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Biosynthesis of 3-Dimethylsulfoniopropionate in *Wollastonia biflora* (L.) DC.[1]

Evidence That *S*-Methylmethionine Is an Intermediate

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The compatible solute 3-dimethylsulfoniopropionate (DMSP) is accumulated by certain salt-tolerant flowering plants and marine algae. It is the major biogenic precursor of dimethylsulfide, an important sulfur-containing trace gas in the atmosphere. DMSP biosynthesis was investigated in *Wollastonia biflora* (L.) DC. [=*Wedelia biflora* (L.) DC., *Melanthera biflora* (L.) Wild, Asteraceae]. After characterizing DMSP and glycine betaine accumulation in three diverse genotypes, a glycine betaine-free genotype was chosen for radiotracer and stable isotope-labeling studies. In discs from young leaves, label from [U-^{14}C]methionine was readily incorporated into the dimethylsulfide and acrylate moieties of DMSP. This establishes that DMSP is derived from methionine by deamination, decarboxylation, oxidation, and methylation steps, without indicating their order. Five lines of evidence indicated that methylation is the first step in the sequence, not the last. (a) In pulse-chase experiments with [^{14}C]methionine, *S*-methylmethionine (SMM) had the labeling pattern expected of a pathway intermediate, whereas 3-methylthiopropionate (MTP) did not. (b) [^{14}C]SMM was efficiently converted to DMSP but [^{14}C]MTP was not. (c) The addition of unlabeled SMM, but not of MTP, reduced the synthesis of [^{14}C]DMSP from [^{14}C]methionine. (d) The dimethylsulfide group of [$^{13}CH_3$,C^2H_3]SMM was incorporated as a unit into DMSP. (e) When [C^2H_3,C^2H_3]SMM was given together with [$^{13}CH_3$]methionine, the main product was [C^2H_3,C^2H_3]DMSP, not [$^{13}CH_3$,C^2H_3]-DMSP or [$^{13}CH_3$,$^{13}CH_3$]DMSP. The stable isotope labeling results also show that the SMM cycle does not operate at a high level in *W. biflora* leaves.

The tertiary sulfonium compound DMSP is accumulated by many marine algae (Blunden and Gordon, 1986) and by certain salt-tolerant higher plants including *Spartina* spp. (Larher et al., 1977; Dacey et al., 1987), sugarcane (Paquet et al., 1994), and *Wollastonia biflora* (Storey et al., 1993). DMSP is structurally analogous to a betaine and, like betaines, can act as a compatible solute or osmoprotectant and hence contribute to osmotic stress tolerance (Paquet et al., 1994, and refs. therein). DMSP differs from betaines in having an atom of S in place of N, and in N-limited environments, algae (Turner et al., 1988; Gröne and Kirst, 1992) and higher plants (Dacey et al., 1987) may compensate for a limited ability to form betaines by producing more DMSP.

In addition to its importance as an osmotic solute, DMSP is the precursor of the gas DMS, a major biogenic source of atmospheric S (Dacey et al., 1987; Turner et al., 1988). Therefore, DMSP is implicated in the biogeochemical S cycle, in acid precipitation, and in the production of atmospheric aerosols that may affect climate.

Little is known about the biosynthetic pathway of DMSP (Rhodes and Hanson, 1993). In the green alga *Ulva lactuca*, radiotracer experiments showed that Met was the source of the S atom and of both methyl groups, and that the α-carbon of Met gives rise to the carboxyl group of DMSP (Greene, 1962; Kahn, 1964). Radiolabeling studies with other algae have confirmed that Met is readily converted to DMSP (Pokorny et al., 1970; Chillemi et al., 1990). Such data led Maw (1981) to propose the biosynthetic pathway shown at the top of Figure 1, in which methylation is the final step (Met → KMTB → MTP → DMSP). However, as the rest of Figure 1 indicates, there are several biochemically reasonable alternatives, none of which are excluded by the available evidence.

We report here a study of the DMSP biosynthesis pathway in *W. biflora*, a common Indo-Pacific strand plant reported to be rich in DMSP (Storey et al., 1993). Because *W. biflora* is widely distributed and highly variable, we examined DMSP accumulation in genotypes from diverse geographic origins before selecting plants and conditions for experiments. We

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Abbreviations: DMS, dimethylsulfide; DMSP, 3-dimethylsulfoniopropionate; FABMS, fast atom bombardment mass spectrometry; KDMSB, 2-keto-4-dimethylsulfoniobutyrate; KMTB, 2-keto-4-methylthiobutyrate; M^+, the molecular ion of SMM or DMSP carrying one net positive charge; MTP, 3-methylthiopropionate; SMM, *S*-methylmethionine.

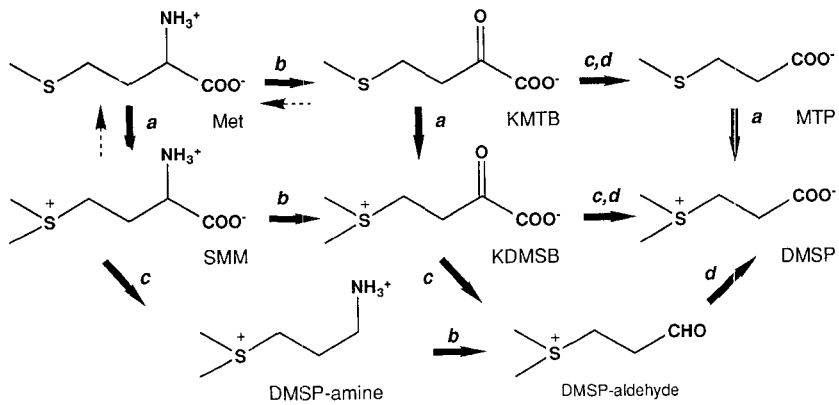

Figure 1. A metabolic grid showing possible pathways of DMSP biosynthesis from Met. The steps involved are methylation (a), oxidative deamination or transamination (b), decarboxylation (c), and oxidation (d), in various orders. Note the broken arrows denoting possible interconversion of Met and SMM via the SMM cycle (Mudd and Datko, 1990), and of Met and KMTB via transamination (Miyazaki and Yang, 1987). Except for KDMSB, all the hypothetical intermediates shown are known from plant, animal, or microbial metabolism; KDMSB would be expected to be unstable (Cooper et al., 1989). DMSP-aldehyde, 3-Dimethylsulfoniopropionaldehyde; DMSP-amine, 3-dimethylsulfoniopropylamine.

then used isotope labeling methods to confirm that DMSP is derived from Met and to show that *S*-methylation is the first step in the pathway and not, as proposed by Maw (1981), the last. As an integral part of this work, we developed FABMS and ancillary methods for DMSP analysis.

MATERIALS AND METHODS

Radiochemicals

L-[U-^{14}C]Met (9.5 MBq μmol^{-1}, NEN-DuPont) was mixed with unlabeled L-Met to give desired specific activities. [U-^{14}C]MTP (0.55 MBq μmol^{-1}) was synthesized from [U-^{14}C]Met by the method of Steele and Benevenga (1979). [^{14}C]SMM (9.5 MBq μmol^{-1}, labeled uniformly except for one ^{12}C-methyl group) was synthesized by a modification of the method of Toennies and Kolb (1945). [U-^{14}C]Met (0.75 MBq) was treated with 10 μL of formic acid:acetic acid:iodomethane (6.4:2:1, v/v/v) for 3 d in darkness at 22°C. [^{14}C]SMM was isolated by TLC on cellulose plates (0.1 mm, Merck) developed with *n*-butanol:acetic acid:water (60:20:20, v/v/v). [^{35}S]DMSP was prepared from nine leaf discs incubated for 6 h with 3.5 MBq ^{35}S-amino acids (>70% L-[^{35}S]Met, 38 GBq μmol^{-1}, ICN). The radiochemical purity of [^{14}C]SMM and [^{35}S]DMSP was shown to be >99% by TLC and thin-layer electrophoresis.

Stable Isotope Compounds

L-[$^{13}CH_3$]Met (96% ^{13}C) and L-[C^2H_3]Met (98% $^2H_3$) were from Cambridge Isotope Laboratories; C^2H_3I (99% $^2H_3$) was from Sigma. [C^2H_3,C^2H_3]DMSP was prepared by a method based on that of Chen and Benoiton (1976). 3-Mercaptopropionic acid (1 mmol, Aldrich) in 20 mL of methanol was stirred with 0.66 mL of C^2H_3I and 1 g of $KHCO_3$ for 24 h at 22°C. The reaction mix was decanted, dried in vacuo, and extracted twice with 3 to 5 mL of acetonitrile:methanol (10:1, v/v); the extracts were pooled and dried in vacuo. DMSP was purified and converted to the HCl form with a 10-mL mixed resin column [Dowex-1 (OH^-):BioRex-70 (H^+), 2:1 (v/v)] followed by a 2-mL Dowex-50 (H^+) column. After washing both columns with water, DMSP was eluted from Dowex-50 with 10 mL of 2.5 M HCl and freeze dried; excess HCl was removed by twice redissolving in water and freeze drying. Yield was 23%. FAB mass spectrum ions [m/z (relative intensity)] were 141(M^+ for the fully labeled compound, 100), 142(10), 143(8), 140(33), 139(8). The peaks at m/z 140 and 139 can be attributed to base-catalyzed ^2H exchange during synthesis (Blackwell, 1981). [C^2H_3,C^2H_3]SMM and [$^{13}CH_3$,C^2H_3]SMM were prepared by the method of Toennies and Kolb (1945) from 1 mmol of [$^{13}CH_3$]Met or [C^2H_3]Met, using C^2H_3I. SMM was purified and converted to the HCl form by ion exchange on BioRex 70 (H^+), eluting with 2.5 M HCl. FAB mass spectra [m/z (relative intensity)] were, for [C^2H_3,C^2H_3]SMM, 170(M^+ for the fully labeled compound, 100), 171(13), 172(9), 169(10), 168(4), and for [$^{13}CH_3$,C^2H_3]-SMM, 168(M^+ for the fully labeled compound, 100), 169(11), 170(8), 167(14), 166(5). Unlabeled DMSP (Paquet et al., 1994) and [$^2H_9$]Gly betaine (Rhodes et al., 1987) were prepared as described.

Plants and Growth Conditions

Flowering material of genotype H was determined by M. Dillon as *Wollastonia biflora*; comparisons of vegetative material of genotypes H, B, and S indicated that they are conspecific. Our nomenclature and synonymy follow Fosberg and Sachet (1980). Provenances and sources of genotypes were as follows. H: provenance, Okinawa, Japan; source, Waimea Arboretum & Botanical Garden, Haleiwa, Hawaii (voucher specimen in the Marie-Victorin Herbarium, Montreal, Canada). B: provenance, Cairns, Australia; source, J. Gorham, University of Wales, Bangor, UK. S: provenance, American Samoa; source, A. Whistler, University of Hawaii, Honolulu. Plants were propagated by cuttings and grown in potting mixture (Pro-Mix-BX, Tourbières Premier, Québec, Canada):loam:perlite (2:2:1, v/v/v) in a growth chamber (8-h day, 23°C, 200–300 μmol PAR quanta m^{-2} s^{-1}/20°C night); 20–20–20 (NPK) fertilizer was applied weekly.

Salinization Experiments

Plants were grown singly in 9-cm pots and irrigated daily with 200 mL of half-strength Hoagland solution (Hoagland and Arnon, 1950) containing 7.5 mM NO_3^- (standard level) or 0.19 mM NO_3^- (balance Cl^-). Plants had four mature leaves when salinization began. Artificial seawater (Flowers et al., 1990) was added to the nutrient solution in 10% steps

every 3 d to a final level of 80% (v/v), which was maintained for 8 d before harvest. Solute potentials were measured on frozen-thawed 9-mm leaf discs from young, expanded leaves, using a Wescor HR-33T dewpoint microvoltmeter with C-52 sample chambers. Blades of the four or six youngest expanded leaves were taken for DMSP analysis and freeze dried, which caused no DMSP loss. Dried leaves were milled and stored in sealed vials at −20°C until analysis. DMSP was stable in these conditions (<5% loss in 4 weeks), but not at 22°C (56% loss in 4 weeks).

Metabolism of Precursors by Leaf Discs

Discs (11 mm diameter) were from young (typically about 70% expanded) leaves of genotype H; each was given eight shallow radial cuts on the abaxial surface. Batches of discs (usually nine discs, 0.25–0.3 g fresh weight) were placed, cut surface down, in 6-cm Petri dishes with a 4.25-cm circle of Whatman No. 1 paper containing 0.5 mL of precursor solution. The HCl form of SMM was neutralized with $KHCO_3$. Incubation was on a rotary shaker (100 rpm) in fluorescent light (150 μmol PAR quanta m^{-2} s^{-1}) at 28 ± 3°C. Water was added to replace that lost to evaporation.

Extraction and Ion-Exchange Separation of Radiolabeled Metabolites

Discs were extracted using a methanol-chloroform-water procedure (Hanson and Gage, 1991) after adding appropriate unlabeled carriers (SMM, MTP, or Met, 0.2–1.0 μmol per nine discs). The aqueous phase was dried at 45 to 50°C in a stream of N_2, dissolved in water, and applied to a series of three 1-mL columns in the order BioRex-70 (H^+), Dowex-1 (OH^-), Dowex-50 (H^+). Each column was washed with 5 mL of water and eluted with 5 mL of 1 M HCl (BioRex-70) or 2.5 M HCl (Dowex columns). Eluates were freeze dried. Before freeze drying the Dowex-1 eluate, MTP and other organic acids were isolated by partitioning twice against 0.5 volume of diethylether and then back-extracting the ether with 0.1 volume of 20 mM NaOH. Radiolabeled compounds added to leaf discs were used to determine recoveries. These were: [^{35}S]DMSP in Dowex-50 eluate, 44%; [^{14}C]MTP in ether extract, 26%; [^{14}C]SMM in BioRex-70 eluate, 21%. For the [^{14}C]Met experiments described in Table III, the residue was re-extracted (5 min, about 80°C) with 1 mL each of water and 5 mM HCl, and the order of the BioRex-70 and Dowex-1 columns was reversed. These changes raised [^{14}C]SMM recovery to 53%.

TLC and Electrophoresis

Biorex-70 and Dowex-50 eluates were analyzed by TLC on Silica Gel G (Polygram, Machery-Nagel, Düren, Germany) developed with methanol:acetone:concentrated HCl (90:10:4, v/v/v); Dowex-50 eluates were also analyzed by thin-layer electrophoresis on silicic acid plates (ITLC SA, Gelman, Ann Arbor, MI) in 1.5 M acetic acid, run at 1.8 kV, 6°C, for 12 min. Organic acids in the ether extract were separated on cellulose plates with *n*-butanol:diethylamine:water (85:1:14, v/v/v). Amino acids in Dowex-1 eluates were separated on cellulose or Silica Gel G with *n*-butanol:acetic acid:water (60:20:20, v/v/v). Labeled zones were detected autoradiographically and quantified by spot intensity or scintillation counting, correcting for recoveries of ^{14}C standards. Because DMSP was unstable on dry TLC plates, label in DMSP was calculated from that in the Dowex-50 eluate, subtracting that in any other TLC zones. DMSP was detected with Dragendorff reagent, MTP with iodoplatinate reagent (Awwad and Adelstein, 1966), and amino acids with ninhydrin. SMM was quantified by area measurement of ninhydrin-positive TLC zones.

Degradation of [^{14}C]DMSP

[^{14}C]DMSP samples were mixed with 1 μmol of unlabeled DMSP and treated in sealed 1.8-mL vials with 200 μL of 17% (w/v) NaOH at 22°C for 30 min to give complete degradation to DMS and acrylic acid. The [^{14}C]DMS released was continuously swept out by bubbling a stream of air containing 7.7% (v/v) carrier DMS through the reaction mixture at about 1 mL min^{-1}. DMS was trapped in 1 mL of 3% (w/v) $HgCl_2$. Trapped [^{14}C]DMS and [^{14}C]acrylate remaining in the reaction vial were assayed by scintillation counting. DMS recovery was 77 ± 4% (mean ± SE), measured with [^{35}S]DMSP.

Isolation of DMSP for FABMS Analysis

Samples (30 mg) of freeze-dried leaf material or batches of nine discs were extracted as above, except that dry material was first rehydrated at 0°C with 0.5 mL of water. The dried aqueous phase was redissolved in water and applied to a 1.5-mL mixed-resin column in series with a 1.0-mL column of Dowex-50 (H^+). Each column was washed with 8 mL of water. DMSP and Gly betaine were eluted from Dowex-50 with 5 mL of 2.5 M HCl; this eluate was freeze dried and used for FABMS analyses. To quantify DMSP and Gly betaine, 1-μmol internal standards of [C^2H_3,C^2H_3]DMSP and [$^2H_9$]Gly betaine were added to samples before extraction. The recovery of [C^2H_3,C^2H_3]DMSP was 77%, that of [$^2H_9$]-Gly betaine was 81%.

FABMS Analysis

Instrumentation was as described by Hanson and Gage (1991). DMSP was analyzed as the HCl salt, routinely using glycerol as matrix. Converting DMSP·HCl to the neutral form by ion exchange caused losses of about 40%. Sensitivity could not be enhanced by converting DMSP to its *n*-butyl ester, as can be done for betaines (Rhodes et al., 1987). After derivatization, no peaks corresponding to DMSP or its ester were found, probably because the ester spontaneously decomposes by elimination of DMS (Wagner and Daniels, 1965).

Hydrogens α to the positive S atom in sulfonium compounds are acidic and undergo base-catalyzed hydrogen-deuterium exchange (Blackwell, 1981). Such exchange would preclude use of methyl-$^2H_3$ label in analytical or metabolic studies. However, it was negligible in our procedures. Leaf disc samples were spiked with 1 μmol of [C^2H_3,C^2H_3]DMSP

(m/z 141) and DMSP was extracted as above and analyzed. The relative intensity ratio of m/z 140:141 for the spike was 0.33 ± 0.01; that for the extracted DMSP was 0.34 ± 0.01 (mean ± SE, $n = 9$).

Endogenous, unlabeled DMSP (m/z 135) was quantified using an internal standard (1 μmol) of [C^2H_3,C^2H_3]DMSP. A calibration plot is shown in Figure 2A. A similar calibration was made for Gly betaine, with [$^2H_9$]Gly betaine as standard (Fig. 2B). DMSP formed from labeled SMM was analyzed using a matrix of hexaethylene glycol (1 μL) mixed with 1 μL of a 1% (w/v) aqueous solution of the K^+ salt of nonafluorobutylsulfonic acid, an anionic surfactant. This matrix solution gave enhanced signals for DMSP and produced no interfering background ions. Glycerol was unsuitable because its disodium adduct (m/z 137) coincided with an analyte peak.

RESULTS AND DISCUSSION

Choice of Genotype and Conditions for Labeling Experiments

W. biflora genotypes B, H, and S were weak shrubs, differing in leaf size and internode length. Their growth, osmotic adjustment, and DMSP accumulation were compared under unstressed and saline conditions (Fig. 3). Salinity treatments included adequate or limiting levels of N. All genotypes tolerated salinization; they adjusted osmotically and continued to grow slowly. All had high DMSP levels (11–13 μmol g^{-1} fresh weight) when unstressed; DMSP levels were raised about 2-fold by salinization, and about 2.5-fold by salinization plus N deficiency. Genotype B also accumulated Gly betaine when salinized; N deficiency decreased the Gly betaine level.

Genotype H was chosen for labeling studies because it lacked the complicating factor of Gly betaine accumulation, grew rapidly when unstressed, and had large leaves separated by long internodes. The relatively high levels of DMSP present in unstressed leaves made it unnecessary to salinize plants before labeling experiments.

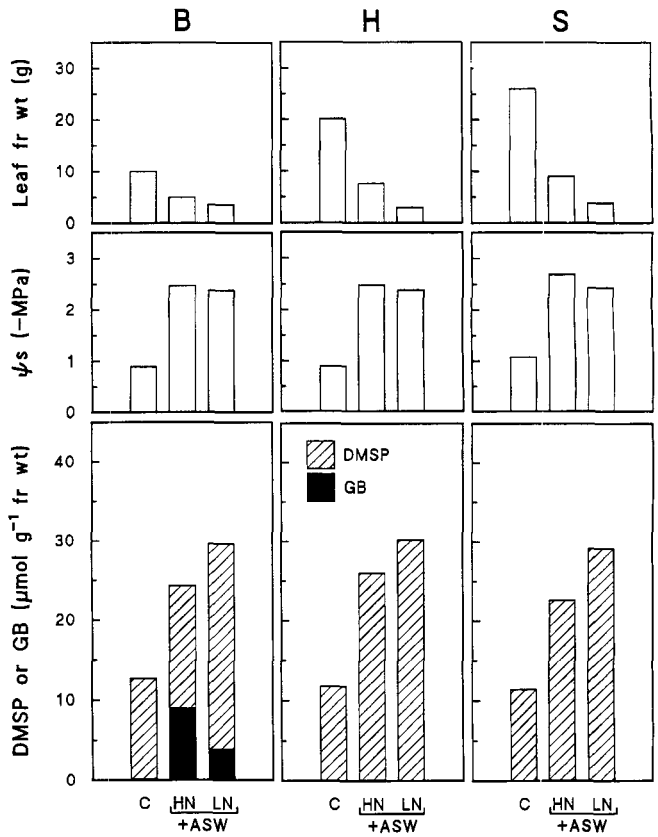

Figure 3. Responses to salinization of *W. biflora* genotypes B, H, and S. Plants were irrigated with normal nutrient solution alone (C) or with 80% artificial seawater (ASW) containing normal (HN) or N-deficient (LN) nutrient solution. Leaf fresh weights are for all leaves alive at harvest; DMSP and Gly betaine data are for the four or six youngest expanded leaves. Gly betaine was below the limit of detection by FABMS (<0.2 μmol g^{-1} fresh weight) for all samples except salinized genotype B. GB, Gly betaine; ψs, leaf solute potential.

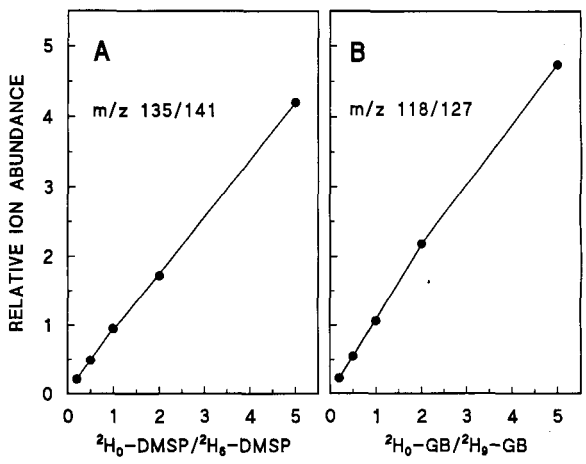

Figure 2. Calibration plots for quantification by FABMS of unlabeled ($^2H_0$) DMSP relative to a 1-μmol standard of [C^2H_3,C^2H_3]-DMSP (A), and unlabeled ($^2H_0$) Gly betaine relative to a 1-μmol standard of $^2H_9$-Gly betaine (B). The horizontal axes are molar ratios.

Conversion of [U-^{14}C]Met to DMSP

When leaf discs metabolized tracer or substrate amounts of [U-^{14}C]Met for several hours, [^{14}C]DMSP was always a major product (Table I). Both the acrylate and DMS moieties of [^{14}C]DMSP were labeled, indicating that the entire DMSP molecule is derived from Met (Table I). No more than 22% of the ^{14}C was found in the DMS moiety. In the simplest case, this moiety would contain 40% of the ^{14}C if both methyl groups as well as the three-carbon chain of DMSP come from [U-^{14}C]Met. However, when [U-^{14}C]Met (as its *S*-adenosyl derivative) serves as methyl donor in DMSP synthesis or any other reaction, the resulting [^{14}C]homocysteine fragment can be recycled to Met by adding an unlabeled methyl group from the folate pool (Giovanelli et al., 1980). With time, such recycling would deplete the methyl-^{14}C content of the

Table I. *Incorporation of radioactivity from [U-^{14}C]Met into DMSP*

Leaf discs were given tracer or substrate amounts of [U-^{14}C]Met. ^{14}C distribution in the [^{14}C]DMSP formed was determined by chemical degradation to acrylate and DMS.

[U-^{14}C]Met Supplied per 9 Discs		Incubation Time	[^{14}C]DMSP Synthesized[a]	^{14}C Distribution in DMSP Moieties	
				Acrylate	DMS[b]
kBq	*nmol*	*h*	*kBq/9 discs*	%	%
66.4	7	8	25.8	88	12
23.9	34	9	8.7		
9.4	2500	24	3.4	78	22

[a] Corrected for 44% DMSP recovery from extraction and ion-exchange procedures. [b] Corrected for 77% DMS recovery in the $HgCl_2$ trapping reagent.

[^{14}C]Met pool, and hence of the [^{14}C]DMSP derived from it. Consistent with this interpretation, methyl-^{14}C labeling of DMSP was greater when the [U-^{14}C]Met dose was large (Table I); an expanded pool of [U-^{14}C]Met should reduce the impact of [^{14}C]homocysteine recycling. Recycling of the methylthio moiety of Met via methylthioribose and KMTB (Miyazaki and Yang, 1987) is unlikely to have been important in these experiments because labeling of polyamines was negligible (not shown) and ethylene synthesis rates were very low [<0.02 nmol h^{-1} (9 discs)$^{-1}$].

^{14}C-Labeling Kinetics of SMM and MTP

The metabolic grid in Figure 1 shows that Met might be converted to DMSP by various routes. To distinguish among them, we investigated the methylation step in a pulse-chase experiment with [U-^{14}C]Met. If the methylation step comes first in the pathway, SMM should initially acquire label and then lose it during the chase period; if methylation comes last, MTP should show such a pattern. The observed labeling kinetics of SMM were consistent with methylation being the first step (Fig. 4). The rapid accumulation of ^{14}C in SMM, the lag in DMSP labeling, and the slow decline in [^{14}C]SMM during the chase all point to SMM as an intermediate in DMSP synthesis. These features further suggest that the metabolic pool of SMM is large, which fits with a measured SMM content of 91 ± 19 nmol/9 discs (about 0.3 μmol g^{-1} fresh weight). The slight, steady accumulation of ^{14}C in MTP is more consistent with MTP being a minor end product of Met metabolism than an intermediate in DMSP synthesis.

^{14}C-Labeling and Trapping Experiments with MTP and SMM

We used two further radiolabeling approaches to probe the roles of MTP and SMM in DMSP biosynthesis. [^{14}C]MTP and [^{14}C]SMM were chemically synthesized and tested as precursors of DMSP; unlabeled MTP and SMM were tested for their ability to lower ^{14}C flux from [U-^{14}C]Met to DMSP by acting as trapping pools.

[^{14}C]MTP was readily taken up, but was a very poor precursor of DMSP compared with [U-^{14}C]Met (Table II). Because [^{14}C]MTP was extensively metabolized, and its metabolites included Met and SMM, the small observed ^{14}C incorporation into DMSP may have been via these compounds. The addition of unlabeled MTP had no effect on [U-^{14}C]Met uptake or metabolism; it did not depress [^{14}C]-DMSP formation or cause ^{14}C to accumulate in MTP (Table II). Taken with the kinetic data in Figure 4, these results make it unlikely that MTP is an intermediate in DMSP synthesis.

In contrast, SMM behaved as expected for an intermediate in both labeling and trapping experiments (Table III). Tracer or substrate doses of [^{14}C]SMM were converted to DMSP at least as efficiently as [^{14}C]Met, and unlabeled SMM strongly reduced the incorporation of ^{14}C into DMSP. About half this reduction can be ascribed to a trapping effect of SMM, and half to another interesting effect: less [^{14}C]Met was metabolized to SMM and DMSP. This suggests that the rate of SMM synthesis may be feedback regulated by the size of the SMM pool.

The Problem of the SMM Cycle

The results presented so far make a good prima facie case that SMM is an intermediate in DMSP biosynthesis. However, the interpretation of radiolabeling experiments with Met and SMM is potentially complicated by the interconversion of these compounds via the SMM cycle (Mudd and Datko, 1990). In the first half of this (futile) cycle, Met is methylated by *S*-adenosylmethionine to SMM; in the second half, SMM combines with homocysteine to give two molecules of Met. Table III shows that the SMM cycle may operate,

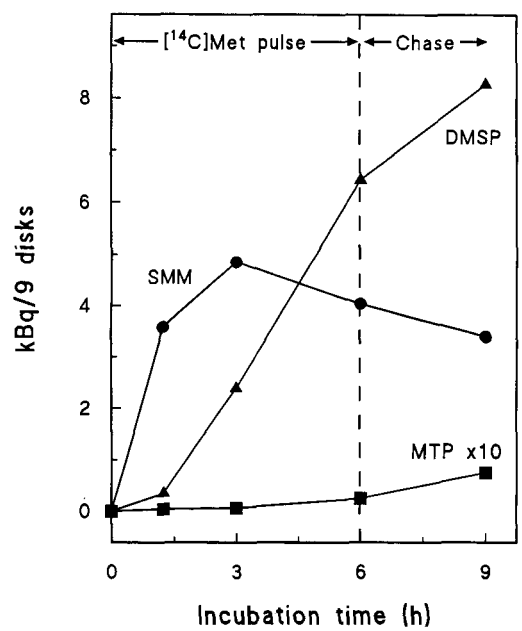

Figure 4. Labeling patterns of SMM, MTP, and DMSP in a pulse-chase experiment with [U-^{14}C]Met. Discs were supplied with [U-^{14}C]Met (73 kBq, 7.7 nmol) for up to 6 h, then transferred to 5 mM L-Met. Data were corrected for recovery from ion-exchange fractionation and TLC. Note that the values for ^{14}C incorporation into MTP have been multiplied by 10.

Table II. *MTP as a precursor or trapping pool in DMSP biosynthesis*

Leaf discs were given either 34 nmol of [U-^{14}C]MTP or 34 nmol of [U-^{14}C]Met with or without a 2.5-μmol trapping pool of unlabeled MTP. Incubation was for 9 h. Labeling of amino acids was estimated from ^{14}C in ether-extracted Dowex-1 eluates. Values shown are kBq per nine discs and have been corrected for recovery.

^{14}C Precursor		MTP Trap	^{14}C Uptake[a]	^{14}C Distribution			
Compound	Dose			Amino acids[b]	MTP	SMM	DMSP
MTP	18.5	−	12.6	3.48	0.40	0.07	0.32
Met	23.9	−	23.1	2.00	<0.05	1.46	8.02
Met	23.9	+	23.0	1.92	<0.05	1.67	8.40

[a] Calculated from disappearance of ^{14}C from the incubation medium. [b] In all samples ≥30% of the ^{14}C activity co-migrated in TLC with Met and Met sulfoxide generated during extraction.

at least to some extent, in *W. biflora*, because when [^{14}C]SMM was supplied, a small amount of label was recovered in Met.

Therefore, our ^{14}C-labeling data do not provide unequivocal support for SMM as an intermediate in DMSP synthesis (reaction sequence a below), since they could also reflect operation of sequence b, alone or in combination with a: (a) Met → SMM → → DMSP; (b) SMM ↔ Met → → DMSP. These alternatives were distinguished by following the fate of the methyl groups of SMM, using stable isotopes.

Stable Isotope-Labeling Experiments

Two experimental designs were used. The principles behind the first may be briefly stated as follows. If SMM gives rise to DMSP indirectly via the SMM cycle, it will first transfer one methyl group to homocysteine, giving two Met. Either can then provide a methyl group or a four-carbon fragment for DMSP synthesis. In this case, if SMM labeled in one methyl group with ^{13}C and in the other with $^{2}H_3$ is supplied, the methyl label in the DMSP product will be randomized, with $^{13}CH_3$,$^{13}CH_3$-, $^{13}CH_3$,C^2H_3-, and C^2H_3,C^2H_3-labeled molecules in a 1:2:1 ratio. Alternatively, if SMM is an intermediate between Met and DMSP, the DMSP synthesized will be entirely $^{13}CH_3$,C^2H_3 labeled.

Table IV shows that at least 93 to 96% of the newly synthesized DMSP was $^{13}CH_3$,C^2H_3 labeled (m/z 139), indicating that SMM is an intermediate and that flux through the SMM cycle is very small compared with that to DMSP. The minor signals at m/z 137 were never equally matched by those at m/z 141 and were absent in one experiment (not shown). This makes it probable that a contaminant ion, as well as [$^{13}CH_3$,$^{13}CH_3$]DMSP, contributes to the observed m/z 137 peak. Although the enzyme-catalyzed methyl transfer from SMM to homocysteine is diastereospecific (Grue-Sorensen et al., 1984), this seems unlikely to have biased these results in any way because the [$^{13}CH_3$,C^2H_3]SMM supplied was chemically synthesized and hence presumably a 1:1 mixture of diastereoisomers.

A second experiment was designed to test the conclusions from the first label-randomization experiment, and to determine whether all the Met flux to DMSP passes via SMM or whether there is also some other pathway. The metabolic grid in Figure 1 makes clear that flux through parallel pathways of DMSP biosynthesis is quite conceivable. SMM labeled with two methyl-$^{2}H_3$ groups was supplied together

Table III. *SMM as a precursor or trapping pool in DMSP biosynthesis*

Leaf discs were given tracer or substrate amounts of [^{14}C]SMM or [U-^{14}C]Met with or without a 2.5-μmol trapping pool of unlabeled SMM. Incubation time was 9 h for tracer [^{14}C]SMM, 24 h for other cases. Amino acid labeling was estimated from ^{14}C in freeze-dried Dowex-1 eluates. Values shown have been corrected for recovery. ND, Not determined.

^{14}C Precursor			SMM Trap	^{14}C Distribution		
Compound	Dose			Amino acids	SMM	DMSP
	kBq	*nmol*		*kBq/9 discs*		
SMM	40.3	4	−	2.07[a]	13.1	20.5
SMM[b]	8.22	2500	−	ND	ND	2.50
Met	8.41	2500	−	1.42[c]	1.77	1.56
Met	8.41	2500	+	2.72[c]	1.26	0.34

[a] Of which 0.21 kBq co-migrated with Met plus Met sulfoxide in TLC. [b] Fractionated by the mixed-bed resin procedure described for FABMS samples. [c] Of which ≥30% co-migrated with Met plus Met sulfoxide in TLC.

Table IV. *Labeling of DMSP synthesized by discs given [$^{13}CH_3$,C^2H_3]SMM*

Leaf discs were incubated with [$^{13}CH_3$,C^2H_3]SMM for 24 or 48 h. Intensities of the labeled DMSP ions are expressed relative to that of endogenous unlabeled DMSP (m/z 135, 100%). All values shown were corrected for background signals in controls not given [$^{13}CH_3$,C^2H_3]SMM. Signals at m/z 137 and 141 were also corrected for the conversion to DMSP of SMM ions at m/z 166 and 170, respectively.

[$^{13}CH_3$,C^2H_3]SMM Supplied	Incubation Time	Relative Intensity of DMSP Ions		
		$^{13}CH_3$,$^{13}CH_3$ m/z 137	$^{13}CH_3$,C^2H_3 m/z 139	C^2H_3,C^2H_3 m/z 141
mM	*h*		%	
5	24	2.6	36.5	0.1
5	48	1.8	40.9	0.1
10	24	2.8	57.6	0.5
10	48	3.1	80.9	1.5

Table V. *Labeling of DMSP synthesized by discs given [C^2H_3,C^2H_3]-SMM plus [$^{13}CH_3$]Met*

Leaf discs were incubated with an equimolar mixture of [C^2H_3,C^2H_3]SMM and [$^{13}CH_3$]Met for 24 or 48 h. Parallel ^{14}C-labeling experiments showed that nearly equal amounts (±10%) of both compounds were taken up. DMSP ion intensities are expressed relative to endogenous DMSP (m/z 135, 100%). All values shown were corrected for background signals in controls not given any precursor. Signals at m/z 139 were also corrected for the conversion to DMSP of the SMM ion at m/z 168.

[C^2H_3,C^2H_3]SMM and [$^{13}CH_3$]Met Supplied	Incubation Time	Relative Intensity of DMSP Ions		
		$^{13}CH_3$,$^{13}CH_3$ m/z 137	$^{13}CH_3$,C^2H_3 m/z 139	C^2H_3,C^2H_3 m/z 141
mM	*h*		%	
5	24	4.7	0.0	24.7
5	48	5.8	0.0	32.4
10	24	3.8	0.0	29.1
10	48	5.3	0.0	37.2

with [$^{13}CH_3$]Met. If there is little or no flux through the SMM cycle, the [C^2H_3,C^2H_3]SMM should be converted to [C^2H_3,C^2H_3]DMSP, and little or no [$^{13}CH_3$,C^2H_3]DMSP should be formed. No formation of [$^{13}CH_3$,C^2H_3]DMSP (m/z 139) was detectable (Table V), confirming that flux through the SMM cycle was negligible relative to that to DMSP. The large added pool of [$^{13}CH_3$]Met might have reduced de novo biosynthesis of homocysteine by inhibiting cystathionine γ-synthase (Giovanelli et al., 1989). However, such an effect seems unlikely to have shut down the SMM cycle in these experiments, inasmuch as the cycle would itself furnish the homocysteine needed for its operation (Mudd and Datko, 1990).

If there is a major pathway from Met to DMSP other than that via SMM, then a large proportion of the DMSP product is expected to be $^{13}CH_3$,$^{13}CH_3$ labeled. There was no clear evidence for this possibility; based on the intensity of the peak at m/z 137, [$^{13}CH_3$,$^{13}CH_3$]DMSP was no more than 12 to 16% of the total (Table V). As discussed above, the true value may be somewhat less. A small amount of [$^{13}CH_3$,$^{13}CH_3$]DMSP synthesis is in any case quite compatible with a pathway via SMM, for even a large pool of SMM could not be expected to trap all the label coming from Met, and indeed did not do so in ^{14}C-labeling experiments (Table III). Therefore, it is improbable that there are alternatives to the pathway(s) via SMM.

CONCLUDING REMARKS

Our results establish that *S*-methylation is the first step in DMSP biosynthesis from Met, and not the last as proposed by Maw (1981). This gives new significance to SMM in plant metabolism. Although SMM is very common among higher plants, its only previously recognized fates were reconversion to Met via the SMM cycle and perhaps cleavage to homoserine and DMS (Giovanelli et al., 1980; Mudd and Datko, 1990). Our data implicate conversion of Met to SMM as a control point in DMSP biosynthesis; salinity almost certainly promotes this conversion because the increase in DMSP level upon salinization (some 15 μmol g^{-1} fresh weight) is 50-fold larger than the SMM pool in unsalinized leaves. N and S nutrition may also control SMM synthesis because N deficiency raises DMSP levels, and S deficiency lowers them (Storey et al., 1993).

The present results do not shed light on the nature of the intermediate(s) after SMM in the pathway. We observed no obvious candidates in labeling experiments with [^{14}C]Met or [^{14}C]SMM. However, because the SMM pool is large in relation to the hourly flux through it, it has a massive diluting effect on radiotracers and so makes small pools of subsequent intermediates hard to detect. Moreover, as indicated in Figure 1, at least one of the possible intermediates (KDMSB) is likely to be unstable.

Because DMSP accumulation occurs in diverse classes of marine algae as well as in two very different families of angiosperms (Asteraceae and Gramineae), it may have evolved independently several times. This suggests caution in generalizing the DMSP pathway in *W. biflora* to other groups. However, the stable isotope-labeling methods we have developed should be applicable to other species, and could permit simple tests of whether or not their DMSP pathways resemble that of *W. biflora*.

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