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Regulation of Ethylene Biosynthesis in Virus-Infected Tobacco Leaves[1]

I. DETERMINATION OF THE ROLE OF METHIONINE AS THE PRECURSOR OF ETHYLENE

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ABSTRACT

The hypersensitive reaction of Samsun NN tobacco leaves to tobacco mosaic virus (TMV) was accompanied by a large increase in ethylene production, just before necrotic local lesions became visible. Normal and virus-induced ethylene production were both largely inhibited by 0.1 millimolar aminoethoxyvinylglycine indicating that methionine is a main ethylene precursor.

The contribution of methionine to ethylene production was estimated by labeling leaves with L-[U-^{14}C]methionine and comparing the specific activities of methionine within and ethylene produced by the leaf. When taken up through the petiole, methionine was largely retained in the veins, leading to production of ethylene with a far higher specific activity in the veins than in the interveinal tissue. After TMV infection, ethylene production increased only in the interveinal tissue, resulting in a decrease in specific activity of the ethylene produced. In the interveinal tissue, the specific radioactivity of the ethylene was lower than expected if methionine were the only precursor. After labeling by vacuum infiltration, the specific activities of the ethylene produced by water- and TMV-inoculated leaves were both identical and in accordance with the specific radioactivity of methionine. Inasmuch as the content of 1-aminocyclopropane-1-carboxylic acid was increased severalfold two days after TMV infection, methionine can be considered to be the only ethylene precursor in healthy and in TMV-infected tobacco leaves.

The increase in ethylene production after TMV-infection was not accompanied by an increased concentration of free methionine within the leaf. Compartmentation of methionine does not appear to be a regulating factor since labeled methionine supplied to the leaf by vacuum infiltration is equilibrated very rapidly with any methionine pool within the leaf cells.

Tobacco cultivars carrying the *N* gene react hypersensitively to TMV[2] (9). Multiplication and spread of the virus are restricted to a zone around the infection site which rapidly necroses in about 48 h. The process is terminated 5 to 8 days later when the slow expansion of these local lesions comes to a halt.

Lesion development is accompanied by a large burst of ethylene emanation (18, 19) which reaches a maximum near the time that lesions become visible, and remains elevated during subsequent lesion growth. Ethylene production does not increase in tobacco leaves infected with viruses that invade the plant systemically and do not cause necrosis (6, 17). The ethylene generated when Samsun NN tobacco reacts hypersensitively to TMV may be responsible for all ensuing physiological and biochemical changes, including the so called "systemic acquired resistance" against further virus infection (22).

Methionine is a main precursor of ethylene in higher plants (14, 24), although additional precursors and pathways may occur in specific situations, *e.g.* fruit ripening (13). Abeles and Abeles (1) found that application of toxic chemicals increased ethylene evolution from tobacco leaves and also increased the conversion of [U-^{14}C]methionine into ethylene. In a pathological situation such as a virus-induced hypersensitive reaction, other precursors might likewise be envisaged. Kato (11) considers that in cowpea reacting hypersensitively to cucumber mosaic virus, ethylene is formed by peroxidation of linolenic acid. By labeling Morning Glory flower tissue with L-[U-^{14}C]methionine, Hanson and Kende (7) demonstrated that all the ethylene evolving during flower senescence was derived from methionine. Such ethylene synthesis is blocked by treatment with AVG, which specifically inhibits the conversion of *S*-adenosylmethionine into ACC, the immediate precursor of ethylene (3, 14). These approaches were adopted to investigate: (a) whether methionine is the only precursor of ethylene in healthy tobacco leaves and (b) as to how far methionine is the precursor of the ethylene emanated in tobacco reacting hypersensitively to TMV.

MATERIALS AND METHODS

Plant Material. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown from seed in a greenhouse; photoperiod was maintained at 16 h by additional illumination from high pressure mercury halide Philips HPI/T lamps at 18 w·m^{-2}. Minimum temperature was 20 C during the day and 19 C at night. When plants were 10- to 11-weeks-old, almost fully-grown leaves, about 18 cm in length, were selected.

In most cases leaves were inoculated on the plant with either TMV or water as a control. Inoculation was carried out by rubbing carborundum-dusted leaves with water or purified TMV WU 1 (100 μg/ml) using a gauze pad, and rinsing with water. The day of inoculation is further referred to as day 0. At regular intervals leaves were detached from the plants, and incubated in growth chambers at 20 C (21).

Labeling of Leaves. Leaves were labeled by either vacuum infiltration or uptake through the petiole. Leaves were infiltrated for 30 s at 1 mm Hg with an aqueous solution of L-[U-^{14}C]-

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[2] Abbreviations: TMV, tobacco mosaic virus; AVG, aminoethoxyvinylglycine; ACC, 1-aminocyclopropane-1-carboxylic acid.

methionine. Labeling through the petiole was carried out by putting a droplet containing the desired amount of methionine on the cut surface of the petiole. After uptake, the leaves were placed in 5 mM KCl for 20 h to avoid excessive adsorption of the labeled amino acid to the conducting vessels (8).

Extraction of Free Amino Acids and Amino Acid Analysis. Preparation of samples for amino acid analysis was carried out by freezing the leaf material in liquid N_2, grinding it in a mortar with pestle, and extracting the resulting powder two times for 30 min with water or 70% methanol. The extracts were clarified by centrifugation at 30,000*g* for 5 min. The combined supernatants were taken to dryness by rotary evaporation at 30 C. The resulting residue was dissolved in 2 ml 0.2 M sodium citrate buffer (pH 2.2) containing 1% thiodiglycol, 0.2% Brij, and 0.01% caprylic acid. After clarification at 30,000*g* for 5 min, the samples were used for amino acid analysis. Recovery of methionine in the final extract was always over 95%, as determined by adding [^{14}C]methionine as an internal standard.

One-half or 1 ml of the amino acid extract was used for amino acid analysis. Analysis was performed with a Beckman amino acid auto-analyzer (type 4255) using sodium citrate buffers of pH 3.22 and 4.25. The retention time for methionine was 84 min. The methionine concentration in the eluate was determined by ninhydrin reagent. Its specific radioactivity was determined by liquid scintillation counting of the methionine-containing fraction. Corrections for counting efficiency were made using an external standard.

Measurement of Ethylene Production. Detached leaves were incubated in water-locked 750 ml Petri-dishes on wetted filter paper under controlled conditions (21). To study the effect of AVG, leaves were cut parallel to the main vein into strips of about 1 cm wide, and incubated on either water (control) or 0.1 mM AVG. At specific times, 1-ml gas samples were withdrawn through a sealed hole in the lid and injected into a gas chromatograph equipped with an alumina column and a flame ionization detector.

To determine the specific radioactivity of ethylene produced by ^{14}C-labeled leaves, a small dish, containing 2 ml 0.25 M mercuric perchlorate in 2 M $HClO_4$, was placed in the Petri dish next to the leaf to absorb the ethylene (2). Efficiency of ethylene trapping was more than 95%. No effect of the presence of mercuric perchlorate on ethylene production or lesion development was noticed. After incubation, radioactivity in 1 ml of the trapping solution was determined by liquid scintillation counting in Lumagel (Lumac Chemicals AG). The other half of the mercuric perchlorate solution was injected into a 40-ml sealed serum flask containing 1 ml of 4 M LiCl. The flask was firmly shaken for 1 min during which time the ethylene was quantitatively released (more than 98%). Two 1-ml gas samples were taken from the flask to determine the ethylene concentration. After the ethylene had been released, the radioactivity of the mercuric perchlorate/LiCl mixture was also counted. This radioactivity consists of CO_2 and other gaseous metabolites that remain bound to mercuric perchlorate. More than 85% of the radioactivity released from the mercuric perchlorate by the addition of LiCl was absorbed by a solution of 0.1 M mercuric acetate in methanol, providing proof that this radioactivity is in ethylene (2).

The specific radioactivity of ethylene was then calculated according to the following formula:

$$\text{Specific radioactivity ethylene} = \frac{\text{Radioactivity } (Hg(ClO_4)_2) - \text{radioactivity } (Hg(ClO_4)_2/LiCl) \text{ mixture}}{\text{Ethylene production}}$$

ACC Assay. Leaf material was frozen in liquid N_2 and ground in a mortar with pestle. Two ml of 5% sulfosalicylic acid were added per g fresh weight and the mixture was stirred for 30 min at room temperature. After centrifugation of the homogenate for 10 min at 10,000*g*, the concentration of ACC in the supernatant was determined by chemical conversion into ethylene according to Lizada and Yang (15).

Chemicals. L-[U-^{14}C]methionine (285 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, England). AVG was from Dr. R. Maag AG, Dielsdorf, Switzerland.

RESULTS AND DISCUSSION

Ethylene Production During Pathogenesis and Effect of AVG on Ethylene Production. Figure 1A shows that inoculation of hypersensitively reacting tobacco leaves with TMV gave rise to a large burst of ethylene just before lesions became macroscopically visible. Maximal production was usually attained between 1 and 2 days after inoculation and was proportional to the number of developing lesions. Ethylene production remained elevated for the rest of the incubation period during which the lesions slowly enlarged. In contrast, ethylene production of water-inoculated leaves remained low during the whole incubation period. The elevated production during the first hours of incubation was caused by wound ethylene produced as a result of the inoculation procedure.

AVG, recently shown to inhibit ethylene production in tobacco leaf discs (4), inhibited ethylene production for more than 75% in water-inoculated, and for about 95% in TMV-inoculated leaves (Fig. 1B). Methionine appears to be the main precursor of ethylene in uninfected as well as in hypersensitively reacting tobacco leaves. AVG did not inhibit lesion formation, but interfered with resulting metabolic alterations. This agrees with the conclusion by Van Loon (23) that ethylene is not responsible for the induction of local lesions but is a causative factor in redirecting plant metabolism during the hypersensitive reaction.

Labeling by Petiolar Uptake. The role of methionine as an ethylene precursor was further studied by labeling leaves with L-[U-^{14}C]methionine and comparing the specific radioactivities of the methionine pool within the leaf and the ethylene produced. Since only two of the five C-atoms of methionine end up in ethylene, the specific radioactivity of ethylene is expected to be 0.4 times that of the methionine if methionine is the only ethylene precursor. If other compounds also serve as a precursor, the specific radioactivity of the ethylene would be correspondingly lower.

Initially, leaves were labeled by uptake through the petiole. After uptake, radioactive ethylene was produced. Such production was completely suppressed by simultaneous feeding of 0.1 mM AVG, confirming that methionine is the main precursor of ethylene in tobacco.

When leaves were labeled 1 day before inoculation with either

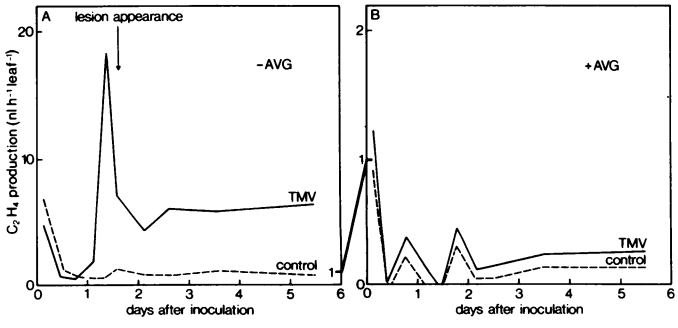

FIG. 1. Ethylene production of Samsun NN tobacco leaves incubated on water (A) or 0.1 mM AVG (B) after inoculation with water (- - -) or TMV (——). Leaf strips were incubated in water locked Petri dishes and ethylene production was measured at 8-h intervals during the first 2 days and at longer intervals during the subsequent period.

water or TMV, the specific radioactivity of the wound ethylene produced during the first day after inoculation was similar in both cases (Fig. 2). In water-inoculated leaves, the return of the ethylene production to a basal level was accompanied by a large increase in specific radioactivity. On the contrary, in TMV-infected leaves, the enhanced ethylene production by the time of lesion appearance was accompanied by a corresponding decrease in specific radioactivity of the ethylene. In both water- and TMV-inoculated leaves the specific activity of the ethylene declined at the same rate during the subsequent days, apparently due to an exhaustion of the label from the methionine pool. Taking into account the amount of labeled methionine taken up by the leaf (1 μCi = about 3.5 nmol) and the pool size of methionine within the leaf (about 100 nmol), the amount of label recovered in ethylene was only about 1% of that expected if all of the applied methionine were available for ethylene production. From autoradiography and extraction it became clear that most of the methionine taken up was held back in the veins, more than 50% of which was irreversibly bound to the cell walls, whereas the free radioactive methionine was quickly metabolized (*cf.* Table II).

Leaves were further labeled some hours after inoculation, and incubated during the subsequent day when local lesions became visible. Since changes in the methionine pool were found to be minor under these conditions, the specific radioactivity of methionine was calculated as the average of the specific radioactivities at the beginning and at the end of the period during which the ethylene was trapped. No differences were found between the specific radioactivities of methionine in water- or TMV-inoculated leaves. The specific radioactivity of the ethylene produced by water-inoculated leaves was slightly higher than expected on the basis of the specific radioactivity of methionine in the leaf. The specific radioactivity of the ethylene produced by TMV-inoculated leaves was far less (Table I).

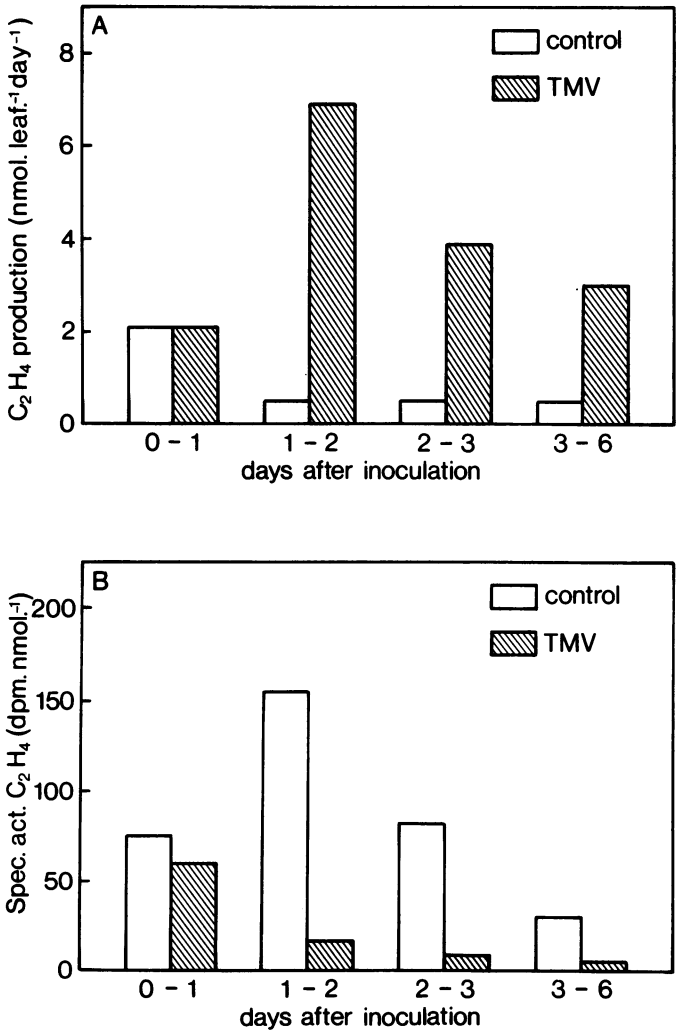

FIG. 2. Comparison of (A) the production and (B) the specific radioactivity of the ethylene produced by tobacco leaves inoculated with water or TMV 1 day after labeling with 1 μCi L-[U-^{14}C]methionine.

After TMV infection, lesions develop only in the interveinal areas and ethylene is produced mainly by the tissue surrounding the local lesions. Therefore the contributions of the main veins and the interveinal tissue to the production of [^{14}C]ethylene were compared after physical separation, immediately following labeling, by cutting out the interveinal tissue alongside the main veins. For water-inoculated leaves, amino acid analysis of extracts from either the vein parts or the interveinal tissue revealed that the concentration of methionine within the veins was two to three times lower than in the interveinal areas. Since most of the labeled methionine taken up was held back in the veins, the specific radioactivity in the veins was 15 to 30 times higher than in the interveinal tissue (Table I). The veins contributed to the ethylene production of the leaf for about one-third. The specific radioactivity of this ethylene was about 50 times higher than that from the interveinal tissue. The specific radioactivity of the ethylene produced by the veins was as expected for methionine being the sole precursor, that of the interveinal tissue was substantially less.

After TMV infection, ethylene production by the veins hardly changed, but ethylene production in the interveinal tissue increased severalfold, reducing the contribution of the veins to only 5 to 10%. As in uninfected leaves, the specific radioactivity in the vein parts was always far higher than in the interveinal tissue. The specific radioactivities of the ethylene produced by either the veins or the interveinal tissue were similar to those of uninfected leaves.

Table I. *Comparison of the Specific Radioactivities of the Methionine Pools Within, and the Ethylene Produced by the Whole Leaf, the Main Veins and the Interveinal Tissue, Respectively*

Leaves were labeled with L-[U-^{14}C]methionine by uptake through the petiole. Specific radioactivity of methionine was calculated as the mean between the specific radioactivities at the beginning and at the end of the incubation period. Ethylene was trapped during a 24-h period when local lesions became visible.

Material		H_2O-Inoculated	TMV-Inoculated
Whole leaf	SA[a] methionine, dpm·nmol^{-1}	240	245
	Expected SA C_2H_4, dpm·nmol^{-1}[b]	96	98
	C_2H_4 production, nmol·leaf^{-1}	1.2	30.0
	SA C_2H_4, dpm·nmol^{-1}	119	16.3
Main veins	SA methionine, dpm·nmol^{-1}	945	1504
	Expected SA C_2H_4 dpm·nmol^{-1}	379	602
	C_2H_4 production, nmol·leaf^{-1}	0.4	1.2
	SA C_2H_4, dpm·nmol^{-1}	349	238
Interveinal tissue	SA methionine, dpm·nmol^{-1}	64	55
	Expected SA C_2H_4, dpm·nmol^{-1}	25.7	22
	C_2H_4 production, nmol·leaf^{-1}	0.81	28.8
	SA C_2H_4, dpm·nmol^{-1}	7.9	7.4

[a] SA, specific radioactivity.

[b] The specific radioactivity of ethylene is expected to be 0.4 times that of methionine.

The decrease in the specific radioactivity of the ethylene produced by the whole leaf after TMV infection, seen in Figure 2 and Table I, can thus be explained by the fact that a far larger part of the ethylene is derived from the interveinal tissue, which produces ethylene with a relatively low specific radioactivity. The same situation appears to hold for the wound ethylene produced immediately upon inoculation (Fig. 2).

Thus, nonhomogeneous distribution of a label taken up through the petiole may give anomalous results with respect to the specific radioactivity of its product. This especially holds for situations in which a treatment such as localized wounding or TMV infection results in a nonhomogeneous stimulation of the reaction, such as the locally increased ethylene production.

The observation that the specific radioactivity of the ethylene produced by the interveinal tissue was substantially less than expected could still result from a nonhomogeneous distribution of the labeled methionine. Since the specific radioactivities in water- and TMV-inoculated leaves are the same, the pathways of ethylene production must be the same in healthy and virus-infected leaves. Whether methionine is the only precursor cannot be assessed on the basis of these results alone. The possibility that fatty acids can function as additional precursors was investigated by labeling leaves with 5 μCi [^{14}C]acetate. Under these circumstances [^{14}C]acetate was rapidly incorporated into lipids, as evidenced by extraction according to Roughan and Batt (20), but no radioactive ethylene was produced during the subsequent 7 days.

Labeling by Vacuum Infiltration. To circumvent unequal distribution of the labeled methionine, leaves were labeled by vacuum infiltration, although this treatment delayed lesion appearance by about 10 h. Leaves were inoculated on the plant and detached just before lesion appearance when ethylene production was rising to its maximum. The midrib was removed, leaf halves were infiltrated with [^{14}C]methionine solution, and ethylene was trapped during the subsequent 3 h. The specific radioactivities of methionine were similar in both water- and TMV-inoculated leaves and decreased rapidly during this period. No differences were found between the specific radioactivities of the ethylene produced by the water or TMV-inoculated leaves (Table II). Although the rapid decrease in specific radioactivity of the methionine pool makes precise determination impossible, the specific radioactivities of the ethylene can be considered in reasonable agreement with those expected if methionine were the only precursor, because the specific radioactivity of ethylene also decreased rapidly during incubation (Fig. 3).

When TMV-inoculated leaves were vacuum-infiltrated with labeled methionine after a 24-h preincubation in 0.1 mM AVG, ethylene production during the subsequent 5 h was inhibited for 98%. The specific radioactivity of the ethylene produced by these leaves was similar to that of controls preincubated in water. It can be concluded that the small amount of ethylene evolved in the presence of AVG is also derived from methionine. This indicates that methionine is the only precursor of ethylene in tobacco leaves.

Radioactive ethylene was produced without a lag phase (Fig. 3). Hence, a physical barrier for methionine between the intercellular space and the site of ethylene production is not apparent. This agrees with the possibility that (part of) the ethylene-producing enzyme system(s) is located at the plasmalemma (14, 16). Since the specific radioactivity of the ethylene produced during the first 3 h after vacuum infiltration was already in accordance with that of the methionine within the leaf, [^{14}C]methionine supplied by vacuum infiltration is equilibrated very rapidly with any methionine pool within the leaf cells. Regulation of ethylene synthesis through effects on the compartmentation of the ethylene precursor, as suggested by Kende and Baumgarter (12), can be questioned.

Endogenous Concentration of Free Methionine. To establish whether the increased ethylene production after TMV infection could result from an increased methionine concentration within the leaf, detached leaves were inoculated with water or TMV. Extracts were made daily to determine the concentration of free

Table II. *Comparison of the Specific Radioactivities of Ethylene Produced by Water- or TMV-Inoculated Leaves After Vacuum Infiltration with [^{14}C]Methionine*

Leaves were detached 2 days after inoculation, labeled and incubated during the subsequent 3 h.

	t = 0 h	t = 3 h
SA[a] methionine, dpm·nmol^{-1}	4450	240
Expected SA, C_2H_4, dpm·nmol^{-1}	1780	96
	H_2O-inoculated	TMV-inoculated
C_2H_4-production, nmol·leaf^{-1}	0.20	1.36
SA C_2H_4, dpm·nmol^{-1}	536	587

[a] SA, specific radioactivity.

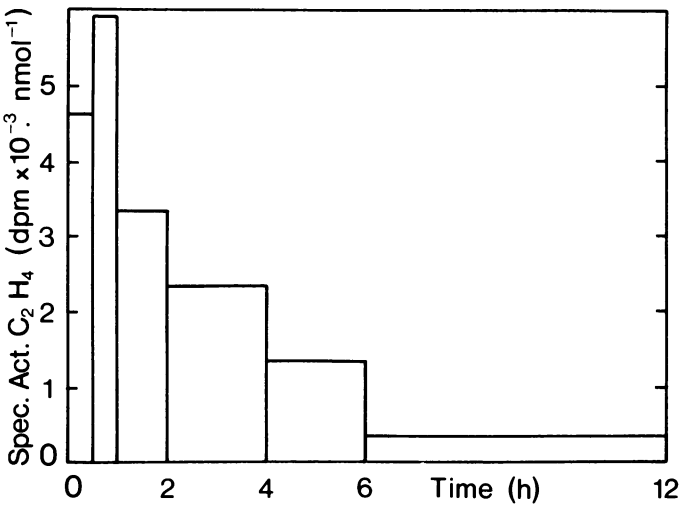

FIG. 3. Course of specific radioactivity of ethylene produced by tobacco leaf halves after vacuum infiltration with L-[U-^{14}C]methionine solution 2 days after TMV-infection.

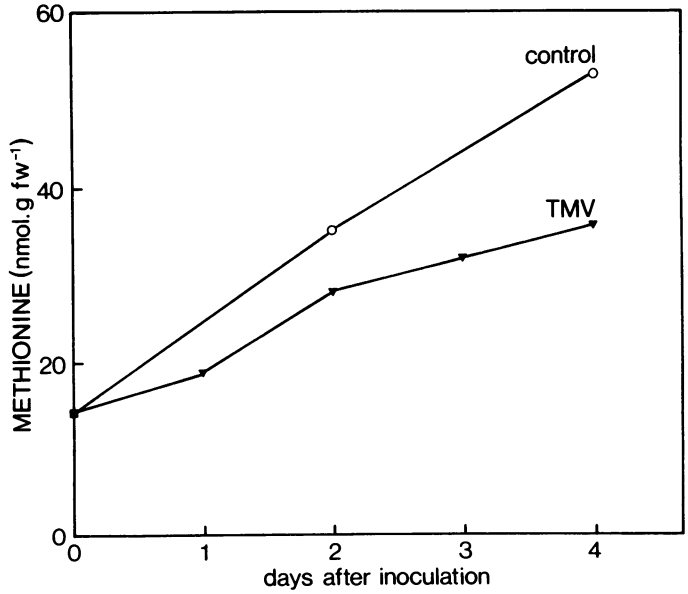

FIG. 4. Methionine concentration in tobacco leaves after water- or TMV-inoculation.

Table III. *Ethylene Production of and ACC Content in Tobacco Leaves 2 Days After Inoculation with Water or TMV*

Data are averages of two or three independent determinations for each experiment.

	Experiment 1		Experiment 2		Experiment 3	
	C_2H_4 (nmol/g fresh weight·h)	ACC (nmol/g fresh weight)	C_2H_4 (nmol/g fresh weight·h)	ACC (nmol/g fresh weight)	C_2H_4 (nmol/g fresh weight·h)	ACC (nmol/g fresh weight)
H_2O-inoculated	0.37	0.13	0.18	0.50	0.32	0.13
TMV-inoculated	2.65	0.52	1.18	2.60	1.51	1.68

methionine (Fig. 4). In water-inoculated leaves, the methionine concentration increased with time, probably due to proteolysis. In TMV-infected leaves the increase in methionine concentration was somewhat less than in the controls. Hence, the increase in ethylene production during the hypersensitive reaction cannot be accounted for by an increase in methionine content.

ACC Determination Experiments. If TMV infection stimulates the conversion of methionine to ethylene, it might be expected that the concentration of ACC, the immediate precursor of ethylene, is increased in infected tissues around the time of lesion appearance (5, 10). Leaf discs were cut from water- or TMV-inoculated leaves. Comparable discs were used either to measure ethylene production or to determine ACC content. As seen in Table III, the content of ACC in TMV-inoculated discs was increased severalfold. The increase in ACC concentration was well correlated with the increase in ethylene production.

It has been suggested (5, 25) that ethylene production is regulated by the activity of the enzymes involved in ethylene biosynthesis, particularly ACC-synthase. This possibility is now further investigated.

Conclusions. The inhibition of ethylene production by AVG, and the accordance between the specific radioactivities of methionine and ethylene after labeling with L-[U-^{14}C]methionine, indicate that methionine is the precursor of ethylene in tobacco leaves. Because similar results were obtained with virus-infected leaves and these leaves showed an increase in ACC content at the onset of the stimulation of ethylene production, virus-induced ethylene production must be likewise derived from methionine.

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