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Stimulation of H_2S Emission from Pumpkin Leaves by Inhibition of Glutathione Synthesis[1]

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HEINZ RENNENBERG[2] AND PHILIP FILNER[3]
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

The effect of inhibitors of glutathione (GSH) synthesis, namely γ-methyl glutamic acid, D-glutamic acid, cystamine, methionine-*S*-sulfoximine (MSX), buthionine-*S*-sulfoximine, and GSH itself, on the emission of H_2S was investigated. All these compounds stimulated H_2S emission from pumpkin (*Cucurbita pepo* L. cv Small Sugar Pumpkin) leaf discs in response to sulfate. MSX and GSH were the most effective compounds, stimulating H_2S emission from leaf discs of mature pumpkin leaves by about 80% in response to sulfate. Both inhibitors did not appreciably enhance H_2S emission in response to L-cysteine and inhibited H_2S emission in response to sulfite.

Treatment with MSX or GSH enhanced the uptake of sulfate by pumpkin leaf discs, but did not affect the incorporation of sulfate into reduced sulfur compounds. Inhibition of GSH synthesis by MSX or GSH caused an increase in the pool size of cysteine, and, simultaneously, reduced the incorporation of labeled sulfate into cysteine. The incorporation of labeled sulfate into the sulfite and sulfide pools of the cells are stimulated under these conditions.

These observations are consistent with the idea that inhibition of GSH synthesis leads to an elevated cysteine pool that inhibits further cysteine synthesis. The H_2S emitted under these conditions appears to arise from diversion of a precursor of the sulfur moiety of L-cysteine. Therefore, stimulation of H_2S emission in response to sulfate upon inhibition of GSH synthesis may reflect a role of H_2S emission in keeping the cysteine concentration below a critical level.

Leaf tissue emits substantial amounts of H_2S when exposed to high concentrations of sulfate (17, 21, 22), SO_2 or sulfite (2–4, 19), or L-cysteine (15, 18). H_2S emission in response to sulfate is a light-dependent process, proceeding at rates comparable to rates of sulfate assimilation into protein (21). The path of sulfate assimilation leading to H_2S emission is heavily dependent on photosynthetic electron transport, but in a way which differs significantly from the dependence of CO_2 fixation (17). Whether free or carrier-bound sulfite is the immediate precursor of the H_2S emitted in response to sulfate still remains unresolved. Desulfhydration of cysteine is the path of H_2S emission from cucurbit leaves in response to L-cysteine in the dark (18). However, upon illumination of cucurbit leaves, H_2S emission in response to this sulfur source becomes a partially light-dependent process (15). In the light, part of the H_2S produced via desulfhydration is oxidized to sulfite, then sulfate, which is reduced again via the light-dependent sulfate assimilation pathway. However, synthesis of cysteine seems to be inhibited in the presence of excess L-cysteine, and the reduced sulfur enters the H_2S pool of the cells again, without being incorporated into cysteine (15). Although the physiological meaning of this intracellular sulfur cycle has not been elucidated, it might be part of a regulatory system to maintain a constant cysteine concentration inside plant cells. Therefore, emission of H_2S might be a mechanism for removing excess sulfur.

Besides incorporation into protein, incorporation into GSH is the major fate of L-cysteine in green tissues (1, 13, 18). One reason for the high incorporation of reduced sulfur into GSH seems to be the function of this peptide as the predominant long distance transport form of reduced sulfur (14): mature tobacco leaves reduce more sulfate than necessary for their own needs and incorporate it into GSH; the excess GSH is translocated from the leaves to the roots and to the growing parts of the stem. Therefore, it is plausible that inhibition of GSH synthesis should either cause cysteine accumulation or cause light-dependent sulfate reduction to be inhibited.

The function of H_2S emission by plants has not yet been determined. If regulation of the cysteine concentration and removing of excess sulfur are functions of the paths of H_2S emission, inhibition of GSH synthesis might result in an enhanced emission of H_2S. The present investigation was undertaken to test this hypothesis.

MATERIALS AND METHODS

Plant Material. The present experiments were performed with *Cucurbita pepo* L. cv. Small Sugar Pumpkin. Plants were grown for 30 to 35 d in an environmental growth chamber under the conditions previously described (21). At this time, plants contain seven to nine leaves. The second and third leaves from the top, which were actively growing, are referred to as young leaves; the fifth and sixth leaves from the top were fully expanded and are referred to as mature leaves.

Measurement of H_2S Emission. Leaf discs (2.65 $cm^2 \times disc^{-1}$) were used for the determination of H_2S emission. Eight discs were punched from one-half of a leaf and floated in 10 ml of a reference solution in a Petri dish; eight leaf discs from the other half of the same leaf were floated on 10 ml of a solution containing the chemical to be tested (17, 18). The pH of each solution was adjusted to 6. The Petri dishes were placed in matched disc chambers and illuminated at 4 mw $\times$ cm^{-2} by cool-white fluorescent lamps at 25 ± 1°C. The disc chambers were connected to an automatic two-channel selector, which was coupled to a CO_2 analyzer (Beckman) and a flame-photometric sulfur detector (Monitor Labs, San Diego, CA; model 8450). Air was pushed through the system at 180 ml $\times$ min^{-1}.

Extraction and Fractionation of ^{35}S Compounds. Leaf discs

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[2] Present address: Botanisches Institut der Universitaet Koeln, Gyrhofstrasse 15, D-5000 Koeln 41, Federal Republic of Germany. Recipient of a Deutsche Forschungsgemeinschaft fellowship.

[3] Present address: ARCO Plant Cell Research Institute, 6905 Sierra Court, Box 2600, Dublin, CA 94566.

were homogenized twice with 5 ml of cold 80% (v/v) ethanol containing 10 mM NEM[4] for 2 min, centrifuged at 12,000*g* for 10 min, and the combined supernatants were fractionated as previously described (18). To the residue, 5 ml H_2O were added, and the mixture was transferred to a boiling water bath for 10 min. The boiled homogenate was centrifuged at 12,000*g* for 10 min and the H_2O extraction was repeated. The residue after the second H_2O extraction was solubilized overnight in 5 ml NCS tissue solubilizer (A. Buchler), and the radioactivity in this fraction was determined by liquid scintillation counting. Electrophoresis and TLC of the fractions obtained from the ethanol and H_2O extractions were performed as described by Sekiya et al. (18).

Quantitative Determination of GSH and Cysteine by Reaction with [^{14}C]NEM. Leaf discs were homogenized, extracted twice with 3 ml of 1 mM [^{14}C]NEM in 80% ethanol (v/v; 1.5×10^6 cpm $\times$ ml^{-1}), and centrifuged at 12,000*g* for 10 min. The residue was extracted with H_2O as described above, and the combined and concentrated ethanol and H_2O fractions were subjected to TLC (18). Alkylated GSH and cysteine were localized on the TLC plates by means of co-chromatographed reference compounds. The spots were scraped, and the radioactivity was determined by liquid scintillation counting. The concentrations of GSH and cysteine were calculated according to the specific activity of the [^{14}C]NEM used, and corrected for recoveries. The recovery of added amounts of GSH and cysteine was 85 ± 9% and 67 ± 12%, respectively.

RESULTS AND DISCUSSION

Effect of Inhibitors of GSH Synthesis on Emission of H_2S. The effects of various compounds, known to inhibit γ-glutamylcysteine-synthetase, which catalyzes the first step in GSH synthesis (cf. 10), on the emission of H_2S from mature pumpkin leaves has been investigated. As shown in Table I, all the inhibitors tested enhance the light-dependent release of H_2S in response to sulfate as sulfur source. Little or no H_2S emission was observed when these inhibitors were present, and sulfate had been omitted in the treatment solutions. Therefore, the stimulation of H_2S emission by those inhibitors of GSH synthesis which contain sulfur, namely MSX, BSX, and cystamine, appears not to be due to a use of these compounds as additional sulfur sources for H_2S production.

The glutamic acid analogs γ-methyl-glutamic acid and D-glutamic acid, which are relatively weak inhibitors of GSH synthesis (7, 20), enhance the emission of H_2S from pumpkin leaves to a smaller extent than does the transition state analog MSX or the allosteric inhibitor cystamine, which are more powerful inhibitors of GSH synthesis than the glutamic acid analogs (6). BSX, the most potent inhibitor of GSH synthesis known so far (8), stimulates H_2S emission at concentrations down to 10 μM. However, the magnitude of the stimulation by BSX is appreciably smaller than that caused by MSX. Furthermore, H_2S emission is enhanced by BSX after a lag period of more than 90 min, whereas stimulation of H_2S emission by MSX is observed immediately upon beginning the exposure of leaf discs to this compound (Fig. 1). Therefore, the differences between the stimulations of H_2S emission by these two closely related compounds may be due to differences in their uptake by leaf cells rather than to different modes of action.

In animal (cf. 10) as well as in higher plant cells (B. Schaer and H. Rennenberg, unpublished results), GSH seems to regulate its own synthesis by feedback inhibition. If compounds known as inhibitors of GSH synthesis stimulate H_2S emission by inhibiting GSH synthesis, as the results suggest, then GSH itself should have an effect on H_2S emission comparable to that of treatment with man-made inhibitors of GSH synthesis. As shown in Table I, GSH is as potent a stimulator of H_2S emission from pumpkin leaves as is MSX, again without serving as additional sulfur source for H_2S synthesis.

Besides GSH, MSX was highest in effectiveness in stimulating H_2S emission from pumpkin leaves (Table I). However, MSX is not a specific inhibitor of GSH synthesis (16); it also acts as an analog of γ-glutamylphosphate in other enzymic processes in which this transition state is involved. Probably, the most important reaction inhibited by MSX is the synthesis of glutamine by glutamine synthetase. Therefore, we investigated whether the stimulation of H_2S emission by MSX is connected to inhibition of glutamine synthetase. Addition of glutamine or/and glutamic acid did not affect the emission of H_2S, neither in the presence nor in the absence of MSX (Table II). In addition, azaserine, an inhibitor

Table I. *Influence of Inhibitors of GSH Synthesis on H_2S Emission from Mature Pumpkin Leaves*

The data were obtained in three replicates of 12 separate experiments, each replicate using discs from a different leaf and a different plant. Eight leaf discs (21 cm^2 leaf area) of one-half of a mature pumpkin leaf were floated for 3 h in the light (4 mw/cm^2) in 10 ml of a treatment solution containing one of the inhibitors indicated and either 25 mM or no sulfate. Eight leaf discs from the other half of the same leaf were floated under the same conditions in 10 ml of a 25 mM sulfate solution as a control. Controls emit 16 to 40 pmol $H_2S \times cm^{-2}$ leaf area $\times$ min^{-1} (100%) and exhibit a CO_2 fixation of 12 to 17 nmol $CO_2 \times cm^{-2}$ leaf area $\times$ min^{-1}.

SO_4^{2-} Concn.	Inhibitor	Concn.	Relative H_2S Emission
mM		*mM*	%
25	—	—	100 ± 9.5[a]
25	MSX	0.1	181 ± 8.9
0	MSX	0.1	ND[b]
25	BSX	0.05	126 ± 9.3
0	BSX	0.05	ND
25	Cystamine	5	153 ± 9.9
0	Cystamine	5	ND
25	γ-Methyl-Glu	5	122 ± 8.6
0	γ-Methyl-Glu	5	ND
25	D-Glu	5	128 ± 8.7
0	D-Glu	5	7 ± 4.3
25	GSH	1	183 ± 9.6
0	GSH	1	ND

[a] ± SE.
[b] ND, H_2S emission not detected.

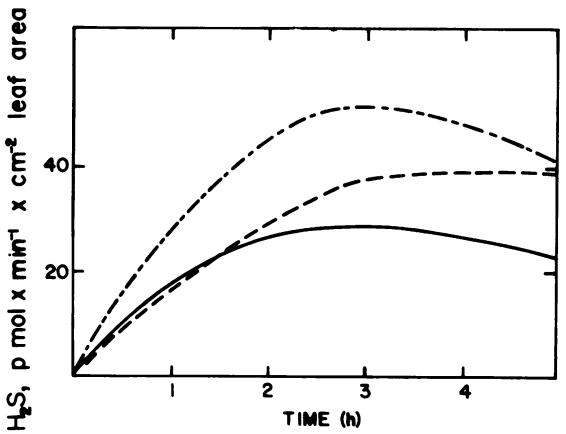

FIG. 1. Changes in the pattern of H_2S emission from pumpkin leaves by MSX and BSX. Experiments were performed as described in Table I. Leaf discs were floated for 5 h in 25 mM sulfate + 0.1 mM MSX, or 25 mM sulfate + 0.05 mM BSX. Leaf discs in 25 mM sulfate alone were used as a control. (——), Control; (— — — —), + MSX; (-----), +BSX.

[4] Abbreviations: NEM, *N*-ethylmaleimide; MSX, methionine-sulfoximine; BSX, buthionine-sulfoximine.

of glutamic acid synthesis by glutamate synthase (cf. 11), did not have a significant influence on the emission of H_2S (Table II). These observations indicate that the enhanced H_2S emission from pumpkin leaves treated with MSX probably does not depend on an effect of this inhibitor on nitrogen metabolism.

The percentage stimulation of H_2S emission by GSH or MSX is much greater in mature leaves than in expanding leaves (Table III). Whereas MSX- or GSH-treated cells of mature leaves emit 80 to 90% more H_2S than untreated controls, H_2S emission from expanding leaves is enhanced by these compounds to about 20% only. However, the absolute increases in the rate of H_2S emission are very similar in young and mature leaves. This is true for either inhibitor. H_2S emission from cucurbit leaves has not only been shown with sulfate, but also with sulfite, SO_2, or L-cysteine as sulfur source (15, 18, 19). A considerable stimulation of H_2S emission by MSX or GSH is only observed when sulfate is used as substrate for H_2S synthesis (Table IV). H_2S emission in response to sulfite is inhibited rather than stimulated by MSX and GSH; these data indicate that the paths of H_2S formation from sulfate and sulfite are different. H_2S emission is response to L-cysteine is only slightly enhanced by MSX and GSH. Therefore, the stimulation of H_2S emission from sulfate can be understood in terms of inhibition of sulfur assimilation beyond L-cysteine, with resultant accumulation of an intermediate which is the precursor of sulfide.

Table II. *Effect of Glutamine and Glutamic Acid on the MSX-Mediated Stimulation of H_2S Emission from Pumpkin Leaves*

Experiments were performed as described in Table I, and H_2S emission integrated over a 2-h period.

Sulfate Concn.	MSX Concn.	Further Additions	Concn.	Relative H_2S Emission
mM			*mM*	%
25	0	—	—	100 ± 9.0[a]
25	1	—	—	178 ± 10.4
0	1	—	—	ND[b]
25	1	Gln	5	192 ± 8.6
25	1	Glu	5	181 ± 10.1
25	1	Gln + Glu	5 each	185 ± 9.0
0	1	Gln + Glu	5 each	8 ± 4.9
25	0	Gln + Glu	5 each	104 ± 7.5
25	0	Azaserine	1	94 ± 9.8

[a] ± SE.

[b] ND, H_2S emission not detected.

Table III. *Influence of Leaf Age on the Stimulation of H_2S Emission by MSX and GSH*

Experiments were performed as described in Table I, and the H_2S emission integrated over a 5-h period.

SO_4^{2-} Concn.	Addition	Concn.	H_2S Emitted	
			Mature leaf	Expanding leaf
mM		*mM*	*pmol × cm² leaf area⁻¹ × min⁻¹*	
25	—	—	21.4	97.6
25	MSX	1.0	39.2	116.7
		Stimulation	17.8 (83%)	19.1 (20%)
25	—	—	28.8	83.3
25	GSH	1.0	54.2	101.9
		Stimulation	25.4 (88%)	18.6 (22%)

Table IV. *Effect of MSX and GSH on the Release of H_2S from Mature Pumpkin Leaves Treated with Different Sulfur Sources*

Experiments were performed as described in Table I, and the emission of H_2S integrated over a 3-h period.

Sulfur Source	Concn.	Addition	Concn.	Relative H_2S Emission
	mM		*mM*	%
Sulfate	25	—	—	100 ± 9.4[a]
Sulfate	25	MSX	0.1	180 ± 10.1
Sulfate	25	GSH	1.0	183 ± 9.8
Sulfite	10	—	—	100 ± 13.2
Sulfite	10	MSX	0.1	69 ± 12.6
Sulfite	10	GSH	1.0	63 ± 14.1
L-Cysteine	10	—	—	100 ± 9.0
L-Cysteine	10	MSX	0.1	113 ± 9.9
L-Cysteine	10	GSH	5.0	114 ± 10.7

[a] ± SE.

Effect of MSX and GSH on Sulfur Assimilation. To prove the hypothesis concerning the action of MSX and GSH on sulfate-dependent H_2S emission, the distribution of radioactivity was analyzed in pumpkin leaf cells, exposed to [^{35}S]sulfate in the presence and in the absence of MSX or GSH. As shown in Figure 2, the uptake of sulfate by discs is enhanced by addition of MSX or GSH to the treatment solution. At the end of a 3-h incubation, 17 μmol ± 6.4% sulfate × g fresh wt^{-1} (5 × 10^7 cpm × g fresh wt^{-1}) were taken up by GSH-treated cells, 13 μmol ± 5.9% sulfate × g fresh wt^{-1} (4 × 10^7 cpm × g fresh wt^{-1}) by MSX-treated cells, but only 10 μmol ± 6.8% sulfate × g fresh wt^{-1} (3 × 10^7 cpm × g fresh wt^{-1}) by untreated controls. Most of the radioactivity found inside the cells was confined to sulfate, and only small amounts were found in reduced suifur. The reduced sulfur fraction repre-

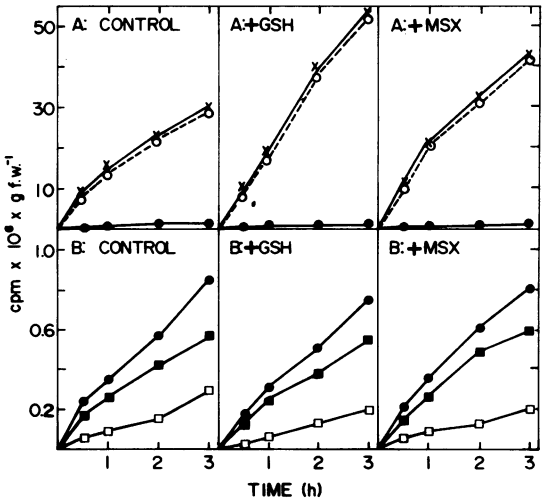

FIG. 2. Uptake and distribution of ^{35}S in pumpkin leaf discs exposed to [^{35}S]sulfate. Three samples of five leaf discs (13 cm^2 leaf area) were punched from a mature pumpkin leaf and floated for 3 h in the light (4 mw/cm^2) in 10 ml of a treatment solution containing 25 mM [^{35}S]sulfate (74 × 10^6 cpm × ml^{-1}) and one of the inhibitors indicated. The incubation was stopped by washing and transferring the discs into liquid N_2. Leaf discs were homogenized and extracted twice with 10 mM NEM in 80% ethanol. The residue of these extractions was extracted twice with distilled H_2O. Combined ethanol and H_2O fractions are referred to as soluble sulfur fraction; sulfate was separated from the other constituents in this fraction by TLC electrophoresis. The residue, remaining subsequently to the ethanol and H_2O extraction, was solubilized in NCS tissue solubilizer (A. Buchler) and referred to as protein fraction. Radioactivity was determined by liquid scintillation counting. MSX, 0.1 mM; GSH, 1 mM. A: (x), total ^{35}S; (○), [^{35}S]sulfate; (●), reduced ^{35}S. B: (●), reduced ^{35}S; (□), protein ^{35}S; (■), soluble reduced ^{35}S.

sents 1.3 to 1.5% of the sulfur taken up by leaf cells exposed to GSH, 1.7 to 1.9% of the sulfur taken up by those exposed to MSX, but 2.5 to 2.8% in controls without inhibitors of GSH synthesis. However, the absolute amount of incorporation of ^{35}S into reduced sulfur compounds, 0.8×10^6 cpm $\times$ g fresh wt^{-1}, is similar in MSX- (GSH-) treated and untreated leaf discs. Therefore, the differences in the relative amounts of ^{35}S in reduced sulfur are due to the enhanced sulfate uptake in GSH- (MSX-) treated leaf discs. Provided that the specific activities of the 'active' sulfate pools in MSX- and GSH-treated pumpkin cells are comparable to those of controls not exposed to these compounds, the observed stimulation of sulfate uptake by MSX and GSH caused an accumulation of sulfate inside the cells, but did not enhance sulfur reduction.

Although the distribution of reduced sulfur between protein and the fraction of low-mol-wt soluble sulfur compounds is not significantly affected by addition of MSX or GSH (Fig. 2), remarkable changes occur in the composition of the fraction of soluble reduced sulfur compounds upon treatment with these inhibitors of GSH synthesis (Fig. 3). The rate of net ^{35}S incorporation into GSH was reduced in MSX- (GSH-) treated cells during the first hour of incubation; thereafter, the amount of ^{35}S present in this peptide remained constant in MSX- (GSH-) treated discs, whereas increasing amounts of ^{35}S were found in the GSH pool(s) of controls. A similar pattern was observed for the net incorporation of ^{35}S into cysteine. The ^{35}S labeling of the methionine pool(s) is reduced upon exposure to MSX (GSH) at the beginning; beyond the first hour of incubation, the rate of net incorporation of ^{35}S into methionine was very similar in controls and MSX- (GSH-) treated discs. After a lag period of 30 to 60 min, where MSX (GSH) did not have a significant influence on the ^{35}S present in sulfide, the amount of label in this compound is doubled within 2 h in MSX- (GSH-) treated cells, whereas only a slight increase is observed in untreated controls. As also the emission of $H_2{}^{35}S$ is enhanced by treatment with MSX (GSH), the values shown in Figure 3 for the labeling of intracellular sulfide even underestimate the difference in the incorporation of ^{35}S into sulfide between

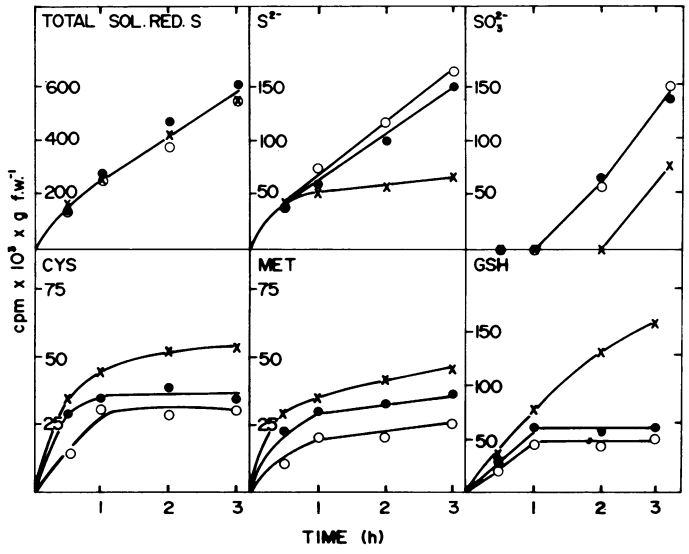

FIG. 3. Influence of MSX and GSH on the incorporation of [^{35}S]sulfate into soluble reduced sulfur compounds. Pumpkin leaf discs were exposed to [^{35}S]sulfate in the presence and absence of MSX (GSH), extracted, and fractionated as described in Figure 2. The fraction of soluble sulfur compounds was subjected to TLC, and the reaction products with NEM were localized on the TLC plates by means of co-chromatographed reference compounds. Radioactivity was determined by liquid scintillation counting. (x), Control; (●), +MSX; (○), +GSH.

Table V. *Effect of MSX and GSH on the Pool Size of Cysteine and GSH in Pumpkin Leaf Discs Exposed to 25 mM Sulfate*

Three samples of five leaf discs (13 cm^2 leaf area) were punched from a mature pumpkin leaf and floated for 3 h in the light (4 mw/cm^2) in 10 ml of a treatment solution containing 25 mM sulfate and one of the inhibitors indicated. The incubation was stopped by washing and transferring the leaf discs into liquid N_2. Leaf discs were homogenized and extracted twice in 1 mM [^{14}C]NEM in 80% ethanol (1.4×10^6 cpm $\times$ ml^{-1}). The residue of this extraction was extracted twice with distilled water. Ethanol and H_2O extracts were combined, concentrated, and subjected to TLC analysis. The reaction products of cysteine and GSH with NEM were localized on the TLC plates by means of co-chromatographed reference compounds; radioactivity was determined by liquid scintillation counting.

	Cysteine		GSH	
	Concn.	Relative pool size	Concn.	Relative pool size
	mM	%	mM	%
Control	0.071	100 ± 12.0	0.141	100 ± 10.9
MSX, 0.5 mM	0.094	132 ± 13.9	0.066	47 ± 15.2
GSH, 5 mM	0.140	198 ± 14.3	0.466	330 ± 12.7

MSX- (GSH-) treated cells and controls. [^{35}S]sulfite was first detected in controls after 3 h of incubation; in MSX- (GSH-) treated discs, considerable amounts of label were found in this compound after 2 h of incubation. At the end of a 3-h experiment, the amount of ^{35}S in sulfite was twice as high in MSX- (GSH-) treated cells as in controls without these inhibitors.

The reduced net labeling of the cysteine and GSH pools of the pumpkin cells upon treatment with MSX was accompanied by a reduced pool size of GSH, and a slightly enhanced pool size of cysteine (Table V). In the presence of GSH in the treatment solution, not only the intracellular GSH, but also the cysteine pool(s) of the leaf discs was (were) expanded, most likely due to uptake and degradation of GSH. These observations show that exposure of pumpkin cells to 25 mM sulfate in the presence of MSX (GSH) reduces the incorporation of reduced sulfur into carbon skeletons; however, this effect of MSX (GSH) seems not to be connected to a decrease in sulfate reduction, but to a redistribution of reduced sulfur among low-mol-wt soluble sulfur compounds.

CONCLUSIONS

The present experiments provide evidence that glutathione synthesis in pumpkin leaves is inhibited by MSX and GSH itself. Inhibition of GSH synthesis by these compounds stimulated the uptake of sulfate, but seems not to have affected sulfate reduction. As GSH synthesis is a major path of reduced sulfur in plants (5, 17), inhibition of this pathway while sulfate reduction is proceeding unchanged should result in an enhanced incorporation of reduced sulfur into other reduced sulfur-containing compounds. However, although the pool size of cysteine is enhanced in MSX- (GSH-) treated pumpkin cells, the incorporation of reduced sulfur in this amino acid is reduced in comparison with untreated controls. This observation might be explained by an inhibitory effect of an elevated cysteine level on cysteine synthesis, as cysteine synthase has shown to be inhibited by L-cysteine (12). Another possible explanation for the reduced incorporation of reduced sulfur into cysteine in the presence of MSX would take the inhibition of glutamine synthetase by this compound (cf. 11) into consideration: inhibition of glutamine production by MSX might reduce the availability of serine for *O*-acetyl-serine synthesis, thereby reducing cysteine synthesis. However, this explanation is unlikely, as GSH treatment also reduces the incorporation of reduced sulfur into cysteine, but is not known to affect the nitrogen

metabolism in plants.

In cucumber cells exposed to inhibitors of GSH synthesis, not only an inhibition of the incorporation of reduced sulfur into cysteine is observed, but also a stimulation of H_2S emission. This stimulation of H_2S emission does not appear to be caused by a desulfhydration of excess L-cysteine (9, 15, 18), as a considerable stimulation of H_2S emission by MSX (GSH) is not observed, when leaf discs were supplied with L-cysteine as sulfur source. This idea is supported by the observation that in leaf discs fed with [^{35}S] sulfate less reduced sulfur is incorporated into cysteine in the presence of MSX (GSH). Therefore, the emission of H_2S may reflect an enhanced amount of carrier bound thiol that cannot be used for cysteine synthesis, because this process is inhibited; the thiol may be split off the carrier under these conditions, and may be emitted as H_2S into atmosphere.

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