNII* AND *LINGLODINIUM POLYEDRA*.

Introduction

Crypthecohdinium cohnii and *Linglodinium polyedra* (formerly known as *Gonyaulax polyedra*) are two unusual DMSP producing organisms. *C. cohnii* is the only known heterotrophic organism that accumulates DMSP. It produces DMSP in the dark as well as in the light (48, 86). For the experiments described herein *C. cohnii* cultures were grown in the dark. The biosynthesis of two sulfonium compounds in addition to DMSP, whose structures are very similar to DMSP is the unique feature of *L. polyedra*. Gonyauline (*cis*-2-(dimethylsulfonio) cyclopropane carboxylate) is a novel compound whose function is associated with period shortening of in the bioluminescent circadian rhythm of *L. polyedra* (12, 63). Gonyol (3S-5-dimethylsulfonio-3-hydroxypentanoate) is also found in some other dinoflagellates such as *Amphidinium carterae* (63). There is no defined physiological function for gonyol.

DMSP biosynthetic studies have been conducted previously in both of these organisms. Uchida et al. authored a paper on the synthesis of DMSP in *C. cohnii* in which they proposed the first step of the pathway is decarboxylation of Met. They described the complete pathway as the following. Met →

methylthiopropylamine (MTP-amine) → methylthiopropionate (MTP) → DMSP. Their evidence was largely based on cold trapping experiments with MTP and the purification of a methionine decarboxylase, an activity that is known in many non-DMSP accumulating organisms. (12, 54, 58, 77). There were no direct labeling studies done, however, no other investigators have done follow-up studies to support or disprove the pathway.

Nakamura et al. confirmed Greene's result (30) that DMSP was synthesized from Met in *L. polyedra*. They also suggested that the carbon skeleton of gonyol was derived from DMSP and acetate, and that gonyauline was also synthesized from DMSP, with it's C1 carbon originating from CO_2 (63).

In this chapter [^{35}S]Met feeding studies are described which are consistent with the pathway: Met → MTOB → MTHB → DMSHB → DMSP in both species.

Materials and Methods

Growth conditions for Linglodinium polyedra. Stocks were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton. (Stock number CCMP 407) Cultures of *L. polyedra* were grown in L1 media at 18°C on a 14:10 light dark cycle with a photon fluence of 390 μE. Cells were used in exponential growth phase or early stationary phase.

Growth conditions for Crypthecodinium cohnii. Stock cultures were obtained from Martek Biosciences. Cultures of *C. cohnii* were grown in three types of media. 50 ml cultures were grown in a yeast-glucose medium prepared from 50 g/L glucose and 6 g/L yeast extract in filtered seawater. The cultures were maintained in 250 ml Erlenmeyer flasks in ambient light at room temperature. Cultures in L1 medium were maintained using the same conditions as those used for the yeast-glucose cultures. In addition a defined medium prepared according to the method described by Guillard and Keller (33) was used. The protocol was modified by reducing the concentration of $(NH_4)_2SO_4$ tenfold to a concentration of 5.0 mg/L. Na_2SO_4 and Na_2EDTA were also eliminated from the stock solution. Na_2SO_4 was added on the fourth day of culture, or was replaced with 2.5 mM cysteine. Na_2EDTA was added on the fourth day of culture. Vitamin levels were augmented to the levels suggested by Tuttle and Loeblich (85) 50 ml cultures were grown in 250 ml Erlenmeyer flasks that were maintained in the dark. Subcultures were started by inoculating 50 ml of the defined medium with 1 ml of cells from a culture not more than 10 days old that was grown in 50 g/L glucose and 6 g/L yeast extract medium. Cell populations were calculated using a hemacytometer with a 0.1 mM cell depth

Media preparations for uptake experiments. The defined medium from above was used as the basis for all experiments. A low nitrogen variant was prepared from the basic media protocol as described above. Complete medium was supplemented with 2.25 mg $(NH_4)_2SO_4$ to raise the nitrogen level to that prescribed in the protocol. High salt medium was made by adding 0.995 g NaCl and 2.25 mg $(NH_4)_2SO_4$. Another variant of the medium was made by replacing SO_4 with 2.5 mM cysteine. A high salt, low nitrogen medium was prepared by adding 0.995 g NaCl. A high salt medium was also prepared in which SO_4 was replaced with 2.5 mM cysteine. This medium was supplemented with 2.25mg $(NH_4)_2SO_4$. A high salt, low nitrogen, 2.5 mM cysteine medium was made exactly as the preceding, except no $(NH_4)_2SO_4$ was added.

Radiochemicals. $[^{35}S]$Met 43.5 TBq/nmol (NEG-009A) was purchased from NEN Life Science products. It was treated with 4% thioglycollic acid at 95°C for 4 h to reduce Met sulfoxide and with AG50 (NH_4^+) to remove basic impurities and AG1(COO^-) to remove acidic impurities (51). Racemic MTHB was purchased from Sigma and was recrystallized from aqueous ethanol.

Labeling with $[^{35}S]$Met. *C. cohnii* cultures were used on the seventh day following inoculation. Seven 12 ml subcultures were prepared from the reduced nitrogen culture. Cells were used in exponential growth phase at a population density of 7.5×10^5 cells/ ml. Cultures were inoculated with 19 μCi

(0.4 nmol Met) and incubated on a 2-D rocker at a low to moderate speed. *L. polyedra* cultures were used in late exponential or early stationary phase. A 52 μCi, 0.8 nmol dose of [^{35}S]Met was added to each of four cultures corresponding to harvest time points of 0, 20, 60, and 120 min. Lighting and temperature conditions were identical to those used in culturing. One culture was used as a 0 time point (control). Cultures were centrifuged to pellet the cells. The supernatant was removed and a dose of [^{35}S]Met was added that was equivalent to that added to the other cultures. An aliquot was removed and assayed by scintillation counting to determine the amount of radioactivity remaining outside the cells. This was done to provide a baseline that could be used to monitor uptake of radioactivity by the other cultures. The amount of radioactivity remaining in the supernatant was assayed, and the percent uptake was calculated using the value obtained from the control culture as a baseline value.

Extraction of metabolites. *L. polyedra* cultures were gently centrifuged after labeling at 4300 rpm using an S4180 rotor in a Beckman GS15R centrifuge. Freezing in liquid nitrogen and adding l.0 ml of 0.1 N HCl lysed the pellets. One μmol of MTP, MTOB, MTHB, SMM, MTP-amine, DMSP, and DMSHB were added as carriers to enhance recovery. The pellet was vortexed and centrifuged at 4300 rpm. The lysate was fractionated into acidic, basic, and zwitterionic components using a series of three columns AG1 (-OH), Bio-Rex 70 (H+), and AG50-X8 arranged in tandem. The cell lysate was washed through the series of columns, and columns were eluted individually. *C. cohnii* cultures were also extracted with 2 ml of methanol:chloroform:water (MCW) in a 12:5:1 mixture. The residue was re-extracted with an additional 2 ml of MCW. The extracts were pooled, and an additional 1ml of chloroform and 1.5 ml of H_2O were added in that order. The aqueous phase was kept and acidified with 25 μmol of HCl. The sample was lyophilized, redissolved in 1 ml of H_2O and added to the ion-exchange column series. Column eluates from both algal species were lyophilized, resuspended in 300 μl of 60% methanol and lyophilized in a vacuum concentrator. The residues were suspended in a minimal volume of water for application to TLC plates.

Isolation of metabolites. MTP, MTOB, and MTHB and Met were isolated using a 1.5 ml column packed with AG1 – X8 ($^-$OH) 200 – 400 mesh resin. Cell lysates were applied in a 1 ml volume. The column was washed with 15 ml dd H_2O, and eluted with 5.0 ml 2.5 N HCl. SMM and MTP-amine were isolated by passing the entire wash fraction of the AG1 column through a 1 ml column packed with Bio-Rex 70 ($^+$H) 100 – 200 mesh resin. The column was washed with 15 ml of dd H_2O and eluted with 15 ml of 1.0 N HCl. DMSHB and DMSP were isolated by passing the entire wash fraction from the Bio-Rex 70 column through a 1.0ml column packed with AG50W-X8 (H^+) 200 – 400 mesh resin. The column was washed with 5.0 ml of ddH2O and eluted with 5.0 ml of 2.5 N HCl.

TLE of cellular metabolites. ^{35}S labeled metabolites were identified by co-migration with authentic standards during thin layer electrophoresis.

TLE was done using a Hunter thin layer peptide mapping system and glass backed 0.1 mm cellulose TLC plates from Merck. Organic acids, amino acids, and bases were separated by TLE in a 1:1:38 mixture of pyridine/acetic acid/H_20

for 20 min at 1.8 kV. Sulfonium compounds were separated in 1.5 N formic acid at 1.8 kV for 20 min.

2-Dimensional TLC/TLE. Zwitterionic fractions isolated from C. cohnii were separated using a 2-dimensional TLC/TLE sytem. TLC plates were glass backed 0.1 mm cellulose plates from Merck. TLC was done first using a 6:2:2 butanol:acetic acid:H_2O solvent system. When TLC was finished the plates were allowed to dry completely overnight. TLE was then done in an orthogonal direction in 1.5N formic acid at 1.8kV for 20 min.

Identification of metabolites. Metabolites isolated from column fractions were spotted onto TLC plates in individual lanes. Authentic standards of expected metabolites within the column fractions were spotted in adjacent lanes. After TLE was carried out an autoradiogram was made. The radiolabeled bands were localized, excised from the plate, and counted by scintillation. The lane containing the authentic standards was then stained with a reagent specific to the type of metabolites isolated from the column fractions. Dragendorff's reagent was used to positively identify sulfonium compounds (DMSHB and DMSP) isolated from the AG50-X8 column (36). Ninhydrin was used to identify basic compounds (SMM, MTP-amine) from the Bio-Rex 70 column. Iodoplatinate was used for organic acids from the AG1 column (KTMB, MTP, MTHB).
The mobilities of the standards were then compared to the bands from the autoradiogram, and the individual metabolites subsequently identified.

Autoradiography. Subsequent to separation by TLE, the electrophoretograms were enclosed in an X-ray film cassette with one sheet of X-ray film. Exposure times were dependent on the quantity of radioactivity in the sample.

Quantitation of radioactivity. The amount of radioactivity recovered from columns was assayed by adding a small aliquot to 5 ml of Ready Gel scintillation fluid and assaying by scintillation counting. Radioactivity recovered from plates was calculated by scraping radioactive bands, and adding the scrapings to 2 ml of H_2O. This was mixed with 2 ml of Ready Gel scintillation fluid and assayed by scintillation counting.

Results

Production of DMSP in *C. cohnii* was strongly influenced by culture conditions. When cells were cultured in a defined medium with limited nitrogen; 70% of a 0.4 nmol dose of [^{35}S]Met was taken up by the cells at the 30 min. time point. This compares to little, or no uptake of Met by cells growing in the same defined medium wherein nitrogen was not limited and a 3.2-fold increase in Met uptake compared to cultures grown in L1 media (Tables IV & IV).

Methionine Uptake experiments

Culture conditions of *C. cohnii* were optimized to maximize Met uptake, population density, and health of cultures as assessed by cell morphology. A defined medium for cell culture was prepared as described by Guillard and Keller (33). The medium was modified by augmenting vitamin concentrations to that specified by Tuttle & Loeblich (85). Seven different versions of the cell medium were made. In one version the nitrogen concentration was reduced ten-fold. A high salt medium was made by increasing the NaCL concentration from 0.35 M to 1 M. The other variable tested was sulfur source. This was changed from sulfate to cysteine. The variables were tested alone and in concert. All of the cultures containing cysteine in lieu of sulfate did poorly. This was marked by a lack of swimming cells. The high salt cultures were distinguished by large rounded cells, but with few swimming cells.

When high salt was combined with low nitrogen, a larger number of swimming cells were observed, however growth was inhibited. All of the cultures in which at least two of the three varied factors were combined did poorly. The low nitrogen cultures were easily the healthiest cultures when division rates (Fig. 21) cell morphology, and metabolic activity (this was associated with swimming) were considered together.

Experiments designed to monitor Met uptake of cultures grown in the various types of media showed the low nitrogen cultures to be far more efficient in assimilating Met. This was evidenced by a 70% depletion from the media of exogenously supplied methionine at 30 mins. and 84% at 60 mins. (Fig. 22)

TableIV. DMSP production by *Crypthecodinium cohnii* grown in L1 medium

time	DMSP (nmols)	DMSP (nmols/nmol Met)
15 mins.	4.2×10^{-4}	7.9×10^{-3}
30 mins	9.7×10^{-4}	1.1×10^{-2}
60 mins.	1.6×10^{-3}	1.8×10^{-2}
120 mins	4.0×10^{-3}	2.6×10^{-2}
240mins.	1.2×10^{-1}	5.2×10^{-1}

TableV. DMSP production by *Crypthecodinium cohnii* grown in reduced N medium

time	DMSP (nmols)	DMSP (nmols/nmol Met)
5 mins.	4.7×10^{-3}	4.3×10^{-2}
15 mins	1.0×10^{-2}	4.8×10^{-2}
30 mins.	1.2×10^{-2}	4.3×10^{-2}
60 mins	2.0×10^{-2}	5.4×10^{-2}
120mins.	2.1×10^{-2}	5.4×10^{-2}

Table IV) Quantitation of DMSP after a 0.4 nmol dose of exogenously supplied [^{35}S]Met. The amount of DMSP is based on the specific activity of the supplied [^{35}S]Met in uCi/nmol. Cultures for this experiment were grown in L1 media.

Table IV) Same as above, except that *C. cohnii* cultures were grown in a reduced nitrogen medium.

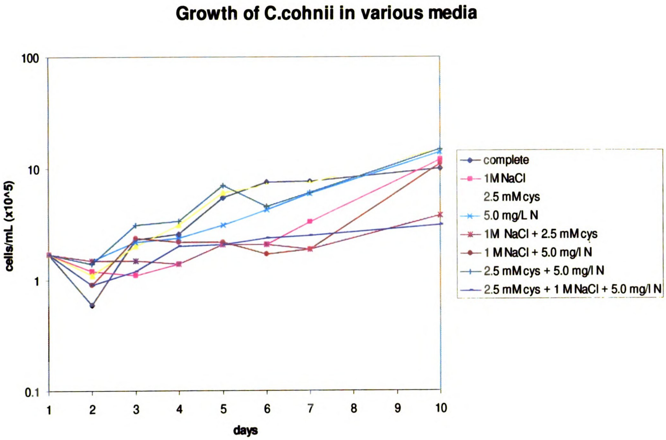
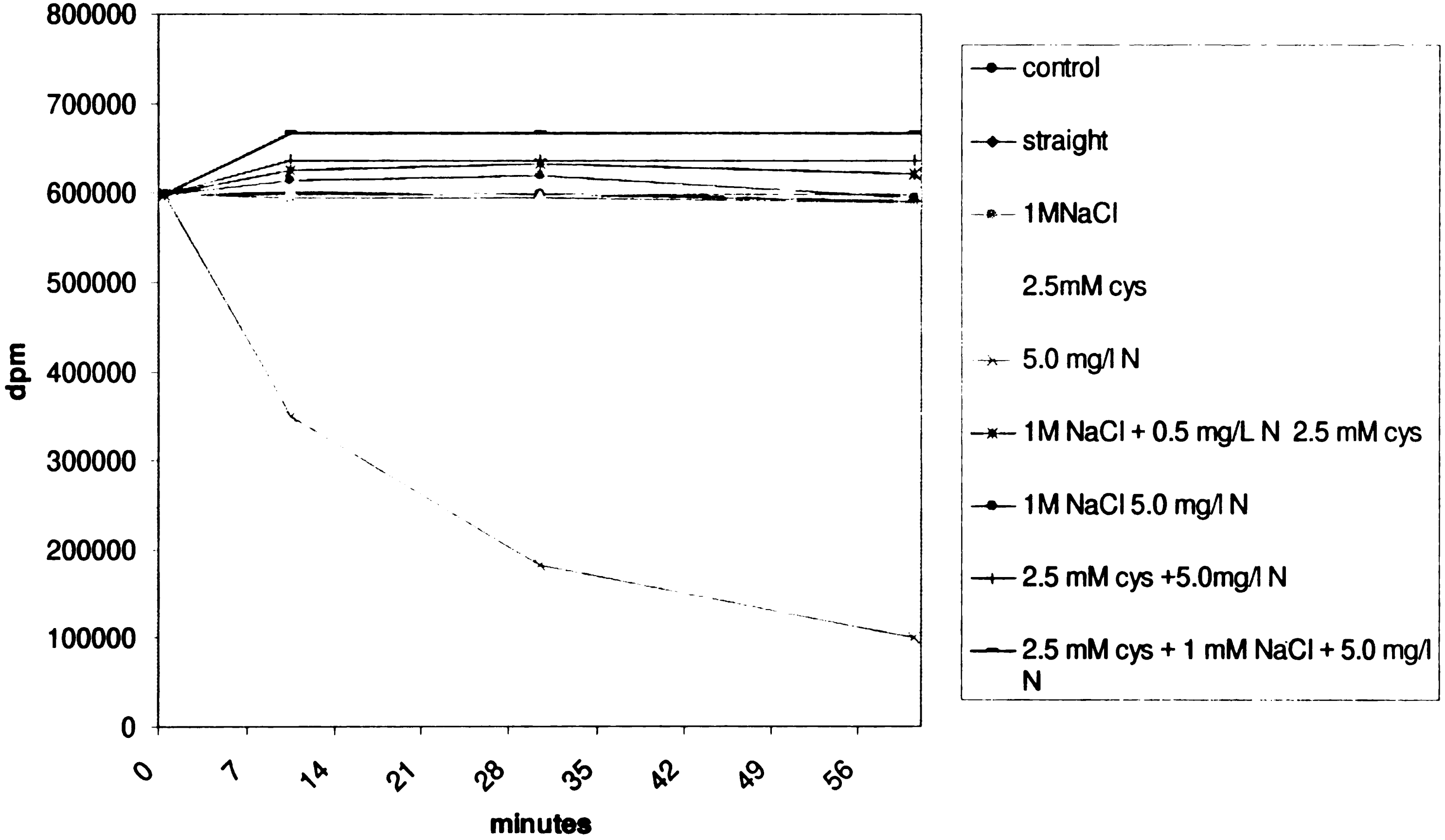


Figure 21) Growth curves for Crypthecodinium cohnii cultured in various media. (Images in this thesis/dissertation are presented in color.)

Figure 22) Uptake of a 0.4 nmol dose of exogenously supplied [^{35}S]Met by *Crypthecodinium cohnii* grown in several variations of a defined medium. Uptake was monitored by sampling a 1 ml aliquot of each 12 ml culture at three time points, 10, 30, and 60 min. The cells were pelleted by centrifugation and the supernatant was assayed by scintillation counting. Uptake is plotted as the amount of radioactivity remaining in the cell medium.



[35S]Met uptake

Glycine Betaine and DMSP in *C. cohnii*

During an early experiment with *C. cohnii* a Dragendorff's positive band was detected in the zwitterionic fraction of cell lysate. The spot had an electrophoretic mobility similar to that of glycine betaine, a functionally equivalent compatible osmolyte. We had earlier observed the presence of both DMSP and glycine betaine in the FABMS spectrum of the zwitterionic fraction of zooxanthellae isolated from Tridacnid clams. There appeared to be a reciprocal relationship between the two peaks (Fig. 23). We therefore looked at the FABMS spectrum of the zwitterionic compounds isolated from *C. cohnii* and we observed a small peak at m/z 118, which could be attributable to the presence of glycine betaine (Fig. 24). It has been reported in the literature that addition of betaine to growth media helps stimulate growth of *C. cohnii* cultures (48). *Tetraselmis subcordiformis*, a Prasinophyte algae and glycine betaine producer, is known to accumulate up to two-fold more DMSP in nitrogen limited conditions. It has been previously suggested in the literature that DMSP, and glycine betaine levels may be inversely related with glycine betaine being produced when nitrogen is available, and DMSP produced when nitrogen is limiting (48). This relationship was another factor in deciding to use nitrogen limited medium for cell culture (32, 48).

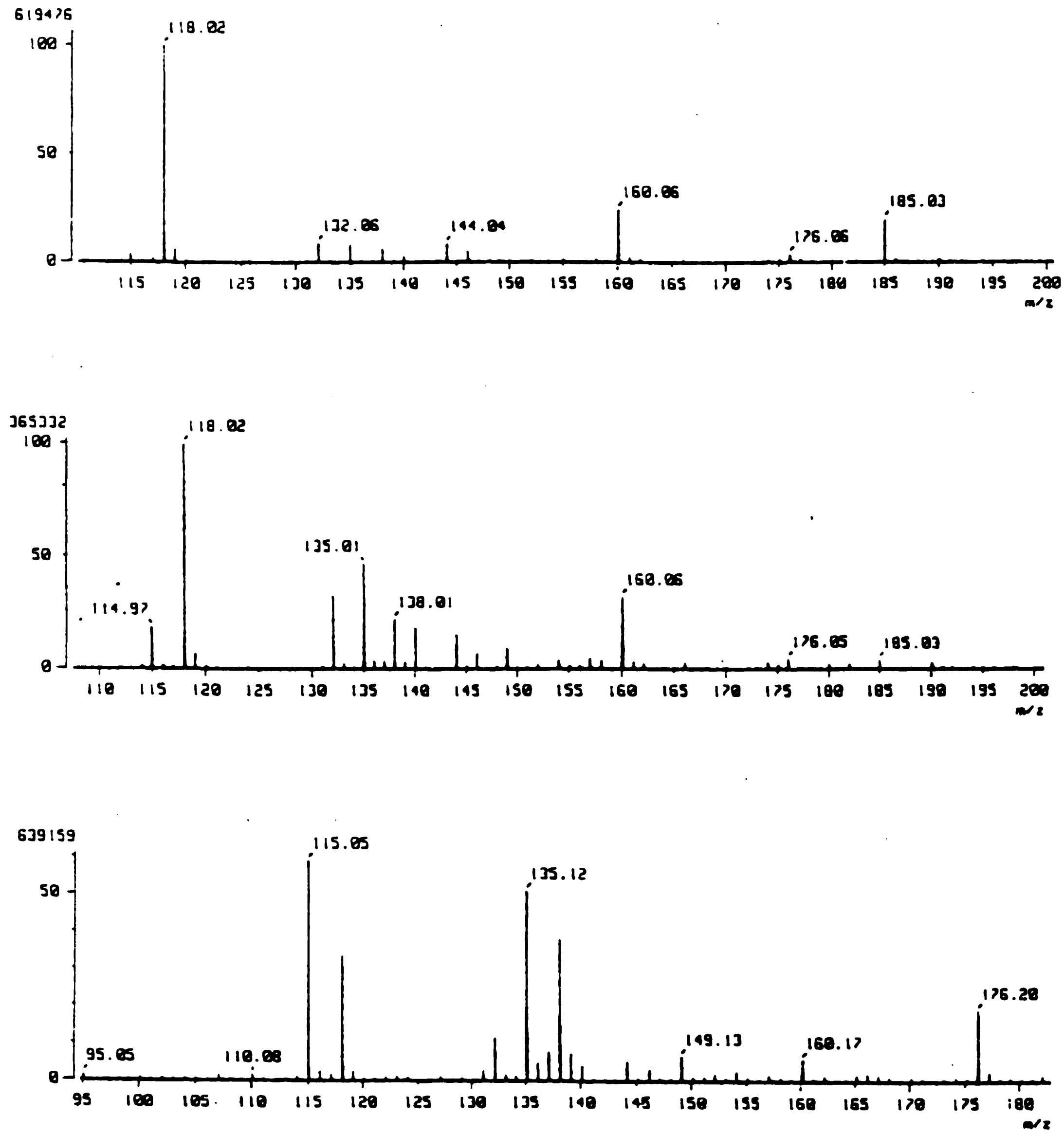


Figure 23) FABMS spectra of zwitterionic fraction isolated from Tridacnid clams. The clams have a symbiotic relationship with zooxanthellae. The ions at m/z 118 and 135 are due to the presence of glycine betaine and DMSP respectively, produced by the zooxanthellae. Note that in the top spectrum the major peak is at m/z 118 (glycine betaine). In the following two spectra the increase in the intensity of the ion at m/z 135 (DMSP) is accompanied by a decrease in glycine betaine (118).

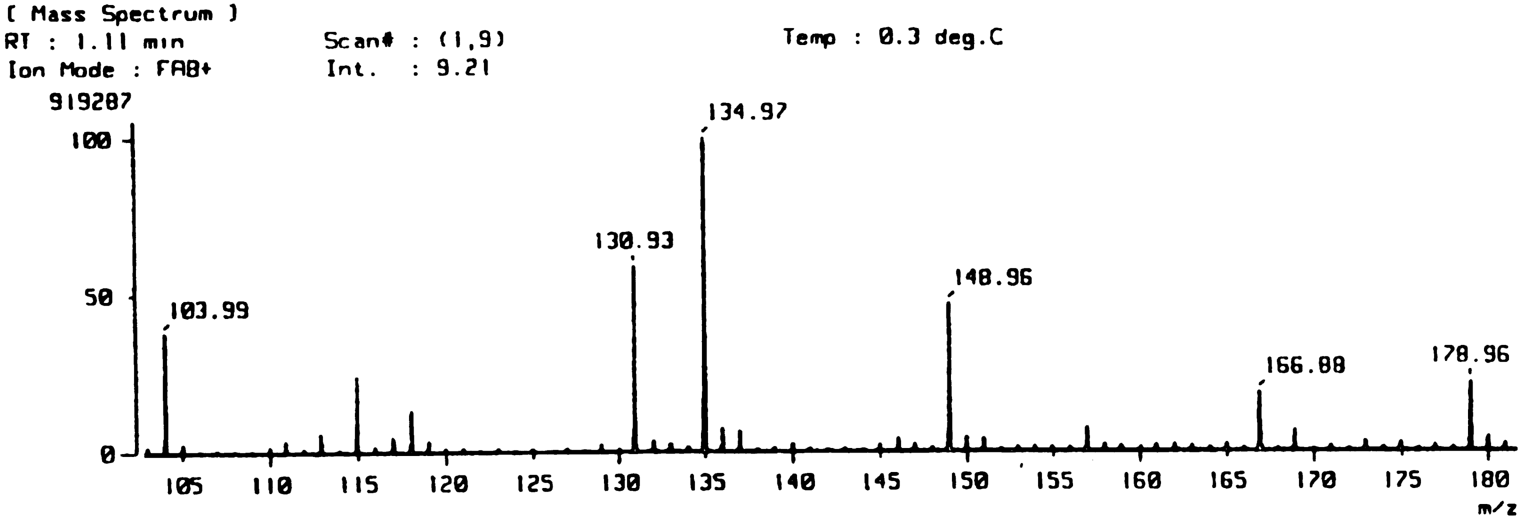


Figure 24) FABMS spectrum of zwitterionic fraction isolated from Crypthecodinium cohnii. The major peak at m/z 135 corresponds to DMSP. The small peak at m/z 118 is due to the presence of glycine betaine.

[^{35}S]Met feeding experiments in *C. cohnii*

Three separate ^{35}S trace labeling studies were carried out with *C. cohnii*. The first experiment was done with *C. cohnii* cultures grown in L1 media. A 0.4 nmol dose of [^{35}S]Met was provided and followed into metabolites. We were able to identify MTOB, MTHB, and MTP as metabolites that assimilated tracer Met (Fig. 25). There was no labeled MTP-amine, which would be expected if the pathway suggested by Uchida et al. were operative. We also saw intensely labeled DMSP. The dark bands seen in the TLE autoradiographs of the column fraction containing DMSP most likely obscured any DMSHB. Because DMSHB was the putative immediate precursor of DMSP, earlier time points with a higher specific activity tracer dose may reveal its presence. The slow rate of Met uptake was another consideration in this experiment. Only 18% of supplied Met was taken up at 15 min with a gradual increase to 39% over the entire 4 h labeling time course. Such a slow rate of uptake may provide insufficient flux to see DMSHB at early time points, if pool sizes are small.

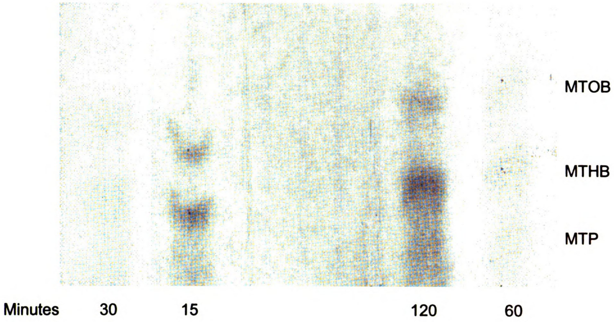


Figure 25) ^{35}S labeling of organic acids extracted from *C. cohnii*

The second [^{35}S]Met labeling experiment was done with a culture grown in a low nitrogen variant of *C. cohnii* media. A seven day old, large single culture was divided into six 12 ml subcultures, corresponding to five time points, and one that was used as a control. A 0.4 nmol dose of [^{35}S]Met was provided. The decrease in nitrogen concentration not only improved the rate of Met uptake significantly; it also increased flux through the DMSP pathway. The low nitrogen media improved the number of moles of DMSP produced/ number of moles Met taken up by 2-fold (Table V). Unfortunately, sampling at earlier time points failed to provide a clear signal for the presence of DMSHB, or MTP-amine. We had identified early precursors of both possible pathways, yet we needed to find either DMSHB or MTP-amine to clearly differentiate between the two possible routes for DMSP biosynthesis. One of the perceived problems was the smearing of bands in the TLE autoradiographs of the zwitterionic fraction. We thought this might be due to oils in the cellular extract. Oils are the major storage product for *C.cohnii*, and low nitrogen conditions are known to trigger an increase in the size of the storage pools (personal communication from Martek Biosciences). It was decided to use an alternative extraction procedure in another labeling experiment.

I supplied a 0.4 nmol dose of tracer Met to six 12 ml subcultures taken from a single large culture of *C. cohnii.* In this experiment the cells were extracted with MCW. We analyzed the zwitterionic fraction of the cell lysate using a 2D-TLC/TLE system. This gave improved resolution between DMSP and DMSHB. We observed a clear spot that corresponded to DMSHB (Fig. 26).

I also analyzed the basic fraction of the cellular lysate and as in previous experiments there was no evidence of MTP-amine. This experiment finally provided the piece of evidence needed to establish the pathway: Met → MTOB → MTHB → DMSHB → DMSP as the operative pathway in *C. cohnii*.

Culture conditions for *L. polyedra*

The challenges in doing feeding studies in *L. polyedra* begin with the difficult task of obtaining high density cultures. *L. polyedra* has a well deserved reputation for being an organism that does not do well at high or low density. I was able to obtain very robust cultures by starting with stock that was derived from a robust culture. We also found that having a sufficient depth of medium in the culture vessel was necessary to support the daily vertical migration of the species. Cultures that we attempted to maintain in Erlenmeyer flask never obtained very high density. I intuited this to be due to the lack of depth of the culture. The superior surface/volume ratio of flask culturing seemed to be of no advantage. When we began using 100 ml of volume in 75 cm^2 tissue culture flask, the cultures became very healthy, and were afterwards exceedingly easy to maintain. Whereas, other dinoflagellate species need to be passaged every third or fourth day, it was no problem for the cultures of *L. polyedra* to remain without being passaged for a period of weeks with no perceived loss of culture vitality.

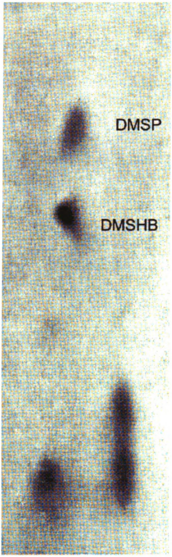


Figure 26) 2-D TLC/TLE of sulfonium fractions form *Crypthecohdinium cohnii* 1200 minutes after addition of [^{35}S]Met.

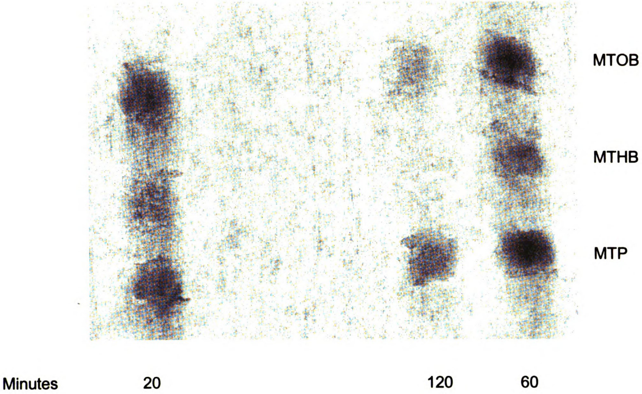


Figure 27) Organic acids extracted from *L. polyedra*

[^{35}S]Met feeding experiments in L. polyedra

The outstanding problem with doing these labeling studies in *L. polyedra* is with the separation of the sulfonium compounds. DMSP, DMSHB, gonyol, and gonyauline are all very similar in structure and separate very poorly during TLE. I also tried a to separate them by TLC using a wide variety of solvent systems. We finally decided on a 2D TLC/TLE separation system. This provided the best results among the alternatives considered. We were able to obtain a clean separation for DMSHB and DMSP, although addition of the other sulfonium products still made it difficult to effect a clean separation.

A single large culture of *L. polyedra* was subdivided into four 24 ml subcultures. The four subcultures corresponded to 3 time points and a control. We added a 0.8 nmol dose of Met. The results indicated labeling of MTOB, MTHB, and MTP in the organic acid fraction (Fig. 27). These results are consistent with those obtained for all of the other species of marine algae that were tested. There was also strong incorporation of label into DMSHB, DMSP, gonyol, and gonyauline. Labeling of DMSHB and DMSP preceded that of gonyol and gonyauline. The quantity of ^{35}S contained in each metabolite was estimated. Because of poor separation quantitation was difficult for 60 min and 120 min as gonyol and gonyauline began to incorporate label. The TLE was divided into 4 zones where standards were known to migrate and label incorporation into each individual metabolite was quantitated by counting these zones (Fig. 28).

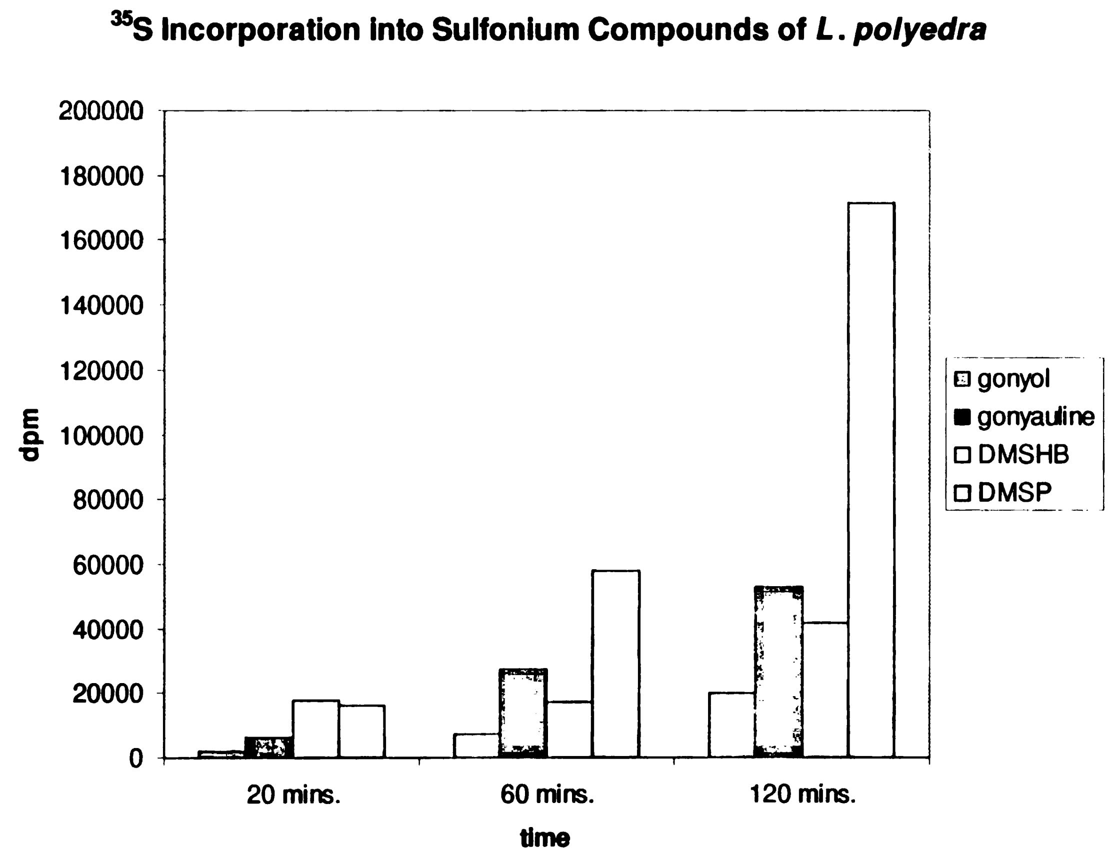


Figure 28) Graph showing [35S]Met incorporation into sulfonium compounds of *L. polyedra*. ^{35}S accumulates in DMSHB and DMSP prior to gonyauline and gonyol. This is consistent with DMSHB and DMSP as precursors to the formation of gonyauline and gonyol. (Images in this thesis are presented in color.)

Discussion

The labeling information garnered from these experiments, together with the data from *Heterocapsa triquetra* establish the biosynthetic pathway in dinoflagellates for the first time. It is clear that this pathway is strongly conserved in marine algae. *C. cohnii* and *L. polyedra* represent two special cases, which might reasonably be considered to diverge from other marine algae. The synthesis of gonyol and gonyauline appear to have evolved from the DMSP pathway. The kinetics of the labeling of sulfonium compounds in *L. polyedra* are consistent with DMSHB and DMSP as precursors of gonyol and gonyauline. The question as to whether DMSP or DMSHB is the immediate precursor of the other two compounds remains unsolved. Nakamura's work suggested that DMSP was the immediate precursor to gonyol and gonyauline. Nothing in our studies contradicts this conclusion. Nakamura harvested cells 24 hrs after feeding labeled precursors and the possibility of recycling of compounds into other compounds is a very real possibility as the labeling kinetics of our studies show that flux through the pathway is very high. This means that DMSHB is still the possible immediate precursor to gonyol or gonyauline, or possibly both.

The study conducted by Uchida et al. was done well in advance of our work with *E. intestinalis*. We now know much more about the three compounds they tested as potential intermediates by their ability to act as cold traps. SMM is clearly not an intermediate in marine algal DMSP biosynthesis. MTOB is a relatively unstable compound and may have been subject to decomposition in these experiments.

We have also found that MTP is an effective cold trap in *H. triquetra*, but this activity is likely the result of recycling back to Met via the Met salvage pathway and not due to it's role as a DMSP precursor. This is further corroborated by the lack of MTP methylating activity in cellular extracts of DMSP producing algae (80). The methionine decarboxylase activity in *C. cohnii* is widespread in algae and other plants which do not produce DMSP.

References

References

1. Aneja, V.P. and W. J. Cooper, *Biogenic sulfur emissions: A review*: 1989. *In* E. S. Saltzman, W. J. Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington D.C. p. 2-13

2. Bates, T. S. Charlson, R. J. and R. H. Gammon, *Evidence for the climatic role of marine biogenic sulfur.* Nature, 1987.**329**: p. 319-320.

3. Balch, W. M. and K. Kilpatrick, *Coccolith production and detachment by Emiliana huxleyi (Prymnesiophyceae).* Journal of Phycology, 1993. **29**: p. 566-575

4. Blunden, G. and S. Gordon, *Betaines and their sulphonio analogues in marine algae.* Progress in Phycological Research. 1986. **4**: p. 40-80

5. Blunden, G., et al., *Betaines and tertiary sulphonium compounds from 62 species of marine algae.* Biochemical Systematics and Ecology, 1992. **20**(4): p. 373-388

6. Borowitzka, L. *Osmoregulation in blue-green algae.* Progress in Phycological Research. 1986. **4**: p. 243-256

7. Burg, M., *Molecular basis of osmotic regulation.* 1995. Walter B. Cannon Lecture. p. F983-F996

8. Challenger, F., et al., *Studies on Biological methylation.* XVII. *The natural occurrence and chemical reactions of some thetins.* 1957. Archives of Biochemistry and Biophysics. **69**: p. 514-523

9. Colmer, T. D., et al., *Interactive effects of salinity, nitrogen and sulphur on the inorganic solutes in Spartina alterniflora leaf blades.* 1996. **296**(47): p. 369-375

10. Cooper, A. J. et al., *Formation of highly reactive vinylglyoxalate (2-oxo-3-butenoate) from amino acids with good leaving groups in the γ-position. Toxicological implications and therapeutic potential.* 1989. Biochemical Pharmacology. **38**: p. 3895-3901

11. Cooper, A. J. L. and A. D. Hanson, *Advances in enzymology of the biogeochemical sulfur cycle.* 1998. Chemtracts-Biochemistry and Molecular Biology. **11**: p. 729-747

12. Cooper, A. J. L., *Chemical and biochemical properties of sulfonium compounds* 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds. Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p. 13-27

13. De Souza, M. P. and D. C. Yoch, *Purification and characterization of dimethylsulfoniopropionate lyase from an Alcaligenes-like dimethyl sulfide-producing marine isolate*. 1995. Applied and Environmental Microbiology. **61**(1): p. 21-26

14. Dickson, D. M., et al., *Steady state osmotic adaptation in Ulva lactua*. 1980. Planta **150**: p. 158-165

15. Dickson, D. M. J. and G. O. Kirst. *The role of β-dimethylsulfoniopropionate, glycine betaine and homoarine in the osmoacclimation of Platymonas subcordiformis*. 1986. Planta **167**: p. 536-543

16. Edwards, D. M., et al., *Organic solute accumulation in osmotically-stressed Enteromorpha intestinalis*. 1987. Marine Biology. **95**: p. 583-592

17. Fall, R., et al., *Laboratory studies of some environmental variables controlling sulfur emissions from plants*. 1988. Journal of Atmospheric Chemistry. **6**: p. 341-362

18. Fuhrman, J. A. and C. A. Suttle, *Viruses in marine planktonic systems*. 1993. Oceanography **6**(2): p. 51-63

19. Gage, D. A. and A. D. Hanson, *Characterization of 3-dimethylsulfoniopropionate (DMSP) and its analogs with mass spectrometry*. 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds. Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p 29-44

20. Gage, D. A., Rhodes, D., Nolte, K. D. Hicks, W. A., Leustek, T., Cooper, a. J. L. and A. D. Hanson, *A new route for the synthesis of dimethylsulfoniopropionate in marine algae*. 1997. Nature. **387**: p. 891-894

21. Gage, D. A. and B. Rathinasabapathi, *Role of glycine betaine and dimethylsulfoniopropionate in water stress tolerance*. 1999. In Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. p. 125-152

22. Galleron, C. *Synchronization of the marine dinoflagellate, Amphidinium carterae in dense cultures*. 1978. Journal of Phycology. **12**: p. 69-73

23. Galleron, C. and a. M. Durrand, *Cell cycle and DNA synthesis in a marine dinoflagellate* Amphidinium carterae. 1979. Protoplasma. 100: p. 155-165

24. Gekko, K. and S. Timasheff, Mechanism of protein stabilization by glycerol: *Preferential hydration in glycerol-water mixtures*. 1981. Biochemistry **20**: p. 4667-4676

25. Gekko, K. and S. Timasheff, *Thermodynamic and kinetic examination of protein stabilization by glycerol*. 1981. Biochemistry **20**: p4677-4686

26. Giovanelli, J. H., S. Mudd, and A. Datko, *Recycling of methionine sulfur in a higher plant by two pathways characterized by either loss or retention of the 4-carbon moiety*. 1981. Biochemical and Biophysical Research Communications. **100**(2): p. 831-839

27. Giovanelli, J. H., S. Mudd, and A. Datko, *Quantitaive analysis of pathways of methionine metabolism and their regulation n Lemna*. 1985. Plant Physiology. 78: p. 555-560

28. Givan, C. V. *Aminotransferases in higher plants*. 1980. *In* B. J. Mifflin ed. The Biochemistry of Plants. **5**: p. 329-357

29. Gold. K. and Barren, C. F. *Growth requirements of Gyrodinium cohnii.* 1966. Journal of Protozoology. **13**: p. 255-257

30. Greene, R. C. *Biosynthesis of dimethyl-β-propiothetin*. 1962. The Journal of Biological Chemistry. **237**(7): p. 2251-2254

31. Grone, T. and G. O. Kirst, *Aspects of dimethylsulfoniopropionate effects on enzymes isolated from the marine phytoplankter Tetraselmis sucordiformis (Stein)* 1991. Journal of Plant Physiology. **138** p. 85-91

32. Grone, T. and G. O. Kirst, *The effect of nitrogen deficiency, methionine and inhibitors of methionine metabolism on the DMSP contents of Tetraselmis subcordiformis (Stein)* 1992. Marine Biology. **497**: p 497-503

33. Guillard, R. R. L. and M. P. Keller, *Culturing Dinoflagellates*. 1984. In David L. Spector ed. Dinoflagellates. Academic Press, Orlando Fla. p 391-441

34. Hanson, A. D. and D. A. Gage, *Identification and determination by fast atom bombardment mass spectrometry of the compatible solute choline-O-sulfate in Limonium species and other halophytes*. 1991. Australian Journal of Plant Physiology. **18**: p. 317-327

35. Hanson, A. D. et al, *Osmoprotectant compounds in the Plumbaginaceae: A natural experiment in metabolic engineering*. 1994. Proceedings of the National Academy of Sciences U.S.A. **91**: 306-310

36. Hanson, A. D., et al., *Biosynthesis of 3-dimethylsulfoniopropionate in Wollastonia biflora* (L.) DC. 1994. Plant Physiology. **105**: p. 103-110

37. Hare, P. D., Cress, W. A. and J. Van Staden, *Dissecting the roles of osmolyte accumulation during stress*. 1998. Plant Cell and Environment. **21**: p. 535-553

38. Harrison, P., J. Waters, R. E. and J. R. Taylor, *A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton*. 1980. Journal of Phycology. **16**: p. 28-33

39. Huang, Z. H., et al., *Analysis of acylcarnitines as their N-demethylated ester derivatives by gas chromatography-chemical ionization mass spectrometry*. 1991. Analytical Biochemistry. **199**: p. 98-105

40. Huang, Z. H. et al., *Characterization of N-ethoxycarbonyl ethyl esters of amino acids by mass spectrometry*. 1993. Journal of Chromatography. **635**: p. 271-281

41. James, F., Nolte, K. D., and A. D. Hanson, *Purification and properties of S-adenosyl-L-methionine:L-methionine S-methyltransferase from Wollastonia biflora leaves*. 1995. The Journal of Biological Chemistry. **270**(28): p. 22344-22350

42. James, F., et al., *Evidence implicating dimethylsulfoniopropionaldehyde as an intermediate in dimethylsulfoniopropionate biosynthesis*. 1995. Plant Physiology. **108**: p. 1439-1448

43. Karsten, U., Wiencke, C., and G. O. Kirst, *The effect of light intensity and daylength on the β-dimethylsulfoniopropionate (DMSP) content of marine green macroalgae from Antarctica*. 1990. Plant Cell and Environment. **13**: p. 989-993

44. Karsten, U., Wiencke, C., and G. O. Kirst, *The β-dimethylsulfoniopropionate (DMSP) content of macroalgae from Antarctica and southern Chile*. 1990. Botanica Marina. **33**: p. 143-146

45. Karsten, U., et al., *Dimethylsulfoniopropionate production in phototrophic organisms and its physiological function as a cryoprotectant*. 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds.
Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p. 143-153

46. Keller, S. E., Hutner, S. H., and D. E. Keller, *Rearing the colorless dinoflagellate Crypthecohdinium cohnii for use as a biochemical tool*. 1968. Journal of Protozoology. **15**(4): p. 792-795

47. Keller, D. M., et al., *Media for the culture of oceanic ultraphytoplankton.* 1987. Journal of Phycology. **23**: p. 633-638

48. Keller, M. D., Bellows, W. K., and Guillard, R. L., *Dimethyl sulfide production in marine phytoplankton*. 1989. *In* E. S. Saltzman, W. J. Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington D.C. p. 167-182

49. Keller, M. D. and Bellows, W. K., *Physiological aspects of the production of dimethylsulfoniopropionate (DMSP) by marine phytoplankton*. 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds.
Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p. 131-142

50. Kinne, R. H., *The role of organic osmolytes in osmoregulation from bacteria to mammals*. 1993. The Journal of Experimental Zoology. **265**: p. 346-355

51. Koscis, M. G., et al, *Dimethylsulfoniopropionate biosynthesis in Spartina alterniflora*. 1998. Plant Physiology. **117**: p. 273-281

52. Lavine, T. F., Floyd, N. F. and M. S. Cammaroti, *The formation of sulfonium salts from alcohols in sulfuric acid.* 1953. p. 107-117

53. Le Rudulier, D., et al., Molecular basis of osmoregulation. 1984. Science. 224: p. 1064-1068

54. Livesey, G., *Methionine degradation: anabolic and catabolic*. 1984. trends in Biochemical Sciences. January 1984. p. 27-29

55. Lund, J. W. G., Kipling, C., LeCren, E. D. T*he inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting*. 1958. Hydrobiology. **11**: p. 143-170

56. Malin, G., *The role of DMSP and DMS in the global sulfur cycle and climate regulation*. 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds. Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p. 177-189

57. Mason, T. G., and Blunden, G. *Quaternary and tertiary sulfonium compounds of algal origin as alleviators of osmotic stress*. 1989. Botanica Marina. **32**: p. 313-316

58. Mazelis, M., and L. Ingraham, *The pyridoxal phosphate-depndent oxidative decarboxylation of methionine by peroxidase*. 1962. The Journal of Biological Chemistry. **237**: p. 109- 112

59. McNeil, S. D. M. L. Nuccio, and A. D. Hanson, *Betaines and Related Osmoprotectants. Targets for metabolic engineering of salt stress Resistance*. 1999. Plant Physiology. **120**: p. 945-949

60. Morel, F. M. M., and R. R. L. Guillard, *Aquil: A chemically defined phytoplankton culture medium for trace metal studies*. 1979. Journal of Phycology. **15**: p. 135-141

61. Mudd, H. S., and A. H. Datko, *Methionine methyl group metabolism in Lemna*. 1986. Plant Physiology. **81**: p. 103-114

62. Mudd, H. S., and A. H. Datko, *The S-methylmethionine cycle in Lemna paucicostata*. 1990. Plant Physiology. **93**: p. 623-630

63. Nakamura. H. et al., *Biogenesis of sulfonium compounds in a dinoflagellate; methionine cascade*. 1997. Tetrahedron. **27**: p. 9067-9074

64. O Brien, M C., and P. A. Wheeler, *Short term uptake of nutrients by Enteromorpha Prolifera (Chlorophyceae)* 1987. Journal of Phycology. **23**: p. 547-556

65. Paquet, L., et al., *Accumulation of the compatible solute 3-dimethylsulfoniopropionate in sugarcane and its relatives, but not other Gramineous crops*. 1994. Australian Journal of Plant Physiology. **21**: p. 37-48

66. Pokorny, M., E. Marcenko, and D. Keglevic, *Comparative studies of L- and D-methionine metabolism in lower and higher plants*. 1970. Phytochemistry. **9**: p. 2175-2188

67. Ranocha, P., et al., *The S-methylmethionine cycle in angiosperms: ubiquity,antiquity and activity*. 2001. The Plant Journal. **25**: .p. 575-584

68. Rhodes, D. and A. D. Hanson, *Quatenary ammonium and tertiary sulfonium compounds in higher plants*. 1993. Annual review of Plant Physiology. **44**: p. 357-384

69. Rhodes, D. et al., *S-Methylmethionine conversion to dimethylsulfoniopropionate: evidence for an unusual transamination reaction*. 1997. **115**: p. 1541-1548

70. Russell, B. L., Rathinasabapathi, and A. D. Hanson, *Osmotic stress induces expression of choline monooxygenase in Sugar Beet and Amaranth*. 1998. Plant Physiology. **116**: p. 859-865

71. Russell, D. W. and A. G. Howard, *The determination of DMSP in marine algae and salt marsh plants*. 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds. Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p. 155-163

72. Scislowski, P. W. D., et al., *Methionine metabolism by rat muscle and other tissues*. 1987. Biochemical Journal. **247**: p. 35-40

73. Sciuto S., Piatelli, M., and Chillemi, R., *(-)-(S)-4-Dimethylsulfonio-2-methoxybutyrate from the red alga Rytiphloea tinctoria*. 1982. Phytochemistry. **21**(1): p. 227-228

74. Shen. B., et al., *Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast*. 1999. Plant Physiology. **121**: p. 45-52

75. Shrift, A., *Nitrogen and sulfur changes associated with growth uncoupled from cell division in Chlorella vulgaris*. 1959. Plant Physiology. p. 505-512

76. Spector, D. L., *Dinoflagellates. In* Dinoflagellates. 1984. Academic Press.

77. Stevenson, D. E., Akhtar, M., and Gani, D., *Streptomyces L-methionine decarboxylase. Enzyme and stereochemical course of substrate decarboxylation*. 1990. Biochemistry. **29**: p. 7660-7666

78. Steudtler, P. A., and B. J. Peterson, *Contributions of gaseous sulfur from salt marshes to the global sulphur cycle*. 1984. Nature. **311**: p. 455-457

79. Storey, R. et. al., *Response of Melanthera biflora to salinity and water stress*. 1993. Journal of Experimental Botany. **44**(267): p. 1551-1560

80. Summers, P. S., et al., *Identification and stereospecificity of the first three enzymes of 3-dimethylsulfonipropionate biosynthesis in a Chlorophyte algae*. 1998. Plant Physiology. **116**: p. 369-378

81. Sunda, W. G., and S. A. Huntsman, *Interrelated influence of iron, light and cell size on marine phytoplankton growth*. 1997. Nature. **390**: p. 389-392

82. Tarczynski, M. C. Jensen, R. G., and H. J. Bohnert., *Stress production of transgenic tobacco production of the osmolyte mannitol.* 1991. Science. **259**: p. 508-510

83. Trossat, C., Nolte, K. D., and A. D. Hanson, *Evidence that the pathway of dimethylsulfoniopropionate begins in the cytosol and ends in the chloroplast.* 1996. Plant Physiology. **111**: p. 1-9

84. Turner, S. M., Malin, G., and P. Liss, *Dimethylsuflide and (dimethylsulfonio) propionate in European coastal and shelf waters.* 1989. *In* E. S. Saltzman, W. J. Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington D.C. p. 183-200

85. Tuttle, R. C., and A. R. Loeblich, *An optimal growth media for the dinoflagellate Crypthecohdinium cohnii.* 1975. Phycologia. **14**: p. 1-8

86. Uchida, A., et al., *Biosynthesis of dimethylsulfoniopropionate in Crypthecohdinium cohnii (Dinophyceae)* 1996. *In* R.Kiene, P. Visscher, G. Kirst, M. Keller eds.Environmental and biological chemistry of DMSP and related sulfonium compounds. Plenum, New York. p. 97-107

87. Walters, D. S. and Steffens, J. C. *Branched chain amino acid metabolism in the biosynthesis of Lycopersicon penneli glucose esters.* 1990. Plant Physiology. **93**: p. 1544-1551

88. Wang, J., et al., *Analysis of amino acids by gas chromatography-flame ionization detection and gas chromatography-mass spectrometry: Simultaneous derivatization of functional groups by an aqueous-phase chloroformate-mediated reaction.* 1994. Journal of Chromatography. **663**: p. 71-78

89. Wolfe, G. and M. Steinke, *Grazing-activated production of dimethylsulfide (DMS) by two clones of Emiliana huxleyi.* 1996. Limnology and Oceanography. **41**(6): p. 1151-1160

90. Wolfe, G. M. Steinke, and G. O. Kirst, *Grazing activated chemical defence in a unicellular marine alga.* 1997. Nature. **387**: p. 894-897

91. White, R. H., *Analysis of dimethyl sulfonium compounds in marine algae.* 1982. Journal of Marine Research. **40**(2): p. 529-535

92. Yancey, P., et al., *Living with water stress: evolution of osmolyte systems.* 1982. Science. **217**: p. 1214-1222

93. Zappia, V., et al., *The specificity of S-Adenosylmethionine derivatives in methyltransfer reactions*. 1969. The Journal of Biological Chemistry. **244**(16): p. 4499-4509