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# Methionine Metabolism and Ethylene Biosynthesis in Senescent Flower Tissue of Morning-Glory[1]

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## ABSTRACT

**In immature rib segments prepared from morning-glory (*Ipomoea tricolor*) flower buds, the major soluble metabolite formed from tracer amounts of L-methionine-U-$^{14}$C was S-methylmethionine (SMM). In segments of senescing ribs, $^{14}$C was progressively lost from SMM and appeared in free methionine. Immature segments contained about 4 nmoles of free methionine and about 16 nmoles of SMM per 30 segments. As the segments senesced, the methionine content increased about 10-fold while the SMM content remained unchanged; during this time about 0.8 nmole of ethylene was produced per 30 segments. Tracer experiments with L-methionine-U-$^{14}$C, L-methionine-methyl-$^{3}$H, and L-homocysteine thiolactone-$^{35}$S indicated that SMM was capable of acting as a methyl donor, and that in senescent segments the methyl group was utilized for methionine production with homocysteine serving as methyl acceptor. Of the 2 molecules of methionine produced in this reaction, 1 was re-methylated to SMM, and the other contributed to the observed rise in the content of free methionine.**

**Internal pools of methionine and SMM were prelabeled (but not significantly expanded) by overnight incubation on 10 $\mu$M L-methionine-U-$^{14}$C. The specific radioactivity of the ethylene subsequently evolved during the senescence of the segments closely paralleled the specific radioactivity of carbon atoms 3 plus 4 of free methionine extracted from the tissue, demonstrating that methionine was the major precursor of ethylene in this system. The specific radioactivity of carbon atoms 3 plus 4 of extracted SMM was about twice that of the free methionine.**

**Based on these results, a scheme for methionine biosynthesis in senescent rib tissue is presented. The operation of this pathway in the control of ethylene production is discussed.**

Methionine has been shown to be a precursor of ethylene in a number of higher plants (for a review, see ref. 24) including climacteric tissues of apple, avocado, banana, and tomato (3, 4, 12, 14), and the metabolism of methionine in climacteric apple has been investigated (1, 2). Although there is good evidence that the total methionine content does not regulate the onset of ethylene production in ripening avocados and apples (2, 3), differences between immature (preclimacteric) and senescent (climacteric) tissues in the compartmentation and metabolism of methionine have not yet been studied. Such differences could be involved in the onset of ethylene production, in a fashion similar to the breakdown in "organization resistance," which has frequently been implicated in the enhanced respiratory metabolism of fruits entering the climacteric (for a review see ref. 19).

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Like ripening fruit, flowers of carnation, orchid, and morning-glory exhibit a sharp increase in ethylene production during senescence and respond to ethylene treatment by premature senescence and ethylene production (5, 8, 17). On the basis of work with morning-glory flowers, Kende and Baumgartner (8) proposed that the regulation of senescence and ethylene production by ethylene may function through an effect of ethylene on the compartmentation of the ethylene precursor: ethylene could act to increase the permeability of the tonoplast, favoring flow of a precursor of ethylene from the vacuole to a cytoplasmic ethylene-generating system. Support for an effect of ethylene on compartmentation came from studies on the effect of ethylene on the efflux of ions and sucrose from rib segments excised from flowers and buds of morning-glory (7). To further test the hypothesis of Kende and Baumgartner (8), we have used excised rib segments in which the pattern of ethylene evolution resembles that of intact flowers (9). We have established here that methionine is the precursor of ethylene in morning-glory flowers, and have compared the metabolism of methionine in immature and senescent flower tissue.

## MATERIALS AND METHODS

**Plant Material and Conditions of Incubation.** Plants of *Ipomoea tricolor* Cav. (cv. Heavenly Blue) were grown in an environmental chamber as described previously (7). Buds were harvested either 1 or 2 days before flower opening, usually between 4:00 and 7:00 PM, and were used for preparation of 10-mm rib segments (9).The abbreviations used in the text to describe the age of flower tissues are as follows: day 0, day of flower opening and fading; day −1, 1 day before flower opening; day −2, 2 days before flower opening. General features of the incubation conditions of rib segments are outlined below; specific details are supplied in the text and figure legends. After excision on day −1 or day −2, rib segments were incubated overnight in the growth chamber. Fresh weights for segments on day −2 were 7 to 8 mg, on day −1, 9 to 11 mg, and on day 0, 10 to 12 mg. Up to 60 segments were floated in a 6-cm Petri dish on 2 ml of 5 mM KCl (pH 5.5-6.2), to which radioactive methionine or homocysteine thiolactone was added in some experiments. The segments were usually removed from the incubation medium on the following morning between 7:30 and 8:30 AM, blotted gently, and washed to remove unabsorbed radioactivity by floating them for 30 min on 30 ml of 5 mM KCl in a Petri dish. The wash solution was stirred slowly with a Teflon-coated magnetic bar. Segments were blotted dry again and transferred to 50-ml Erlenmeyer flasks containing 10 ml of agar medium (5 mM KCl and 1% agar, w/v). Batches of up to 90 segments were placed in each flask. The flasks were closed with a serum cap and incubated in darkness at 27 C. Evolution of ethylene was measured by gas chromatography (9), and rolling up of segments was scored using angular measurements as described previously (7).

**Radiochemicals.** L-Methionine-U-$^{14}$C (260 mCi/mmole) was purchased from Schwarz/Mann and both L-methionine-methyl-$^{3}$H (11 Ci/mmole) and L-methionine-$^{35}$S (220 Ci/mmole) from New England Nuclear. Stock solutions were regularly checked for radiochemical purity and were repurified by TLC (solvent 2, below) if more than 10% methionine sulfoxide was detected. L-Homocysteine thiolactone-$^{35}$S (94.7 mCi/mmole) was prepared from L-methionine-$^{35}$S by treatment with HI (11): 95 $\mu$Ci of L-methionine-$^{35}$S stock was mixed with 1 $\mu$mole of carrier L-methionine in a total volume of 40 $\mu$l; 200 $\mu$l of HI (57% w/v) were added, and the mixture was incubated in a sealed vial under $N_2$ for 4 hr at 127 C. Conversion of methionine to homocysteine thiolactone and a trace of homocysteine was quantitative under these conditions. The reaction mixture was evaporated to dryness in a stream of $N_2$ at 40 to 50 C, and the homocysteine thiolactone was separated by TLC (solvent 1, below). The purity of the product was assessed by paper electrophoresis in sodium acetate buffer; a small amount of homocysteine (<10%) was present.

**Extraction Conditions.** Batches of 10 to 30 strips were ground with a little sand in 3 ml of 80% (v/v) ethanol for 3 min. This ethanol extract was passed through a 0.45 $\mu$m Millipore filter, and the residue was re-extracted twice more with 1 ml of 80% ethanol. The filtered extracts were pooled; in some experiments with $^{14}$C and $^{3}$H, the insoluble residue and the filter were retained for combustion. The pooled extracts were evaporated to dryness at 40 to 50 C in a stream of $N_2$ and were redissolved in 100 or 150 $\mu$l of water. This concentrated extract was then stored under $N_2$ at −15 C until use.

To ascertain whether methionine could be recovered quantitatively and without oxidation by this procedure, 0.25 $\mu$Ci (1 nmole) of L-methionine-U-$^{14}$C was added to a typical extract of unlabeled rib segments (containing about 25 nmoles of methionine) at the start of the extraction procedure. The final recovery of $^{14}$C was 89%, with less than 5% conversion of the tracer methionine to methionine sulfoxide. Methionine was oxidized slowly during prolonged storage of the extracts. Therefore, analysis of experiments was always completed within 3 days.

**Amino Acid Analysis.** The free amino acids of extracts were determined with a modified Technicon autoanalyzer (10); the retention time for methionine was about 170 min and for SMM[2] about 270 min. A volume of extract corresponding to 20 rib segments was used for each analysis. To check whether this technique accurately estimated the small amount of methionine present in extracts, 33 nmoles of authentic L-methionine were added to a sample of a typical extract that already contained 36 nmoles of methionine, as determined with the autoanalyzer. On analysis of the mixture, 94% recovery of the added methionine was obtained.

**Chromatography and Electrophoresis.** Thin layer chromatography of extracts was carried out with precoated TLC plates (0.1 mm MN-cellulose, Brinkmann Instruments, Inc., Westbury, N. Y.) using two solvents: 1-butanol-glacial acetic acid-water (60:15:25, v/v, solvent 1), and 1-butanol-acetone-diethylamine-water (30:30:6:15, v/v, solvent 2). Development times of 1.5 to 2 hr were used. Developed plates were occasionally stored under $N_2$ at −15 C before elution and processing of radioactive zones. For high voltage paper electrophoresis, acetate buffer (0.1 M, pH 4.5) or tris buffer (0.1 M, pH 7.6) was used with Whatman 3MM paper. A potential of 13 v/cm was applied for 2 to 4 hr, at an operating temperature of 6 C. Radioactivity was located on TLC plates and electrophoretograms with a Packard radiochromatogram scanner (model 7201) and by reference to authentic marker compounds.

**Measurement of Radioactivity.** Radioactivity in samples of extracts was determined with a Packard Tricarb scintillation spectrometer, using a dioxane-based scintillant (100 g of naphthalene, 5 g of PPO, 0.3 g of dimethyl POPOP, 730 ml of dioxane, 135 ml of toluene and 35 ml of methanol); efficiencies for $^{14}$C, $^{35}$S, and $^{3}$H were approximately 94%, 71%, and 36%, respectively. Ethanol-insoluble $^{14}$C and $^{3}$H in residues from extraction were determined following combustion in a Packard sample oxidizer (model 306). In experiments with $^{14}$C and $^{35}$S, radioactivity in zones of TLC plates was measured by cutting the plates into sections which were placed in the base of scintillation vials, cellulose face up. Ten ml of a toluene-based scintillant (4 g of PPO and 0.1 g of dimethyl POPOP/liter toluene) were added; counting efficiencies for this procedure were 79% for $^{14}$C and 61% for $^{35}$S. In dual label experiments with $^{3}$H and $^{14}$C, the two isotopes were determined separately following combustion of dried extract samples, insoluble residues, and zones of electrophoretograms in the Packard sample oxidizer. The efficiency for $^{14}$C counting by this method was about 60%, for $^{3}$H about 25%. In experiments where the specific radioactivity of $^{14}$C-ethylene evolved by senescent segments was determined, $^{14}CO_2$ was removed from the atmosphere of the incubation flask with 50 $\mu$l of trapping reagent (1 M NaOH saturated with $Ba(OH)_2$) on a filter paper wick. The $^{14}CO_2$ absorbed was measured by drying the wicks and placing them in 10 ml of the scintillant used for counting TLC sections.

**Purification of $^{14}$C-Ethylene and Determination of Its Specific Activity.** The $^{14}$C-ethylene evolved by senescing rib segments was withdrawn at intervals from the 50-ml incubation flask with 50-ml disposable syringes. The needle of a 50-ml syringe was pushed through the serum cap closing the flask, and the atmosphere of the flask was drawn into the syringe while ethylene-free air was introduced into the flask through a second needle. When the syringe was filled, it was removed, and its own needle was replaced with a serum cap. This procedure was repeated with a second 50-ml syringe. After gas samples were withdrawn, the incubation flask was opened, flushed with ethylene-free air, and resealed for further collection of $^{14}$C-ethylene. About 60% of the ethylene in the flask was usually recovered in the first syringe, about 30% in the second. The two samples were processed separately for determination of the specific radioactivity of $^{14}$C-ethylene as described below.

Two 1-ml samples were taken from each 50-ml syringe, and the concentration of ethylene was determined by gas chromatography as detailed previously (9). One-half ml of 0.1% (v/v) mixture of ethylene in air was added to each syringe, to give a final concentration of about 10 $\mu$l/l. The exact amount of carrier added was determined with two 1-ml samples, and 5 ml of ice-cold mercuric perchlorate solution (0.25 M $Hg(ClO_4)_2$ in 2 M $HClO_4$) were added. After a 10-min agitation of the syringe on a wrist-action shaker at 5 C, ethylene absorption was quantitative (>99.9%). The gases remaining in the syringe were expelled and replaced with ethylene-free air. The syringe was resealed with a serum cap. Ethylene was released from the mercuric complex by injecting 4 ml of 4 M LiCl solution followed by agitation of the syringe for 20 min at room temperature. Two 1-ml samples were taken to determine the recovery of ethylene, which was normally about 95%. The regenerated ethylene was then quantitatively transferred to an evacuated 60-ml Thunberg tube containing 1 ml of ice-cold 0.1 M mercuric acetate in methanol. The side arm of the Thunberg tube had previously been closed with a serum cap and a double-ended needle was used to connect the side arm and the syringe. The tube was agitated for 1.5 to 2 hr on a wrist-action shaker at 5 C, by which time over 99% of the ethylene was absorbed in the mercuric acetate solution. The solution was then mixed with 10 ml of a scintillant (100 g of naphthalene, 5 g of PPO, 0.3 g of dimethyl POPOP, 600 ml of dioxane, 135 ml of toluene, and 165 ml of methanol); counting efficiency for $^{14}$C was about 84%. In calculations of the specific radioactivity of $^{14}$C-ethylene, corrections were applied for the losses due to sampling and during the regeneration step with LiCl.

[2] Abbreviation: SMM: S-methylmethionine.

**Photochemical Production of $^{14}$C-Ethylene from Methionine and SMM.** The specific radioactivities of carbon atoms 3 plus 4 of methionine and SMM were determined using a microscale modification of the flavin-mononucleotide (FMN)-mediated light degradation of methionine described by Yang *et al.* (26). Because SMM is not itself an active substrate in this reaction, it had first to be converted to methionine by treatment with concentrated HCl (11).

Aliquots of extracts equivalent to five to six rib segments were applied to 5-cm wide TLC plates and developed in solvent 2. Radioactive zones corresponding to methionine ($R_F$ about 0.6–0.7) and SMM ($R_F$ about 0.02–0.18) were located by scanning and were scraped from the plate. Corresponding control zones from a blank TLC plate developed in solvent 2 were always prepared and processed with the radioactive methionine and SMM zones. The uneluted zone of methionine was used directly for ethylene generation. The SMM zone was eluted with 1.5 ml of 30% (v/v) ethanol; the eluate was then evaporated to dryness in a stream of $N_2$ at 40 to 50 C, redissolved in 40 $\mu$l of $H_2O$ and mixed with 200 $\mu$l of concentrated HCl. The mixture was heated to 110 C in a sealed vial under $N_2$ for 18 to 24 hr, dried again in a stream of $N_2$, and dissolved in 20 $\mu$l of $H_2O$. Under these conditions about 60% conversion of SMM to methionine was achieved with the quantities of SMM normally present (2–5 nmoles). The redissolved reaction mixture, containing some unreacted SMM, was then used for generation of ethylene.

For ethylene generation, the TLC zones of methionine or the SMM reaction mixtures were transferred to 10-ml disposable syringes and then 200 or 250 $\mu$l of 50 mM potassium phosphate buffer (pH 8.5) were added. The syringes were closed with serum caps and flushed thoroughly with $N_2$. To start the reaction, 10 $\mu$l of 10 mM FMN were injected through the serum cap; the syringes were incubated for 1 hr at room temperature on a light box (fluorescent light, $4 \times 10^3$ ergs cm$^{-2}$ sec$^{-1}$). Methionine standards (0.5–2 nmoles) gave a yield of ethylene of 45 to 55% using this procedure; this yield is similar to the value reported by Yang *et al.* (26) for $\mu$mole amounts of methionine. For methionine separated from extracts by TLC, overall ethylene yield was about 30%; for SMM separated by TLC and partially converted to methionine, the yield was about 10%.

The ethylene evolved from methionine zones, SMM reaction mixtures, and control samples containing blank TLC zones was transferred from the 10-ml syringes to 50-ml syringes via double-ended needles, and was then diluted to 50 ml with ethylene-free air. The specific radioactivity of the $^{14}$C-ethylene in these samples was determined exactly as described above for $^{14}$C-ethylene samples produced *in vivo* by senescing tissues. In calculating specific radioactivities, a correction was made for any ethylene produced in control samples that contained blank TLC zones only; such "background" ethylene never exceeded 10% of the concentration in the actual samples.

A number of tests that were made of the combined TLC separation and FMN-light system are summarized in Figure 1. Samples of an extract, each equivalent to six rib segments, were chromatographed either alone, with 2 nmoles of L-methionine added, or after $H_2O_2$ treatment. The TLC plates were divided into 2-cm zones which were then scraped from the backing material and reacted in the FMN-light system. It is clear from Figure 1 that only zones corresponding to the $R_F$ value of authentic methionine produced significant amounts of ethylene, and that following treatment with $H_2O_2$ no ethylene was produced from those zones. The $H_2O_2$-treatment oxidizes methionine to methionine sulfoxide, which is unreactive in the FMN-light system. To check for zones on the TLC plates capable of inhibiting the photochemical reaction, 1 nmole of authentic L-methionine was added to the scrapings from each zone (Fig. 1E). Although a broad inhibitory zone with maximum activity at $R_F$ 0.3 to 0.4 was apparent, it did not seem to complicate results with either methionine or the SMM reaction mixture.

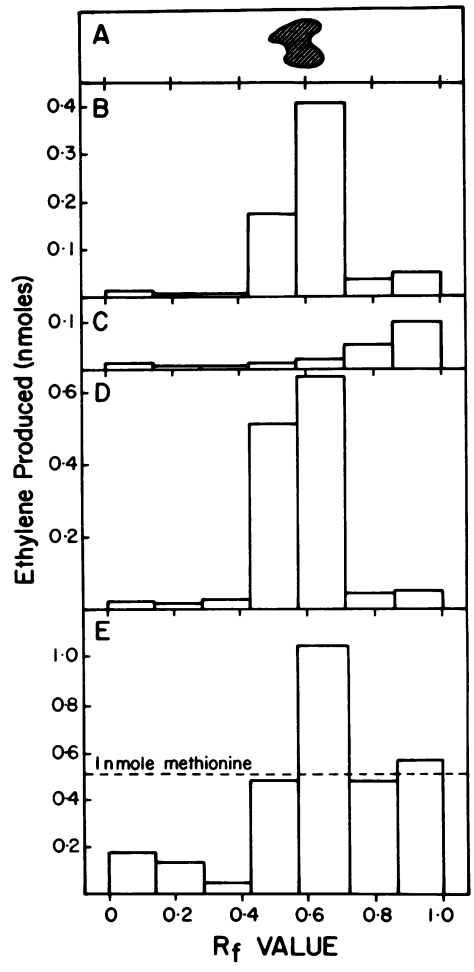



FIG. 1. Photochemical production of ethylene from TLC zones. All separations were of a volume of extract equivalent to six segments. A: Extract co-chromatographed with 20 $\mu$g of L-methionine and sprayed with ninhydrin; B: extract only; C: extract pretreated for 10 min at room temperature with an equal volume of 3% w/v $H_2O_2$; D: extract co-chromatographed with 2 nmoles of L-methionine; E: extract only, with addition of 1 nmole of L-methionine to each zone after chromatography; the horizontal broken line indicates the amount of ethylene evolved by 1 nmole of methionine alone.

## RESULTS AND DISCUSSION

**Identification of SMM and Methionine.** Following overnight exposure to tracer methionine, the principal metabolite of L-methionine-U-$^{14}$C and L-methionine-$^{35}$S that was detected in ethanol extracts of rib segments remained close to the origin in TLC with solvent 2 and moved with an $R_F$ of about 0.25 in solvent 1; the $R_F$ of methionine in both solvents was close to 0.65 (Figs. 2 and 3). The unknown metabolite, labeled with $^{14}$C or $^{35}$S, moved rapidly towards the cathode during paper electrophoresis at pH 4.5 and 7.6. Therefore, the unknown was a cationic sulfur compound of low mol wt. The unknown radioactive metabolite was isolated by preparative TLC with solvents 1 and 2 from extracts of segments exposed overnight to L-methionine-U-$^{14}$C, and was identified as SMM in the following manner. The $^{14}$C-labeled compound had the chromatographic and electrophoretic properties of SMM (bromide salt, Sigma), and the products of its chemical and enzymic degradation were identical to those of

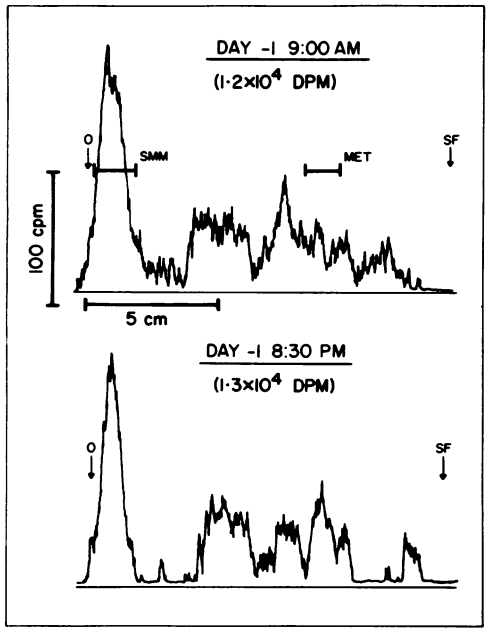



FIG. 2. Scans of radiochromatograms of segment extracts separated by TLC in solvent 2. Segments were excised on day −2, incubated overnight on 12 μM $^{14}$C-methionine and extracted on day −1 at once (9:00 AM) or after incubation on agar until 8:30 PM. The positions of the origin (O) and solvent front (SF) are marked with arrows, and the $R_f$ zones corresponding to SMM and methionine are indicated. The amount of radioactivity applied to each TLC plate is shown in parentheses.

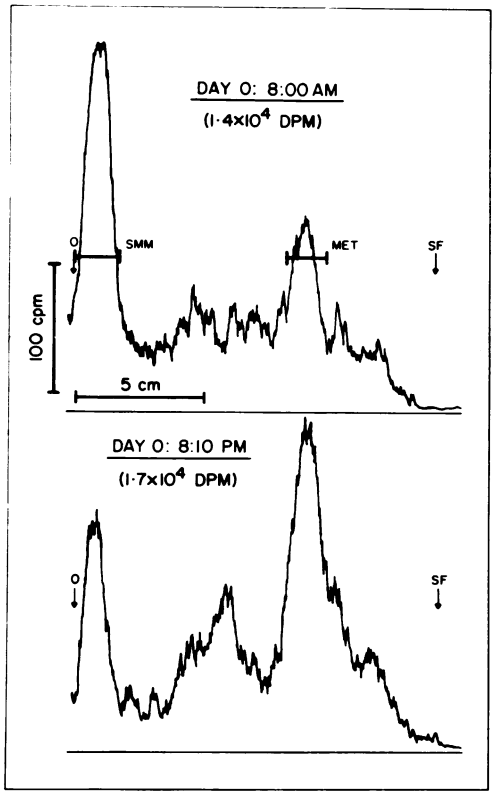
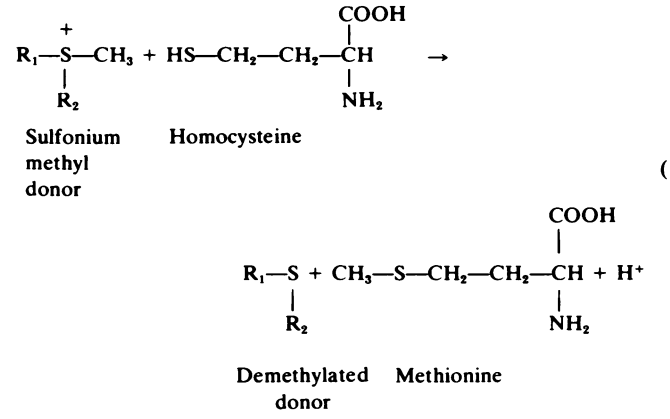



FIG. 3. Scans of radiochromatograms of segment extracts separated by TLC in solvent 2. Segments were excised on day −1, incubated overnight on 5 μM $^{14}$C-methionine and extracted at 8:00 AM and 8:10 PM on day 0. Otherwise as in Fig. 2.

carrier SMM (Table I). The presence of two methyl groups attached to the sulfur atom was established by the use of the radioactive unknown as a methyl donor in the reaction catalyzed by thetin-homocysteine transmethylase (EC 2.1.1.10):




If SMM were the sulfonium methyl donor, the products of demethylation and concomitant methylation would both be methionine, which would therefore be the only reaction product. Any other methyl donor would yield, in addition to methionine, some other radioactive compound as a result of demethylation. With radioactive unknown as methyl donor, only labeled methionine was formed in the reaction. This methionine formation was strictly dependent upon the presence of homocysteine (Table I).

The presence of $^{14}$C- or $^{35}$S-labeled methionine in reaction mixtures and tissue extracts was confirmed by co-chromatography with authentic L-methionine and by oxidation with 3% (w/v) $H_2O_2$, after which the radioactivity co-chromatographed with authentic methionine sulfoxide.

Labeled S-adenosylmethionine was detected in some experiments with $^{14}$C- and $^{35}$S-methionine, but the amounts formed were very small (<2% of the total ethanol-soluble radioactivity) and showed no relationship to the rate of ethylene production.

**Effect of Segment Age on Metabolism of $^{14}$C-Methionine.** When segments excised on day −2 and day −1 were floated overnight on tracer concentrations of L-methionine-U-$^{14}$C, the amounts of methionine taken up were small compared to the endogenous methionine content (<10%) and had no effect on the rolling up of, and ethylene production by, the segments.

Segments cut on day −2 and extracted in the morning of day −1 had incorporated about 60% of the total $^{14}$C absorbed into ethanol-insoluble products. The major labeled ethanol-soluble product was SMM; only a small proportion of the $^{14}$C was in free methionine. During subsequent incubation of these immature segments on agar, the distribution of $^{14}$C within the ethanol-soluble fraction did not change significantly (Figs. 2 and 4); further incorporation of $^{14}$C into the insoluble fraction did not occur, and about 2% of the total label was recovered in $^{14}CO_2$.

Segments cut on day −1 and extracted early in the morning of day 0 showed a pattern of distribution of $^{14}$C similar to that found in immature segments; about 45% of the $^{14}$C was in ethanol-insoluble form, and in the soluble fraction SMM contained more radioactivity than methionine. As these segments senesced during incubation on agar, $^{14}$C was lost from SMM and appeared in free methionine (Figs. 3 and 4). The proportion of $^{14}$C in the insoluble fraction did not change during senescence, and about 5% of the total label was released as $^{14}CO_2$.

SMM has been isolated from several plant tissues (16) and has been shown to be a major metabolite of exogenous $^{14}$C-methionine in seedlings of several species (6, 22). As the thetin-homocysteine transmethylase-catalyzing reaction 1 has also been identified in plant tissues (23), it is possible that SMM could

Table I. *Identification of S-Methylmethionine*

For the chemical degradations, about 2 nCi of radioactive unknown isolated by TLC was mixed with 50 to 200 μg of authentic SMM and treated in sealed vials under $N_2$, using the conditions shown. For the enzymatic degradation, thetin-homocysteine transmethylase was prepared from bakers' yeast by ethanol precipitation (21). Samples of radioactive unknown were mixed with authentic SMM and incubated in the methyl transfer reaction mixture (21) in the presence or absence of homocysteine.

| Treatment | Reference | Labelled Products | Yield (%) | Methods of Separation and Identification |
|---|---|---|---|---|
| pH 3, 121C, 2 hr | 16 | Homoserine and Homoserine lactone | >95 | TLC, solvents 1 and 2 |
| 1N $NH_4OH$, 100C, 3 hr | 6 | Homoserine | >95 | TLC, solvents 1 and 2 |
| 57% w/v HI, 127C, 5 hr | 11 | Homocysteine thiolactone | >95 | TLC, solvent 1 Electrophoresis, pH 4.5 |
| Conc. HCl, 110C, 24 hr | 11 | Methionine | 60 | TLC, solvents 1 and 2 |
| Thetin-homocysteine transmethylase reaction mix | 21 | | | |
| (a) without homocysteine | | None | -- | TLC, solvent 2 |
| (b) with homocysteine | | Methionine | 60 | |

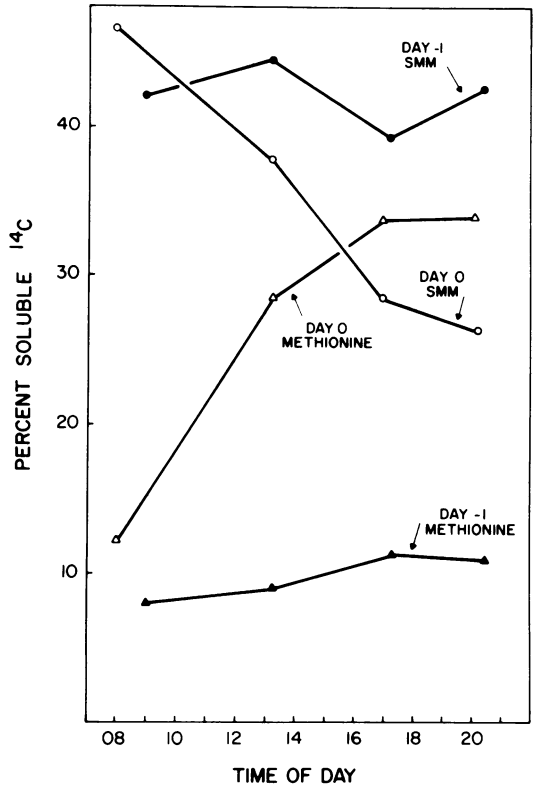
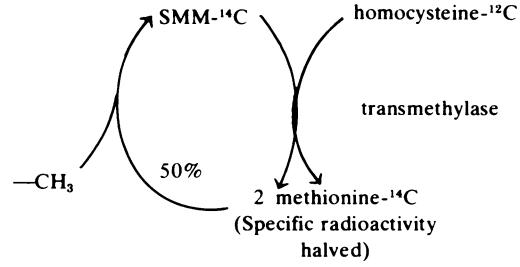



FIG. 4. Distribution of $^{14}C$ in the ethanol-soluble fractions of segments cut on day −2 and day −1, incubated overnight on $^{14}C$-methionine and extracted at various times during the following day. The concentration of $^{14}C$-methionine was 6 μM (day −1) and 12 μM (day −2); radioactivity distribution was determined by scintillation counting of zones cut from TLC plates developed with solvent 2.

function in plants like S-adenosylmethionine as a methyl donor. The observed changes in the pattern of $^{14}C$-labeling during the aging of the tissue could therefore reflect *(a)* the utilization of SMM as a methyl donor for methionine biosynthesis, *(b)* a simple degradation of SMM to methionine, or *(c)* a combination of these pathways. To investigate these possibilities, the concentrations of methionine and of SMM in senescing tissues were determined.

**Methionine and SMM Contents of Segments before and during Senescence.** As rolling up and ethylene evolution occurred, the levels of most free amino acids rose 2- to 4-fold; data for leucine, isoleucine, and valine are shown in Figure 5C. This general increase was probably due to the protein breakdown that occurs during the senescence of morning-glory flowers (15). The level of methionine, very low before the start of senescence (about 4 nmoles/30 segments), rose more than 10-fold as aging occurred while the SMM concentration remained fairly constant at about 16 nmoles/30 segments (Fig. 5B). Similar results were obtained in experiments in which the segments were exposed overnight to tracer concentrations of $^{14}C$-methionine.

The combined chemical and tracer data cannot be explained by a simple breakdown of SMM to methionine, with loss of one of the methyl groups. They can, however, be accounted for if SMM acts as a methyl donor for methionine synthesis from homocysteine, thus:




During aging, SMM labeled with $^{14}C$ by overnight incubation could donate one methyl group to unlabeled homocysteine, giving rise to 2 molecules of methionine. If only 1 of these were remethylated to SMM, the size of the SMM pool would remain constant, and the free methionine content of the tissue would increase by an amount equivalent to the size of the SMM pool for each turn of the cycle. At the same time, one-half of the $^{14}C$-label would be transferred from SMM to free methionine, assuming that the remethylation reaction does not discriminate between the 2 methionine product molecules. The operation of such a pathway, in addition to protein breakdown, could explain most of the large increase in the content of free methionine compared to the increases of other free amino acids, which are presumably derived mainly from proteolysis. The existence of this pathway was verified using homocysteine thiolactone-$^{35}S$, methionine-methyl-$^{3}H$, and methionine-U-$^{14}C$.

**Metabolism of Homocysteine Thiolactone-$^{35}S$ in Immature and Senescent Rib Segments.** If methionine biosynthesis in senescent segments occurs as shown in equation 2, three predictions can be made about the metabolism of tracer quantities of homocysteine: *(a)* labeled homocysteine should be converted to methionine, at least in senescent tissue; *(b)* a proportion of the methionine so formed should be further metabolized to SMM; *(c)* label in SMM synthesized by this route would be expected to be progressively transferred back to free methionine during

aging. Table II summarizes the results obtained by incubating segments overnight on 10 μM L-homocysteine thiolactone-$^{35}$S. Preliminary experiments showed that the segments were able to convert homocysteine thiolactone to homocysteine readily; no $^{35}$S label remaining as the thiolactone form was detectable in extracts. Homocysteine thiolactone was preferable to homocysteine, which autoxidized rapidly to homocystine in the incubation medium. It is clear from Table II that the metabolism of $^{35}$S in senescent segments follows the pattern predicted; it is also apparent that immature segments were able to convert homocysteine thiolactone to methionine and SMM. As the enzymes involved must therefore be present and active before senescence, some other factor, such as the availability of homocysteine, must limit the rate of methionine production by this route in immature tissue.

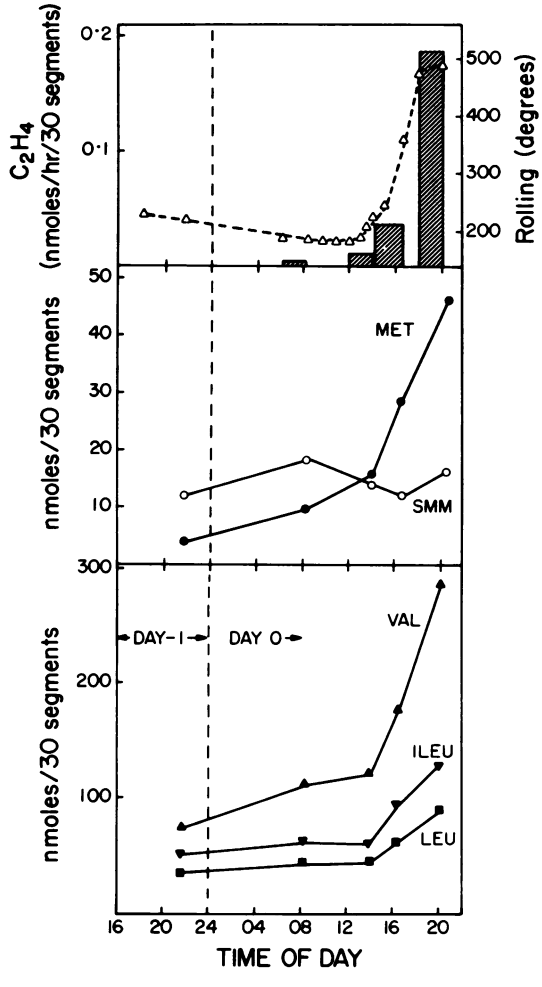



FIG. 5. Rolling up, rates of ethylene evolution, and levels of free amino acids in senescing rib segments. Batches of 30 segments were prepared between 4:00 and 6:00 PM on day −1 and incubated in 50-ml flasks on 10 ml of 1% w/v agar containing 5 mM KCl. Rolling up was scored continuously. At intervals the flasks were closed for 2 to 3 hr with serum caps to determine the rates of ethylene evolution, after which a sample of segments was taken for amino acid analysis. A: Rolling up (△-----△) and rate of ethylene production (histogram); B, C: contents of methionine (●), SMM (○), valine (▲), isoleucine (▼), and leucine (■) in rib segments. Similar results were obtained in two other experiments.

**Effects of Homocysteine Thiolactone and Methionine on Metabolism of $^{14}$C-SMM.** To test the possibility that the pathway of equation 2 is present in immature tissue but limited by the supply of homocysteine, an experiment with immature segments was performed in which a pulse of $^{14}$C-methionine was chased with excess unlabeled methionine or homocysteine thiolactone. In control segments incubated on 5 mM KCl after exposure to $^{14}$C-methionine, the proportions of $^{14}$C in methionine and in SMM remained constant (*cf.* Fig. 4). During incubation on unlabeled homocysteine thiolactone, a loss of $^{14}$C from SMM and a corresponding increase of label in methionine was observed (Fig. 6); these changes closely resembled those occurring in senescent tissues. This effect was much less marked after a chase with methionine.

The results of Figure 6 demonstrate that SMM can donate one of its methyl groups to homocysteine in immature tissue and are consistent with such a role for SMM in senescent tissue. They do not indicate whether SMM is normally metabolically inactive in immature tissue or whether it is an actively turning-over pool from which methyl groups are withdrawn by acceptors other than homocysteine.

**Dual Label Experiments with L-Methionine-Methyl-$^{3}$H and L-Methionine-U-$^{14}$C.** To investigate the fate of the methyl groups of SMM in immature and senescent tissues, segments were cut on day −2 and day −1 and exposed for 4 hr to a medium containing both L-methionine-U-$^{14}$C and L-methionine-methyl-$^{3}$H ($^{3}$H/$^{14}$C = 17.1). The ratio of $^{3}$H/$^{14}$C in extracted SMM was then determined after various periods of incubation of segments on agar (Fig. 7). In immature segments excised on day −2, the $^{3}$H/$^{14}$C ratio in extracted SMM immediately after feeding was already less than one-third of the ratio of the incubation medium. During subsequent incubation, the $^{3}$H/$^{14}$C ratio continued to fall, suggesting that SMM was metabolically active in immature tissue and that it was serving continuously as a methyl donor. In segments cut on day −1, the $^{3}$H/$^{14}$C ratio had also fallen after the labeling period, and declined further during subsequent incubation, although to a lesser extent than in segments cut on day −2. The relative stability of the $^{3}$H/$^{14}$C ratio during senescence might be expected if the major methyl acceptor were homocysteine. If the 2 molecules of methionine arising from the reaction between SMM and homocysteine were equally accessible to the remethylation step, the SMM resynthesized would have a $^{3}$H/$^{14}$C ratio equal to the average of those of the 2 methionine molecules. This

Table II. *Metabolism of L-Homocysteine Thiolactone-$^{35}$S in Immature and Senescent Rib Segments*

Segments were cut on day −2 and day −1, floated overnight on L-homocysteine thiolactone-$^{35}$S (10 μM, 94.7 mCi/mmole), and extracted in the morning and evening of the following day. Extracts were separated by TLC with solvent 1 and the distribution of radioactivity determined by scintillation counting. Methionine zones were checked for purity by TLC in solvent 2 and by $H_2O_2$ treatment; SMM zones were checked for purity by paper electrophoresis at pH 4.5.

| Excision of Segments | Segment Extraction | Soluble $^{35}$S (nCi/15 segments) | Distribution of $^{35}$S in Soluble Fraction (percent of total) | | |
|---|---|---|---|---|---|
| | | | Methionine | SMM | Homocysteine + Homocystine |
| Day -2 | Day -1, 10:15 AM | 190 | 8.2 | 39.4 | 26.8 |
| | Day -1, 8:30 PM | 205 | 10.2 | 43.9 | 25.0 |
| Day -1 | Day 0, 10:15 AM | 296 | 22.8 | 43.4 | 14.7 |
| | Day 0, 8:30 PM | 294 | 40.4 | 24.9 | 16.7 |

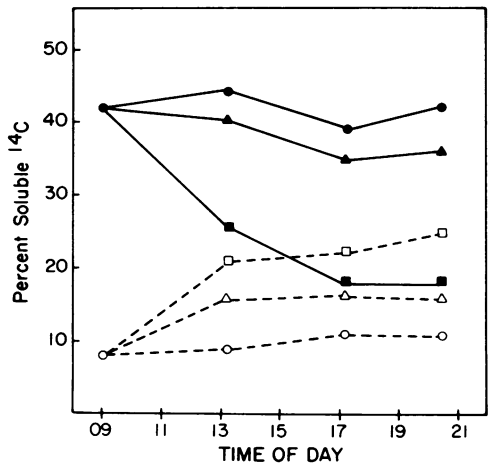



FIG. 6. Effect of chases with substrate concentrations of L-methionine and DL-homocysteine thiolactone on the metabolism of $^{14}$C-methionine and $^{14}$C-SMM. Rib segments were cut on day −2 and incubated overnight on L-methionine-U-$^{14}$C (12 μM, 41.9 mCi/mmole). One batch of segments was then transferred to 5 mM KCl solution (control), a second batch to a 1 mM solution of L-methionine in 5 mM KCl, and a third batch to a 1 mM solution of DL-homocysteine thiolactone in 5 mM KCl. Segments were extracted at various times and the per cent $^{14}$C distribution in the ethanol-soluble fraction determined by scintillation counting of TLC plates developed with solvent 2. O-----O: control, $^{14}$C-methionine; Δ-----Δ: methionine chase, $^{14}$C-methionine; □-----□: homocysteine thiolactone chase, $^{14}$C-methionine; ●——●: control, $^{14}$C-SMM; ▲——▲: methionine chase, $^{14}$C-SMM; ■——■: homocysteine thiolactone chase, $^{14}$C-SMM. The experiment was repeated once, with similar results.

average ratio would be the same as that for the SMM from which they were derived.

These results indicate that SMM acts in immature segments as a general methyl donor and that, as senescence proceeds during day 0, an increasing proportion of the methyl groups are accepted by homocysteine. The methyl-acceptor molecules of immature segments were not investigated in detail, but in preliminary experiments the $^3$H/$^{14}$C ratio of the ethanol-insoluble fraction was always higher than that of the incubation medium. This could be explained by a role of SMM in the methylation of cell wall pectins (20) and other ethanol-insoluble products.

**Specific Radioactivities of $^{14}C_2H_4$ and C3 plus C4 of Methionine and SMM.** To establish whether methionine is a precursor of ethylene in excised rib tissue, segments were incubated overnight on 9 μM L-methionine-U-$^{14}$C. The ethylene produced during senescence was trapped in three fractions, corresponding to that evolved as rolling up began, as rolling up occurred most rapidly, and as rolling up ceased. The specific radioactivities of these three fractions were compared to the activities of carbon atoms 3 plus 4 of both methionine and SMM extracted from the tissue as determined by the FMN procedure (Fig. 8). The specific radioactivity of ethylene fell during senescence, following almost exactly the specific radioactivity of C3 plus C4 of extracted methionine. This close agreement indicates that virtually all of the ethylene evolved as the segments senesced was derived from methionine. The production of $^{14}$C-ethylene occurred with a physiological concentration of methionine present in the tissue; comparison of the specific radioactivity of C3 plus C4 of the $^{14}$C-methionine fed (determined to be 66.6 nCi/nmole) with that of methionine extracted from the tissue (about 2 nCi/nmole) demonstrated that supply of $^{14}$C-methionine raised the endogenous methionine level only by about 3%.

It is also clear from Figure 8 that the specific radioactivity of C3 plus C4 of SMM was always about double that of methionine, which might be expected if methionine in senescing tissues arises from labeled SMM and unlabeled homocysteine (equation 2). Because the specific radioactivity of SMM was about 2-fold higher than that of methionine, it is very unlikely that SMM is a closer precursor of ethylene than methionine itself. Furthermore, Yang and Baur (25) have presented evidence suggesting that, in apple tissue, SMM is less effective as an ethylene precursor than is methionine.

The data of Figure 8 provide no indication of the existence of a discrete methionine pool available for ethylene synthesis, although they do not exclude this possibility, because such a pool could have equilibrated with other pools during the experiment. To investigate this possibility, segments were incubated overnight with tracer $^{14}$C-methionine and then exposed to a massive chase of unlabeled methionine just before ethylene production began (Table III). Preliminary experiments showed that such treatment did not significantly affect ethylene evolution, and that the resulting decrease in the specific radioactivity of free methionine was greater than that of SMM for several hours. The re-equilibration of $^{14}$C between methionine and SMM was therefore fairly slow, enabling sufficient ethylene for specific radioactivity measurement to be collected before re-equilibration was complete. Table III shows that the chase with methionine reduced the specific radioactivity of C3 plus C4 of methionine to 38% of the control value during the period of ethylene collection, whereas that of SMM was reduced to 57% of the control. Because the specific radioactivity of the ethylene evolved decreased only to 50% of the control value, it is clear that the methionine available for ethylene synthesis comprised a distinct fraction with relatively high specific radioactivity. Such a fraction could be a pool of methionine synthesized from labeled SMM and unlabeled homocysteine, and separated from methionine of exogenous origin by compartmentation. The results of the experiments presented below can be accounted for readily by this model.

**Ethylene Evolution and Control of Methionine Biosynthesis.**

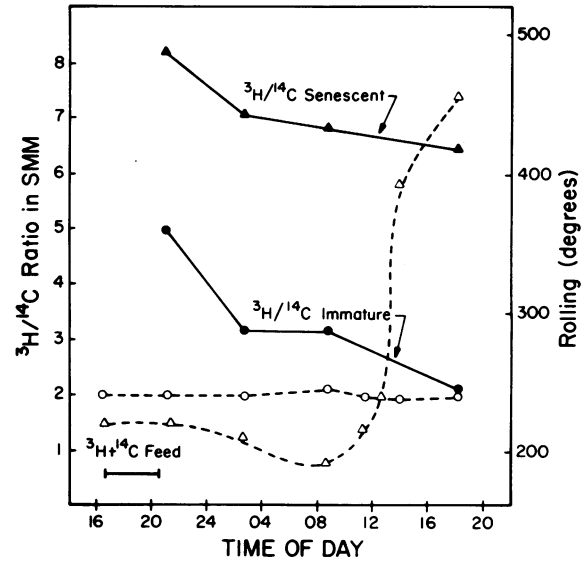



FIG. 7. $^3$H/$^{14}$C ratios in SMM extracted from immature and senescent rib segments; rolling up is shown for comparison. Segments cut on day −2 and day −1 were incubated for 4 hr on 11 μM methionine containing L-methionine-U-$^{14}$C (75.7 mCi/mmole) and L-methionine-methyl-$^3$H (1296 mCi/mmole); $^3$H/$^{14}$C ratio of medium = 17.1. Segments were then transferred to agar and incubated for up to 21 hr; at intervals samples were taken for extraction. SMM was separated by paper electrophoresis at pH 4.5 and the $^3$H/$^{14}$C ratio in SMM was determined after combustion in the sample oxidizer. O-----O: rolling up of −2 day segments; Δ-----Δ: rolling up of −1 day segments; ●——●: $^3$H/$^{14}$C ratio of SMM in −2 day segments; ▲——▲: $^3$H/$^{14}$C ratio of SMM in −1 day segments. The experiment was repeated, using a longer incubation with labeled methionine, with similar results.

Unlike in apple and avocado tissues (2, 3), there is sufficient free methionine in rib segments on the evening before senescence to account for the total amount of ethylene produced during aging (about 4 nmoles of methionine/30 segments and up to 0.8 nmole ethylene/30 segments produced over the experimental period, Figs. 5 and 8). Furthermore, because the free methionine content of segments increases about tenfold during senescence, only about 2% of the total free methionine is utilized for ethylene production. Two lines of evidence, however, suggest that the concentration of methionine available at the site of ethylene synthesis may be a rate-limiting factor in ethylene evolution, and that this concentration may depend mainly on endogenous methionine production from SMM and homocysteine.

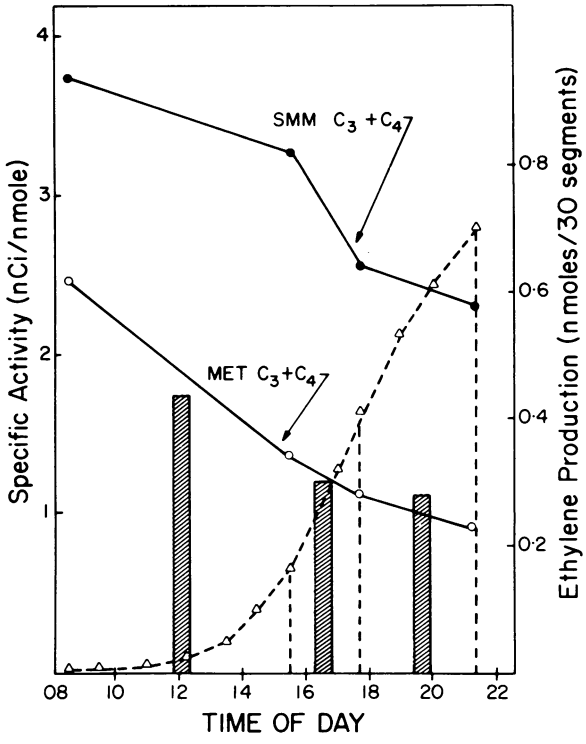



FIG. 8. Specific radioactivities of $^{14}C_2H_4$ and C3 + C4 of methionine and SMM during senescence. On day −1, 115 segments were cut and floated overnight on L-methionine-U-$^{14}$C (9 $\mu$M, 260 mCi/mmole). After incubation, 25 segments were taken for extraction and the remainder placed on agar in a 50-ml flask. The ethylene evolved during senescence was collected during three consecutive periods, and at the end of each period 30 segments were taken from the flask for extraction. Δ-----Δ: time course of ethylene production; the 3 collection periods are indicated by vertical broken lines, and the specific radioactivities of ethylene collected in each period are shown as bars; O——O: specific radioactivity of C3 + C4 of methionine; ●——●: specific radioactivity of C3 + C4 of SMM. Similar results were obtained in two further experiments.

First, overnight treatments of segments cut on day −1 with methionine concentrations up to 1 mM failed to hasten the onset, enhance the rate or increase the amount of ethylene production and had a slight retarding effect on rolling up of rib segments. When segments excised on day −1 were incubated overnight on $3 \times 10^{-4}$ M homocysteine thiolactone, both ethylene evolution and rolling up occurred prematurely. This effect was observed in five experiments, although its magnitude varied; representative data are shown in Figure 9. The results of Figure 6 demonstrate that an effect of incubation on homocysteine thiolactone was to increase methionine synthesis from SMM in immature segments. This effect was also observed in senescing segments (results not shown). In contrast to the results with segments excised on day −1, treatment with homocysteine thiolactone did not provoke ethylene production and rolling up in the immature segments of Figure 6. We ascribe the lack of ethylene production by immature tissue, even when rolling up occurs as a result of treatment with exogenous ethylene, to the inability of the segments to produce ethylene at this juvenile stage (9).

Second, we have previously reported that treatment with ethylene or propylene causes premature senescence (rolling up and

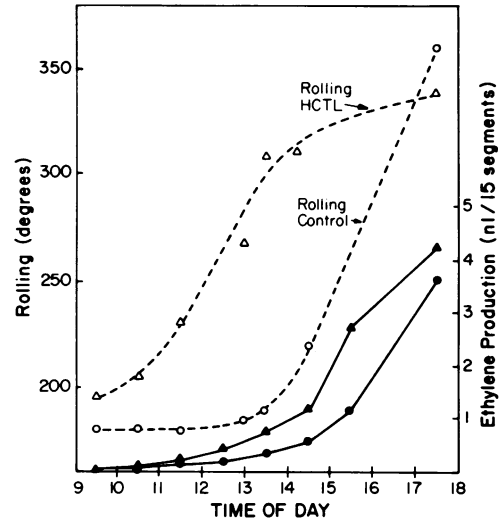



FIG. 9. Effect of homocysteine thiolactone on rolling up and ethylene evolution. Segments cut on day −1 were incubated overnight on a 5 mM solution of KCl (control) or a solution containing 5 mM KCl and 1 mM DL-homocysteine thiolactone (HCTL) and transferred to agar for measurement of rolling up and ethylene production. O-----O: rolling up of control segments; Δ----Δ: rolling up of HCTL-treated segments; ●——●: ethylene production by control segments; ▲——▲: ethylene production by HCTL-treated segments.

Table III. *Effect of a Methionine Chase on the Specific Activity of Ethylene and C3 + C4 of Methionine and SMM*

Two batches of 60 segments were cut on day −1, floated overnight on L-methionine-U-$^{14}$C (9 $\mu$M, 260 mCi/mmole), washed, and then transferred for 2 hr to either 5 mM KCl (control) or 5 mM KCl containing 1 mM L-methionine. Segments were then washed again. Twenty segments were taken at this time for extraction and the remainder placed on agar. After 4.7 hr, the ethylene evolved was collected and the remaining segments extracted. The values in parentheses show the specific radioactivities in the samples chased with methionine expressed as a percentage of the respective control values.

| Treatment | Total $C_2H_4$ evolved (nmoles/40 segments) | Specific Activities (nCi/nmole) | | |
|---|---|---|---|---|
| | | $C_2H_4$ evolved | C3+C4 methionine[1] | C3+C4 SMM[1] |
| Control | 0.352 | 1.45 | 1.47 | 3.83 |
| 1mM Methionine Chase | 0.416 | 0.72 (50%) | 0.56 (38%) | 2.20 (57%) |

[1]Values at the time when one-half of the ethylene had been evolved, calculated from the specific activities before and after the period of ethylene collection.

Table IV. *Effect of Ethylene Treatments on Methionine Metabolism in Immature Rib Segments*

Batches of 10 to 15 segments were cut on day −2 and floated overnight on L-methionine-U-$^{14}$C (14 $\mu$M, 72.2 mCi/mmole). One batch was then extracted; the remaining batches were transferred to agar, incubated in air (control) or air to which ethylene was added, and extracted at the end of day −1. The distribution of $^{14}$C in the ethanol-soluble fraction was determined by scintillation counting of zones cut from TLC plates developed with solvent 2.

| Experiment | Treatment | Time of Extraction | Rolling up (degrees) | Soluble $^{14}$C (nCi/10 segments) | % soluble $^{14}$C in methionine |
|---|---|---|---|---|---|
| 1 | None | 8:30 AM | 236 | 69.0 | 6.0 |
| | Air control | 8:30 PM | 228 | 61.5 | 8.2 |
| | Ethylene 10 $\mu$l/l | 8:30 PM | 550 | 62.2 | 13.3 |
| | Ethylene 100 $\mu$l/l | 8:30 PM | 476 | 60.0 | 13.2 |
| 2 | None | 9:30 AM | 198 | -- | 9.3 |
| | Air control | 8:30 PM | 203 | -- | 16.5 |
| | Ethylene 10 $\mu$l/l | 8:30 PM | 609 | -- | 27.0 |
| 3 | None | 9:30 AM | 200 | 44.9 | 7.6 |
| | Air control | 8:00 PM | 200 | 49.8 | 13.4 |
| | Ethylene 10 $\mu$l/l | 8:00 PM | 593 | 43.8 | 19.5 |

ethylene production) of rib segments (9). The data of Table IV show that treatment with ethylene also activated the synthesis of methionine from SMM in immature tissue. Inasmuch as the ethylene evolved during senescence was probably derived mainly from methionine synthesized in this way (Fig. 8 and Table III), it is tempting to speculate that the activation of methionine production from SMM brings about the stimulation of endogenous ethylene synthesis. Because the results of Figure 6 show that homocysteine availability limits methionine production in immature segments, ethylene treatment may enhance methionine production through an effect on homocysteine synthesis or compartmentation. We have previously shown (7) that ethylene treatment causes a breakdown of the compartmentation of inorganic ions, organic acids and sucrose in rib segments. It is therefore possible that ethylene stimulates methionine synthesis by increasing the flow of homocysteine to a compartment containing SMM and/or the transmethylase. If this compartment is also the site of ethylene synthesis, the increased availability of methionine might lead to a rise in the rate of ethylene production, explaining the phenomenon of ethylene-induced ethylene synthesis.

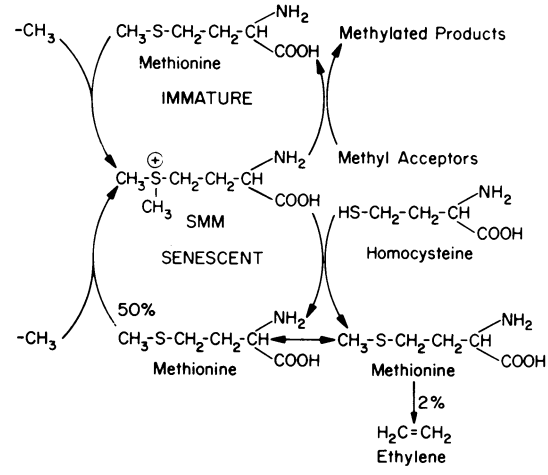



FIG. 10. Scheme of methionine metabolism in immature and senescent flower tissue of morning-glory.

## CONCLUSION

The experiments with radioactive tracers show that the methionine pathway is the main, if not the only, route of ethylene biosynthesis throughout senescence of morning-glory flower tissue. This conclusion is based on the comparison of the specific radioactivity of carbon atoms 3 plus 4 of endogenous methionine with that of ethylene evolved by the tissue. Our earlier results with the ethoxy analog of rhizobitoxine further support this conclusion; evolution of ethylene in aging rib segments was inhibited by over 99% (9). Rhizobitoxine and its analogs are thought to inhibit the utilization of methionine in the pathway of ethylene synthesis (13, 18).

In senescing segment tissue, the endogenous level of free methionine increases more than 10-fold, mainly due to a change in the catabolism of SMM; SMM synthesis from methionine by an uncharacterized methylation reaction is not affected. In aging tissue, SMM donates a methyl group to homocysteine to yield 2 molecules of methionine; this reaction may be restricted in immature tissues by the supply of homocysteine, the methyl group of SMM being utilized in other transmethylation reactions. Baur and Yang (1) have reported SMM as a minor metabolite of methionine-methyl-$^{14}$C in short (4 hr) experiments with ripe apple tissue, but did not establish its chemical concentration and its relationship to methionine synthesis. Our present data indicate that a re-evaluation of the importance of SMM in apples and other climacteric fruits would be of interest.

To explain the observed changes in the metabolism of methionine and their role in the synthesis of ethylene, we suggest the following working hypothesis. Ethylene production is dependent on methionine derived from SMM and homocysteine, possibly because ethylene-generating and methionine-synthesizing systems are located in the same cellular compartment. During aging, the supply of homocysteine to this system increases, either through direct stimulation of homocysteine production or due to a breakdown in the compartmentation between stored homocysteine and the methionine-synthesizing system. Ethylene treatment can provoke a premature increase in homocysteine availability and thus lead to earlier ethylene production. This hypothesis is represented schematically in Figure 10.

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