Phytochemistry, Vol. 24, No. 6, pp. 1181–1185, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

FORMATION OF METHANETHIOL FROM METHIONINE BY LEAF TISSUE

AHLERT SCHMIDT*, HEINZ RENNENBERG†, LLOYD G. WILSON‡ and PHILIP FILNER§

Botanisches Institut der Universität München, Menzinger Str. 67, D-8000 München 19, West Germany; †Botanisches Institut der Universität Köln, Gyrhofstr. 15, D-5000 Köln 41, West Germany; ‡MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.; §ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94566, U.S.A.

(*Received* 6 *July* 1984)

Key Word Index—*Cucurbita pepo*; Cucurbitaceae; sulphur metabolism; methanethiol; methionine; S-methylcysteine; volatile sulphur.

Abstract—Leaf discs, but not detached leaves, exposed to L-methionine or *S*-methyl-L-cysteine emitted a volatile sulphur compound identified as methanethiol by different trapping systems and by GC. Methanethiol emission was analyzed using pumpkin (*Cucurbita pepo*) leaf discs. Emission was observed in darkness or light, however methanethiol emission was greatly stimulated by light. Light-dependent emission started after a lag-time of 5–6 hr with an emission peak after 36–40 hr. Maximum rates obtained were in the range of 200 pmol methanethiol/min/cm^2 leaf area. After a period of 42 hr about 60–80% of total methionine sulphur added was released as methanethiol. Addition of chloramphenicol did not alter the induction period nor the maximum emission rate of methanethiol in response to L-methionine. Emission was also observed in response to *S*-methyl-L-cysteine; however, the shorter lag-period for methanethiol formation suggests metabolism via a different enzyme system. In a cell-free system of pumpkin leaves methanethiol formation occured in response to L-methionine. Feeding experiments with L-[^{35}S]methionine to leaf discs showed that more than 80% of methanethiol emitted was derived from the labelled methionine fed. These findings suggest that plants have the capacity to degrade L-methionine to methanethiol. Whole leaves fed L-methionine by the petiole system do not emit methanethiol, but this compound is formed and transported into the feeding solution. Thus, methanethiol is also produced by the intact leaf, but, in contrast to sulphide, is not released into the atmosphere. It is suggested that translocation of methanethiol may function as a signal for the regulation of sulphate uptake.

INTRODUCTION

Leaf tissues of higher plants are able to emit into the atmosphere hydrogen sulphide formed from sulphate [7, 16–19, 31, 35, 41, 42], sulphite [5, 6, 8, 17, 27, 31, 33], cysteine [15, 17, 19, 20, 25–27, 32] and glutathione [19]. The different systems of sulphide production in response to these sulphur sources have been analysed in cucurbit plants [cf. 7, 17] and an enzyme system for sulphide release from cysteine has been described [15, 25, 26]. Only few data concerning the emission of volatile sulphur compounds derived from methionine, however, are available for plants [28]. Possible volatile degradation products of methionine have been observed in natural environments. Dimethyldisulphide (DMSD) and methanethiol (MS) are emitted into the atmosphere by biogenic sources [1] and dimethylsulphide (DMS) is thought to be a major part of the biogenic sulphur emission [2, 9]. Recently DMS has been observed in the headspace of potato suspension cultures and in culture fluids of algae [4, 11, 13, 40].

The present study was initiated by the finding that methionine-treated leaves did not emit volatile sulphur compounds, however, volatile sulphur accumulated in the treatment solution when leaves were fed L-methionine through the petiole. This observation led to a study of the formation, and to the identification, of the volatile sulphur compound(s) released by methionine-treated leaves and leaf discs from pumpkin.

RESULTS

The emission of volatile sulphur in response to treatment with L-methionine was measured by flame photometry. Practically no emission was detected when a petiole of a pumpkin leaf was placed into a 10 mM L-methionine solution. Leaf discs, however, emitted substantial amounts of a sulphur compound smelling like methanethiol when placed on a solution of 10 mM L-methionine (Fig. 1). The emission started after a lag-period of about 5–6 hr and lasted for over 40 hr with maximum emission rates observed after 35–39 hr (Fig. 2). Maximum rates of emission by discs from pumpkin leaves were in the range of 200 pmol/min/cm^2 of leaf area.

Methionine-induced emission of volatile sulphur was found either in the light or in the dark, but light clearly enhanced sulphur emission (Fig. 2). Emission of volatile sulphur in response to L-methionine was not influenced by addition of 0.1 mM chloramphenicol, thus excluding bacterial origin of this volatile sulphur compound. This sulphur emission was specifically induced by L-methionine; D-methionine caused only little induction of volatile sulphur (Fig. 3).

*To whom correspondence should be addressed.

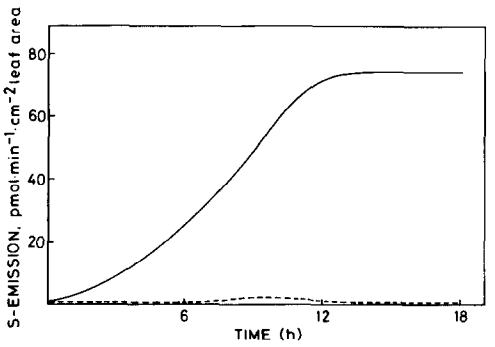

Fig. 1. L-Methionine induced emission of volatile sulphur by whole leaves and leaf discs. – – –, whole leaf (area 120 cm^2) fed 10 mM L-methionine, pH 7.5, via the petiole; ——, 10 leaf discs (area 26.5 cm^2) floated on 10 mM L-methionine, pH 7.5, and illuminated with 8 mW/cm^2. Volatile sulphur emitted was monitored as described in Experimental.

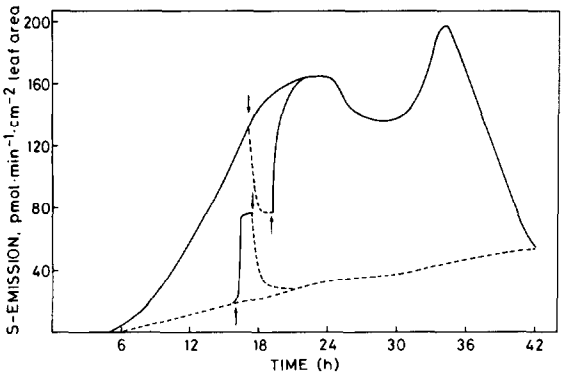

Fig. 2. Effects of light and darkness on L-methionine induced emission of volatile sulphur. Ten leaf discs (upside up) were floated on 10 mM L-methionine pH 7.5. ——, group kept in the light; – – –, group kept in dark. The arrows indicate a switch from light to dark or dark to light.

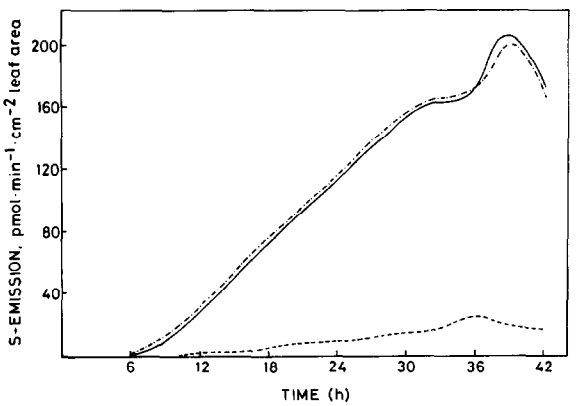

Fig. 3. Effectiveness of D- and L-methionine for volatile sulphur emission by leaf discs. Conditions as in Fig. 2. ——, L-methionine; – · – · –, L-methionine + 100 μM chloramphenicol; – – –, D-methionine.

Different trapping systems were used to collect and to identify the volatile sulphur compound emitted. Distilled water or 1 M NaOH did not trap the volatile sulphur compound, indicating that it was not SO_2 or dimethylsulphide. The volatile sulphur was not retained in a Zn-acetate trap, which showed that the compound was not hydrogen sulphide; on the other hand, DTNB-reagent trapped about 28% of the volatile sulphur, suggesting that a thiol group was involved (Table 1). The compound was completely absorbed by Purafil, which excludes carbonyl sulphide, carbon disulphide, and dimethyl disulphide. This left us with a compound having a thiol group attached to a carbon. Thus, the simplest compound possible was methanethiol.

Cupric salts of methanethiol are precipitated as Cu(I)SMe. Thus a trap of Cu(I)Cl was tried. It is evident from Table 1 that this trap efficiently removed the volatile compound.

For further identification the volatile sulphur emitted in response to L-methionine was trapped by freezing with liquid oxygen in a glass-trap of 1 ml volume. After a collection time of 1 hr the trap was closed and warmed up to room temperature for 15 min. An aliquot was subjected to GC analysis (Fig. 4). From this data it was obvious that only methanethiol and a trace of dimethyldisulphide, the oxidized product of methanethiol were present with 96% of the total volatile sulphur being methanethiol. This demonstrated that the methionine-induced volatile sulphur compound emitted by pumpkin leaf discs was exclusively methanethiol.

To establish if the methanethiol emitted was derived directly from the L-methionine fed, two groups of 8 discs were exposed to L-[^{35}S]methionine. After 18 hr of incubation one group of discs was analysed for total methanethiol emission by flame photometry; the second group of discs was connected to a Cu(I)Cl trap. After 1 hr the amount of methanethiol, emitted, as determined by sulphur analysis, was compared with the radioactivity

Table 1. Trapping of the L-methionine induced volatile sulphur compound(s) by different chemical treatments

Trapping device	Trapping efficiency (% control)
Water	16
Zn-acetate*	17
1 *M* NaOH	18
Ellman's reagent†	28
Cu(I)Cl‡	98
Purafil§	98

The gas stream from the leaf discs actively emitting the volatile sulphur compound was either measured directly or passed through 20 ml of trapping solution via a Pasteur pipett and the efficiency was calculated from the difference.

*0.1 mM.

†1.5 μmol/ml DTNB in 0.1 mM Tris–HCl buffer, pH 7.0.

‡1 g of CuCl/10 ml, stirred solution.

§Purafil INC, Atlanta, Georgia.

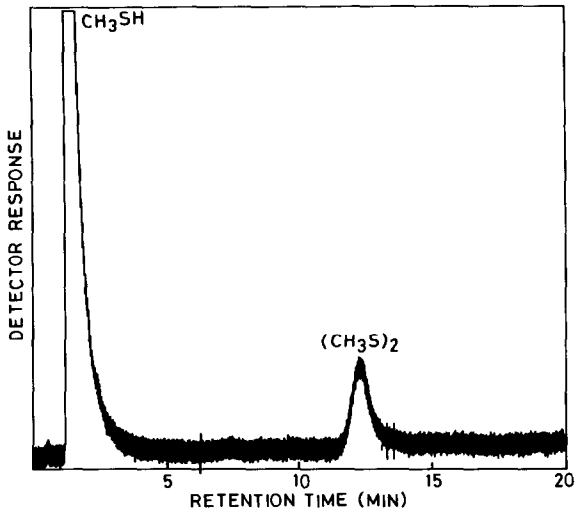

Fig. 4. GC analysis of trapped volatile sulphur emitted by leaf discs after L-methionine treatment. For details see Experimental.

collected in the Cu(I)Cl trap. Based on the specific activity of the L-[^{35}S]methionine used for the incubation the amount of methanethiol found in the Cu(I)Cl trap was calculated to be 63 nmole, whereas the sulphur analysis gave 77 nmole. Therefore at least 82% of the methanethiol emitted is derived from the L-methionine added, if dilution of the label by the methionine pools inside the leaf discs is neglected. This observation clearly demonstrated that the L-methionine added to the leaf discs was metabolized directly to methanethiol.

The presence of an inducible enzymatic system degrading L-methionine to methanethiol was demonstrated by cell-free system derived from pumpkin leaves. In this system the emission of methanethiol was clearly dependent on addition of substrate (L-methionine) and the enzyme system from pumpkin leaves. A low level of the activity converting L-methionine to methanethiol was present in non-induced controls; pretreatment with L-methionine for 40 hr enhanced this activity about 18-fold (Table 2).

From this data it was evident that the leaf had the capacity to form methanethiol from L-methionine. However, methanethiol was not emitted from the intact leaf, suggesting that it might be transported to other parts of the plant. To test this hypothesis, a leaf was fed L-methionine through the petiole for 18 hr with continuous monitoring of the emission of volatile sulphur. Volatile sulphur was not emitted, but methanethiol accumulated in the feeding solution. This accumulation was shown by passing air through the feeding solution, which liberated some of the methanethiol accumulated. In order to discount bacterial production of this compound over the 18 hr period, the feeding solution was replaced once and then exchanged with water at the time indicated. At the time intervals shown 10 ml of air was bubbled through this solution to liberate methanethiol accumulated in the treatment solution. The results of such an experiment indicated a time-dependent excretion of methanethiol from the petiole into the treatment solution (Fig. 5). Thus, methanethiol was not emitted from the intact leaf, but transported out of the leaf through the petiole.

Table 2. Formation of methanethiol from L-methionine in a cell-free system from pumpkin-leaves

	(nmol Volatiles/ min/g Fr. wt)
Without enzyme fraction	Not detected
Without L-methionine	Not detected
Preincubation in water	0.25
Preincubation in L-methionine	4.45

For further details see Experimental.

The finding, that methanethiol in contrast to hydrogen sulphide was not emitted by intact leaves was verified by another possible precursor of methanethiol, *S*-methyl-L-cysteine. Leaves fed *S*-methyl-L-cysteine by the petiole were compared with leaf discs (Fig. 6). Again there was no

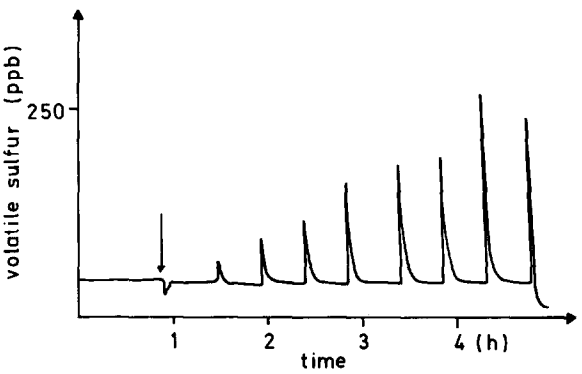

Fig. 5. Transport of methanethiol through the petiole into the treatment solution. A pumpkin leaf was fed 10 mM L-methionine, pH 7.5, for 18 hr via the petiole. At the time indicated by the arrow the L-methionine solution was replaced by water, which was immediately flushed with 10 ml of air. At the intervals shown 10 ml air was passed through the solution to liberate the methanethiol excreted.

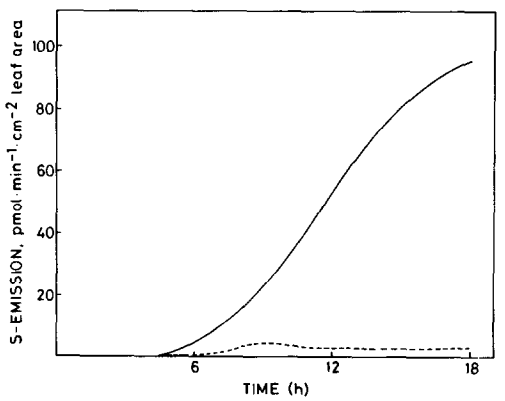

Fig. 6. Methanethiol formation by whole leaves and leaf discs from *S*-methyl-L-cysteine. Conditions as in Fig. 1, except *S*-methyl-L-cysteine was used instead of L-methionine. ——, leaf discs; – – –, whole leaf.

emission of volatile sulphur from the intact leaf, but an intensive emission from leaf discs. The time kinetics were different for the emission of volatile sulphur observed in response to L-methionine and *S*-methyl-L-cysteine. The emission of methanethiol from *S*-methyl-L-cysteine started immediately after exposure of leaf discs to this compound. A lag-period of about 5 hr, however, was observed for methanethiol emission in response to L-methionine. This observation suggested that degradation of *S*-methyl-L-cysteine and L-methionine involved different enzyme systems.

DISCUSSION

Methionine plays an important role as a metabolic precursor of several groups of compounds in higher plants. Methylation reactions, polyamine formation, ethylene production, and protein synthesis are dependent on the availability of methionine. To meet the plant's need for all these processes, not only methionine synthesis by itself, but also the catabolism of methionine via different metabolic pathways has to be coordinated.

There are, however, no data available concerning the decomposition of methionine to volatile sulphur compounds in higher plants. Trudinger and Loughlin stated in a recent review [39] that even our ideas regarding the decomposition of methionine to volatile substances in general are rudimentary.

The data presented in this publication indicate that pumpkin leaves have the capacity to degrade methionine to methanethiol. This degradation seems to be induced by L-methionine, since non-treated leaves had little capacity for L-methionine degradation in a cell-free system. About 5 hr are needed for the onset of methanethiol emission from leaf discs *in vivo* compared with about 1 hr needed for sulphide emission from cysteine [32]. These observations suggest that different enzyme systems are involved in the generation of hydrogen sulphide and methanethiol. It is obvious from the data presented that methanethiol is not emitted from the intact leaf, although it is produced in the intact leaf. This can be deduced from the observation that methanethiol is excreted into the feeding solution if a whole leaf is fed methionine via the petiole. However, if a leaf is cut into discs (i.e. the veins are opened) methanethiol is actively emitted and up to 80% of the L-methionine added can be converted to, and emitted as, methanethiol. This methanethiol formation is not brought about by bacterial contamination, since addition of chloramphenicol, an inhibitor of bacterial growth, did not change either the amount of methanethiol formed nor the time kinetics measured.

The volatile sulphur compound emitted from methionine-treated pumpkin leaf discs was identified as methanethiol by its odour, its trapping behaviour and by GC using a flamephotometric sulphur analyser or a photoionization detector. Formation of methane[^{35}S]-thiol from L-[^{35}S]methionine was observed with 82% of the methanethiol collected originating from L-methionine added.

There is one important difference between cysteine degradation and methionine degradation to volatile sulphur: If L-cysteine is used as precursor, hydrogen sulphide is formed and emitted from whole leaves or leaf discs [20, 32], whereas methanethiol formation from L-methionine is detected only using leaf discs. This indicates, that methanethiol is specifically retained in the leaf and that its emission is only detected if the veins are damaged. Thus, plants, whilst retaining methanethiol, seem to have a sulphur releasing system specifically designed for hydrogen sulphide release.

The finding that methanethiol is formed, but retained by the intact leaf has been tested by using *S*-methyl-L-cysteine as a source of volatile sulphur. *S*-Methyl-L-cysteine is also degraded to methanethiol by pumpkin leaves. However, its emission is again found only by leaf discs, not by intact leaves. The enzyme system for *S*-methyl-L-cysteine degradation seems to be different from the L-methionine degrading system, since methanethiol formation by pumpkin leaf discs starts after about 1 hr in response to *S*-methyl-L-cysteine compared with about 5 hr in response to L-methionine.

Trapping experiments with ^{35}S-labelled methionine clearly indicate, that the methanethiol collected is derived from L-methionine fed, thereby demonstrating a direct degradation of L-methionine to methanethiol. It is known that soil microorganisms degrade methionine to methanethiol and dimethyl disulphide [12, 21, 29], and formation of dimethyl disulphide has been demonstrated by fungi, algae and plant tissue cultures [11, 13, 21, 40]. Formation of methanethiol from L-methionine by an α,γ-elimination reaction is catalysed by the enzyme 'methioninase' (L-methionine γ-lyase: EC 4.4.1.11) found in *Escherichia coli* [14], *Pseudomonas putida* [10, 36], *P. ovalis* [37, 38] and several fungi [22, 34]. Formation of methanethiol from L-methionine has been postulated in higher plants for apple tissue, since *S*-methyl-L-cysteine was formed from labelled L-methionine leaving the methanethiol group intact; however no volatile S-compounds were detected [3]. The simplest explanation for methanethiol formation in pumpkin leaves would be an inducible methioninase similar to the systems found in bacteria, and our data of the cell-free system could be explained in such a way.

At the present state of knowledge one can only speculate about the role of L-methionine degradation to methanethiol. It appears as if methanethiol could function as a signal for sulphur flux regulation. Plants do take up sulphate by the root system and transport sulphate to the chloroplasts for reduction and further metabolism [23]. There has to be a signal-compound produced in the leaves and transported to the roots to regulate the uptake of sulphate by the root according to sulphur needed for growth. Since hydrogen sulphide is emitted from leaves, if the sulphate concentration around the root system is suddenly increased [7, 17, 32, 35, 41] or if the leaf is fed cysteine [20, 25–27] hydrogen sulphide emission appears to be one possibility to regulate sulphur flux in a changing environment. However, in the long term a controlling system reducing the uptake of sulphate appears to be more favourable than the emission of the excess sulphur taken up as hydrogen sulphide. Methanethiol derived from L-methionine might be synthesized in the leaves and transported to the root system to function there as a controlling signal for sulphate uptake. Since methanethiol is a volatile sulphur compound which is exported from the leaves and which is not easily trapped, it might have escaped the analytical procedures used in earlier experiments.

EXPERIMENTAL

Plant material. Experiments were performed with pumpkin plants (*Cucurbita pepo* L. cv. Small Sugar Pumpkin). Seeds were planted directly into a steam-sterilized mixture of sand, soil, peat and Perlite [2] and watered with a modified Hoagland nutrition

solution [2]. Plants were grown for 25–30 days in an environmental growth chamber operating for 16 hr at 33° and full light (7.5 mW/cm², fluorescent and incandescent lamps) and 8 hr at 15° with 2 hr light at the beginning and end of the cool period (2.4–4.7 mW/cm², incandescent lamps). At the end of the growth period the plants had developed 6–8 visible leaves.

Continuous measurement of volatile sulphur emission. Leaf discs (2.65 cm²/leaf disc) were used to determine emission of sulphur compounds. A group of leaf discs was punched from each half of the leaf. The leaf discs were floated on 10 ml soln either in petri dishes in the two-channel-system described by Rennenberg *et al.* [20] or in 250 ml Erlenmeyer flasks in the four-channel-system described by Sekiya *et al.* [32]. Each system was connected to a flame photometric sulphur analyser (Monitor Labs, San Diego, Ca; Model 8450). Illumination was provided at 8 mW/cm² (four-channel-system) by 400 W phosphor-coated metal halide lamps or at 4 mW/cm² (two-channel-system) by cool white fluorescent lamps. Light intensity was measured with a radiometer (Yellow Springs Instruments, Model GSA). During all measurements the temperature was set to 26 ± 1°; the pH of each soln was adjusted to 6.0 ± 0.1.

In vitro *determination of volatile sulphur emission.* Two groups of 8 leaf discs (6.25 cm²/leaf disc) were punched from a leaf, each group from one half of the leaf. One group of discs was floated for 40 hr on distilled water, the other group was exposed for the same time to 10 ml of 10 mM L-methionine. The discs were homogenized for 2 min in an ice-cold mortar with 6 ml of 0.1 K-phosphate buffer, pH 7.5, containing 1% ascorbic acid. 3 ml of the homogenate was placed at 25° in a 25 ml Erlenmeyer flask which was connected to a flame-photometric sulphur analyser in such a way that room air was drawn through the head space of the flask and into the sulphur analyser. The reaction was started by the addition of 0.6 ml 100 mM L-methionine. Volatile sulphur emission was calculated as an average emission rate for the first 5 min.

GC-analysis. A Varian 3700 GC equipped with a photoionization detector (HNU, Newton, Ma, model P 152) was used for the GC-analysis of the volatile sulphur emitted by pumpkin leaf discs in response to L-methionine. Chromatography was performed on a chromasil 330 column, 6 ft × 1/8 teflon. The column was operated at 65° and a flow rate of 30 ml N_2/min. The inlet port was maintained at 80°, the detector at 85°. The photoionization detector was operated at lamp intensity '4' and 10^{-10}/mV.

Degradation of L-[^{35}S]*methionine.* L-[^{35}S]Methionine was obtained from New England Nuclear. Two groups of 8 leaf discs were each floated in 10 ml 10 mM methionine, pH 6. 1.8 uCi/10 μmol L-methionine was added and the discs were incubated for 18 hr. Afterwards the vessels were connected to a CuCl trap and to the flame emission sulphur analyser for 1 hr. Total volatile sulphur measured was integrated over the 1 hr period, and total volatile radioactivity was determined as the CuCl-trap in a liquid scintillation counter; from the sp. radio activity of the L-methionine in the vessel the amount of methanethiol formed was calculated.

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