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Methionine Metabolism and Ethylene Formation in Etiolated Pea Stem Sections[1]

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ABSTRACT

Stem sections of etiolated pea seedlings (*Pisum sativum* L. cv. Alaska) were incubated overnight on tracer amounts of L-[U-^{14}C]methionine and, on the following morning, on 0.1 millimolar indoleacetic acid to induce ethylene formation. Following the overnight incubation, over 70% of the radioactivity in the soluble fraction was shown to be associated with S-methylmethionine (SMM). The specific radioactivity of the ethylene evolved closely paralleled that of carbon atoms 3 and 4 of methionine extracted from the tissue and was always higher than that determined for carbon atoms 3 and 4 of extracted SMM.

Overnight incubation of pea stem sections on 1 millimolar methionine enhanced indoleacetic acid-induced ethylene formation by 5 to 10%. Under the same conditions, 1 millimolar homocysteine thiolactone increased ethylene synthesis by 20 to 25%, while SMM within a concentration range of 0.1 to 10 millimolar did not influence ethylene production. When unlabeled methionine or homocysteine thiolactone was applied to stem sections which had been incubated overnight in L-[U-^{14}C]methionine, the specific radioactivity of the ethylene evolved was considerably lowered. Application of unlabeled SMM reduced the specific radioactivity of ethylene only slightly.

Tissues of higher plants produce ethylene when subjected to stress, during ripening and senescence or after application of auxin (for a review see ref. 1). Methionine has been shown to be the precursor of ethylene in a number of higher plants (for reviews see refs. 1 and 10). Recently, *S*-adenosylmethionine was proposed as an intermediate in the conversion of methionine to ethylene in apple tissue (3, 9). Results of Hanson and Kende (5) indicated that ethylene production in flower tissue of *Ipomoea tricolor* might be dependent on methionine derived from SMM[3] and homocysteine.

This investigation using etiolated pea stem sections was carried out to determine: (*a*) whether all ethylene evolved as a result of IAA treatment is derived from carbon atoms 3 and 4 of methionine; (*b*) whether SMM is a metabolite of methionine; and (*c*) whether induction of ethylene synthesis is accompanied by formation of methionine from SMM and homocysteine as found earlier in flower tissue of *Ipomoea tricolor* (5).

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[3] Abbreviation: SMM: *S*-methylmethionine.

MATERIALS AND METHODS

Plant Material and Conditions of Incubation. Seedlings of *Pisum sativum* L., cv. Alaska (Vaughan's Seed Co., Downers Grove, Ill.), were grown in the dark as described earlier (6). Stem sections (1.0 cm) were cut below the plumular hook in dim green light according to Lieberman and Kunishi (8). Incubation of plant material was performed as described earlier (6).

Determination of Ethylene. Ethylene production was measured by GC according to Hanson and Kende (5).

Plant Extraction. Stem sections were ground in 1 ml of 80% (v/v) ethanol for 1 min. The suspension was clarified by centrifugation at 1,000*g* for 5 min. The residue was reextracted twice more with 1 ml of 80% ethanol. The pooled extracts were evaporated to dryness at 40 to 50 C in a stream of N_2 and were redissolved in 0.2 ml water and either chromatographed immediately or stored under N_2 at −15 C until use.

Amino Acid Analysis. Methionine and SMM in extracts were determined with a modified Technicon autoanalyzer as described by Lamport (7).

Chromatography and High Voltage Electrophoresis. TLC of extracts was performed using precoated TLC plates (0.1 mm cellulose and 1-butanol-glacial acetic acid-water (60:15:25, v/v) as solvent. The chromatograms were run for 5 h at room temperature. High voltage thin layer electrophoresis was performed on cellulose plates with sodium acetate buffer (0.1 M, pH 4.5). A potential of 3 kv was applied for 8 min at a temperature of 6 C. Radioactivity was located on the plates with a Packard radiochromatogram scanner, model 7201 (Packard Instruments, Downers Grove, Ill.).

Measurement of Radioactivity. ^{14}C and ^{3}H in the soluble fraction of extracts, in ethanol-insoluble residues, and in zones of TLC plates were determined as described earlier (5). The specific radioactivities of ethylene evolved by stem sections and of carbon atoms 3 and 4 of methionine and SMM extracted from the tissue were determined according to Hanson and Kende (5), based on the method of Yang *et al.* (12).

Homocysteine-Methyltransferase from Jack Bean Meal. This enzyme was prepared from the soluble fraction of Jack bean meal by precipitation with ammonium sulfate between 35 and 45% saturation and by dialysis overnight against 0.01 M K-phosphate at pH 6.9 as described by Abrahamson and Shapiro (2). The 0.515-ml assay mixture contained 100 mM K-phosphate (pH 6.9), 1 mM $ZnSO_4$, 10 mM DTT, [^{14}C]SMM isolated from pea stem sections, 4 mM L-homocysteine, and 0.1 ml enzyme solution. L-Homocysteine was prepared just prior to use by adding 0.5 ml of cold 0.3 M NaOH to 40 μmol of L-homocysteine thiolactone; this solution was neutralized after 10 min with 0.5 ml 0.3 M KH_2PO_4. The assay mixture was incubated at 37 C for 1 and 10 h. The reaction was stopped by cooling in an ice bath. A 0.2-ml aliquot of the reaction mixture was placed on an ion exchange column (5 × 16 mm, 0.3 ml Dowex 50W-X8, 200–400 mesh, in the Li^+ form), and the [^{14}C]methionine formed during the reaction was eluted in

three washes of 0.2 ml distilled H_2O each. When the assay mixture was incubated without the enzyme, less than 5% of the radioactive substrate was eluted from the column.

Chemicals. L-[U-^{14}C]Methionine (250–260 mCi/mmol) was purchased from New England Nuclear, L-methionine from Nutritional Biochemical Corp. (Cleveland, Ohio), DL-methylmethionine sulfonium chloride (SMM) from United States Biochemical Corp. (Cleveland, Ohio), L-homocysteine thiolactone hydrochloride from Calbiochem, and IAA from Sigma.

RESULTS AND DISCUSSION

Identification of SMM. When stem sections were incubated overnight on tracer amounts of L-[U-^{14}C]methionine, over 70% of the radioactivity in the ethanol-soluble fraction was associated with one metabolite. The chromatographic and electrophoretic properties of this metabolite were identical to those of authentic SMM. Abrahamson and Shapiro (2) described a methyltransferase from Jack bean meal which catalyzed the transfer of the methyl group from SMM to homocysteine, resulting in the formation of methionine. The methyltransferase of Jack bean meal was partially purified, and the enzyme was used to test whether the unidentified, radioactive compound could act as a methyl donor. During incubation of the presumed [^{14}C]SMM with the Jack bean enzyme and homocysteine, a labeled compound was formed which was eluted from the ion exchange column with water and which co-chromatographed with authentic L-methionine (Table I). Therefore, the major ethanol-soluble metabolite of [^{14}C]methionine in pea stem sections appears to be SMM.

Metabolism of [^{14}C]Methionine. In rib segments excised from flower buds of *I. tricolor,* SMM was also the major ethanol-soluble metabolite of [U-^{14}C]methionine, with only a small portion of the radioactivity remaining in methionine. When the flower tissue underwent aging, radioactivity was lost from SMM and reappeared in methionine (5).

When pea stem sections were floated overnight on [U-^{14}C]-methionine, the distribution of soluble radioactivity between SMM and methionine was very similar to that found in *Ipomoea* (Table II). During IAA-induced ethylene synthesis, no net conversion of SMM to methionine could be detected. The radioactivity in methionine was reduced and the radioactivity in SMM continued to increase slowly. The incorporation of ^{14}C into the ethanol-insoluble fraction was 45 to 55% of the total ^{14}C in the tissue and did not change during the experiment (Table II).

Etiolated pea stem sections contained 837 nmol of SMM and 425 nmol methionine per g fresh weight as determined with an amino acid analyzer.

Specific Radioactivities of $^{14}C_2H_4$ and of C-3 and C-4 of Methionine and of SMM. The specific radioactivity of ethylene produced by senescent flower tissue of *I. tricolor* was very close to the specific radioactivity of C-3 and C-4 of extracted methionine (5). The specific radioactivity of C-3 and C-4 of SMM was always higher than that of methionine. For comparative purposes, it seemed worthwhile to determine the specific radioactivities of ethylene, methionine, and SMM in etiolated pea stem sections treated with IAA.

The specific radioactivities of ethylene and of C-3 and C-4 of methionine extracted from pea stem sections were very close (Fig. 1). Assuming that the applied [^{14}C]methionine was in equilibrium with the internal pool of free methionine, this indicates that all ethylene formed by IAA-treated pea stem sections was derived from C-3 and C-4 of methionine. Since the specific radioactivity of C-3 and C-4 of SMM was always lower than that of ethylene, it appears unlikely that SMM is a closer precursor of ethylene than methionine (Fig. 1). After 5.5 h of incubation in IAA, less than 0.5% of the methionine present in the sections was converted to ethylene.

Effect of Methionine, Homocysteine Thiolactone, and SMM on Ethylene Evolution. In some cases, added methionine was found to enhance ethylene synthesis in plant tissues (4). However, methionine had little effect upon ethylene production in flower tissue of *I. tricolor* (6) in which ethylene synthesis was enhanced and occurred prematurely in the presence of homocysteine thiolactone (5). This treatment also advanced aging of the flower tissue. In pea stem sections, ethylene production was, on the average, only slightly promoted by treatment with methionine (5–10%). Addition of homocysteine thiolactone to the incubation medium enhanced ethylene synthesis consistently by 20 to 25% while SMM

Table I. Formation of methionine from SMM-^{14}C and homocysteine by an enzyme preparation from Jack bean meal.

Substrates	Radioactivity in methionine (dpm)	
	1 h	8 h
SMM-^{14}C + L-homocysteine	2,400	5,813
SMM-^{14}C	99	137

SMM-^{14}C was obtained from pea seedlings. In each experiments, 14,166 dpm were added. The specific radioactivity of SMM was not determined.

Table II. Distribution of radioactivity in the ethanol-soluble fraction, in methionine, in SMM and in the ethanol-insoluble fraction.

Forty-eight pea stem sections were floated overnight on L-methionine-U-^{14}C (12 μM, 127.6 mCi/mmol). On the following morning, the sections were rinsed and 12 were taken for immediate extraction, TLC and determination of radioactivity. The remaining sections were placed in batches of 12 into three 25-ml Erlenmeyer flasks containing 0.1 mM IAA, and were analyzed after 2.5, 4, and 5.5 h.

Time h	Soluble fraction (dpm x 10^{-5})	Methionine (dpm x 10^{-5})	SMM (dpm x 10^{-5})	Insoluble fraction (dpm x 10^{-5})
0	5.11	1.30	3.71	5.63
2.5	5.96	0.92	4.91	5.45
4.0	6.01	0.84	5.10	5.56
5.5	5.95	0.62	5.21	5.81

did not increase ethylene formation (results not shown).

Reduction of the Specific Radioactivity of Ethylene by Unlabeled Methionine, Homocysteine Thiolactone, and SMM. Burg and Clagett (4) showed that unlabeled methionine applied to banana slices was highly effective in reducing the specific radioactivity of ethylene derived from [^{14}C]methionine. They concluded that methionine was a close precursor of ethylene. In our experiments with peas, the specific radioactivity of ethylene was reduced about equally when either methionine or homocysteine thiolactone was applied to IAA-treated stem sections which had been incubated overnight on [U-^{14}C]methionine (Table III). SMM was much less effective in reducing the specific radioactivity of ethylene (Table III). These data further indicate that SMM is not as close a precursor of ethylene as is methionine or homocysteine.

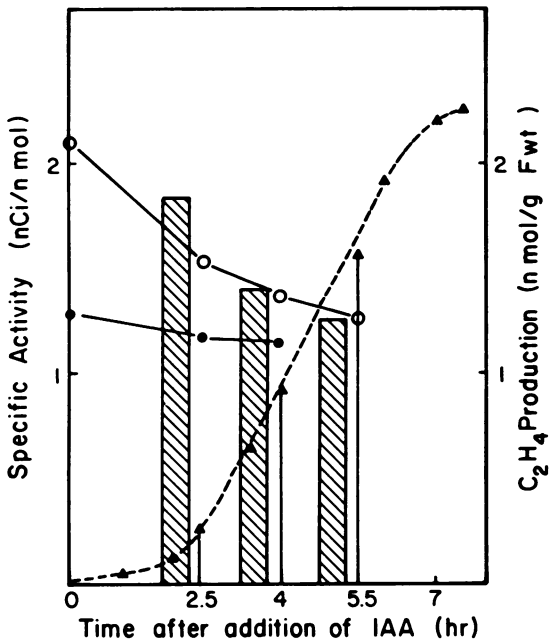

FIG. 1. Specific radioactivities of $^{14}C_2H_4$ and of C-3 + C-4 of methionine and SMM. Time course of ethylene production (▲- - -▲). The three collection periods are indicated by vertical lines, and the specific radioactivities of ethylene collected in each period are shown as bars. Specific radioactivity of carbon atoms 3 + 4 of methionine (○——○); specific radioactivity of carbon atoms 3 + 4 of SMM (●——●).

Dual Label Experiments with L-[methyl-^{3}H]Methionine and L-[U-^{14}C]Methionine. To test the possibility that SMM acts as a methyl donor for the biosynthesis of cell wall pectins and other polymers, stem segments were incubated for 3 h in a solution containing L-[methyl-^{3}H]methionine and L-[U-^{14}C]methionine (^{3}H/^{14}C = 9.2). After the labeling period, the segments were floated on a 0.1 mM solution of IAA, and the ratio of ^{3}H/^{14}C was determined in extracted SMM and in the ethanol-insoluble fraction after various periods of incubation (Table IV). Immediately after the 3 h of labeling, the ^{3}H/^{14}C ratio in SMM was only one-half of that in the incubation medium. During subsequent incubation, the ^{3}H/^{14}C ratio declined further, but at a decreasing rate. The ^{3}H/^{14}C ratio in the ethanol-insoluble fraction was always higher than that of SMM in the extract and in the incubation medium, suggesting that SMM was metabolically active and that it was serving as the methyl donor for ethanol-insoluble products.

CONCLUSIONS

The data represented in Figure 1 are fully consistent with the hypothesis that all of the ethylene evolved from IAA-treated pea stem segments originates from carbon atoms 3 and 4 of free methionine. Further evidence that methionine is an intermediate

Table III. Reduction of the specific radioactivity of ethylene by methionine, homocysteine thiolactone and SMM.

Forty-eight pea stem sections were floated overnight on L-methionine-U-^{14}C (12 μM, 127.6 mCi/mmol). After radioactive feeding, the stem sections were washed and distributed in batches of 12 to four 25-ml Erlenmeyer flasks containing 0.1 mM IAA. The respective unlabeled amino acids were added at 1 mM. The specific radioactivity of ethylene was determined after 4 h.

Unlabeled amino acid	Specific activity of ethylene (mCi/mmol)	% Decrease in specific activity of ethylene
-	1.54	-
L-methionine	0.43	72.1
L-homocysteine thiolactone	0.61	61.4
D,L-SMM	1.20	22.1

Table IV. ^{3}H/^{14}C Ratios in SMM and in the ethanol-insoluble fraction.

Forty-eight pea stem sections were incubated for 3 h on 12 μM L-methionine containing L-methionine-U-^{14}C (86.9 mCi/mmol) and L-methionine-methyl-^{3}H (799.4 mCi/mmol). The ^{3}H/^{14}C ratio of the medium was 9.2. Following incubation, the sections were washed, one batch of 12 was analyzed immediately, and the remaining sections were transferred to three 25-ml Erlenmeyer flasks. Samples were taken for extraction 2, 4, and 6 hr following incubation on labeled methionine.

Time of sampling h	^{3}H/^{14}C Ratio	
	SMM	Insoluble fraction
0	4.5	16.7
2	3.7	12.6
4	3.5	12.3
6	3.3	9.9

in ethylene biosynthesis can be derived from the dilution experiments (Table III). Methionine was most effective in reducing the specific radioactivity of ethylene but homocysteine thiolactone was almost as effective. Stimulation of ethylene synthesis was consistently higher with homocysteine thiolactone than with methionine. It is not known whether homocysteine can serve as a direct precursor of ethylene or whether it is converted to methionine prior to ethylene formation.

Our results with radioactive tracers show that SMM was the main soluble metabolite of methionine (Table II), just as in flower tissue of *I. tricolor* (5). However, the metabolism of SMM in pea stem sections was unlike that in *I. tricolor*. In peas, no net conversion of SMM to methionine was observed when ethylene synthesis occurred. The specific radioactivity of carbon atoms 3 and 4 of SMM was always lower than that of methionine and of ethylene (Fig. 1). SMM did not enhance ethylene evolution and was not very effective in reducing the specific radioactivity of ethylene (Table III). Provided that uptake of SMM into the cell is not limiting, these results indicate that SMM is not a close precursor of ethylene in IAA-treated pea stem sections. A similar conclusion has also been reached by Yang and Baur (11) using apple tissue.

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