Effects of L-Methionine-S-sulfoximine on Growth and Glutathione Synthesis in Tobacco Suspension Cultures

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Z. Naturforsch. **35 c**, 945–951 (1980); received June 6/August 8, 1980

L-Methionine-S-sulfoximine, Glutathione, Tobacco, Tissue Culture

Tobacco cells grown photoheterotrophically with high ammonium and sulfate concentrations are able to cope with small amounts of methionine-sulfoximine. The observed methionine-sulfoximine tolerance of tobacco suspensions is due to an extracellular glutathione and an intracellular glutamine reservoir; both reservoirs are considerably reduced by treatment with methionine-sulfoximine concentrations that do not affect the growth of the cells. In tobacco suspension cultures grown with nitrate as sole nitrogen source that do not contain high amounts of glutathione and glutamine, growth inhibition by methionine-sulfoximine can be prevented by addition of these substances to the growth medium. These data indicate that synthesis of glutathione and glutamine are both inhibited by mentionine-sulfoximine; furthermore they show evidence that – in contrary to animal cells – the whole glutathione molecule is taken up by tobacco cells. Synthesis of glutathione from the consisting amino acids is inhibited by methionine-sulfoximine in crude cell homogenates to a similar extent than observed in tobacco suspensions *in vivo;* therefore, the activity, and not the amount of enzymes of glutathione synthesis seems to be reduced by treatment with methionine-sulfoximine. As tobacco suspensions are able to recover from methionine-sulfoximine treatment with respect to accumulation of glutathione in the medium as well as with respect to growth, detoxication of methionine-sulfoximine has to be assumed.

Introduction

Photoheterotrophically grown suspension cultures of *Nicotiana tabacum* produce high amounts of glutathione and release it into their culture media [1, 2]. Surplus production of glutathione is restricted to chloroplast-containing cells [2] and induced by high ammonium and sulfate concentrations [3]. When the sulfate supply in the medium is exhausted, released glutathione is taken up again and reutilized as sulfur source for protein synthesis [3]. These data indicate a function of glutathione as storage and transport form of cysteine. This function of glutathione holds also true for the whole plant, since investigations of the long-distance transport of sulfur showed that glutathione is the main transport form of reduced sulfur in tobacco plants [4].

Despite of the observed mobility of glutathione in tobacco suspension cultures and in tobacco plants, the glutathione level inside tobacco cells remains at a quite constant level [2, 5]. It has to be expected that this constant intracellular glutathione concentration is the result of a regulation of synthesis and efflux as well as uptake and degradation. In order to get informations about such a regulation, work with metabolic inhibitors is a common approach. From animal cells L-methionine-S-sulfoximine is known to be a potent inhibitor of glutathione synthesis [6]. As no data about the action of this compound on synthesis of glutathione in higher plants have yet been published, the influence of methionine-sulfoximine on growth and efflux of glutathione in tobacco suspension cultures has been investigated.

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0341-0382/80/1100-0945 $ 01.00/0

Materials and Methods

The tobacco suspensions used in the present experiments were obtained from a callus culture isolated by Bergmann in 1959 [9]. Cells were subcultured in a modified liquid M+S medium [10] and grown at 25 °C and 60–70% air humidity under continuous illumination (3000 lx). In suspensions grown with nitrate as sole nitrogen source NH_4NO_3 was replaced by equimolar amounts of $Ca(NO_3)_2$. Experiments were conducted with exponential phase tobacco cells, 9–11 days old, in 100 ml erlenmeyer-flasks containing 30 ml growth medium. Methionine-sulfoximine (Sigma)-, glutathione (Boehringer)- and glutamine (Merck)-solutions were adjusted at pH 5.6 to 5.8, sterilized by filtration through Millipore filters

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(0.2 µm pore size), and added to the suspensions under sterile conditions.

Glutathione content of the culture media, amino acid content of the cells, and dry weight of the suspensions were measured as described before [2, 11]. Enzymatic synthesis of glutathione from the consisting amino acids was carried out *in vitro* by a modified procedure of Carringer *et al.* [12]: Tobacco cells (2 g fresh weight) were suspended in 15 ml 0.1 M potassium phosphate buffer, pH 6.8, together with 1 g Polyclar AT (Serva) and prehomogenized in a potter-homogenizer. The suspension was incubated in a cell disruption bomb (Parr, model 4635) for 5 min at a N_2-pressure of 2000 psi. By release to atmospheric pressure cells and organelles were completely disrupted. The homogenate was centrifuged for 20 min at 40000×*g*, and 3 ml of the supernatant were passed through a Bio-gel P-2 (Bio-Rad) column (12×0.9 cm ∅). Fractions of 20 drops were collected and the combined fractions 5–8 were diluted 1:50 with 0.1 M potassium phosphate buffer pH 6.8. 1 ml of this solution was incubated for 3 min at 25 °C in a reaction mixture that contained the following substances in a total volume of 2.1 ml: 2 µM glutamic acid (Merck), 2 µM cysteine (Merck), 2 µM (^{14}C-U)-glycine (A. Buchler), 4 µM ATP (Boehringer), 0.1 µM $MgSO_4$, and methionine-sulfoximine concentrations of 10^{-12} to 10^{-6} mol/l (all in 0.1 M potassium phosphate buffer, pH 6.8); in controls methionine-sulfoximine was omitted. The reaction was stopped by incubation in boiling water (15 min). After cooling down to room temperature, 0.2 ml N-ethyl-maleimide (Sigma) solution (10 mg/ml) were added for 1 h to alkylate SH-compounds [13]. Separation of labeled substrate and product was performed by TLC using precoated cellulose plates (Macherey, Nagel & Co: CEL-300/0.5 mm) and BuOH/AcAc/H_2O (60/15/25; v/v/v) as solvent system. Radioactive areas were localized and quantitated by a TLC-Scanner (Berthold); co-chromatographed reference compounds were localized using an o-phthalaldehyde spray reagent [2]. The protein content of the samples was determined by the Biuret-Phenol method described by Layne [14].

Results

Influence of methionine-sulfoximine on growth and efflux of glutathione

Growth and efflux of glutathione in photoheterotrophically grown tobacco suspension cultures are both inhibited by methionine-sulfoximine. As shown in Fig. 1 smaller amounts of methionine-sulfoximine are sufficient to reduce the accumulation of of glutathione in the culture medium than to reduce growth: The amount of glutathione in the medium of tobacco suspensions, cultured for 10 days in the presence of 3×10^{-9} M methionine-sulfoximine, reached only 50% of controls without methionine-sulfoximine, although growth has not been affected by this methi-

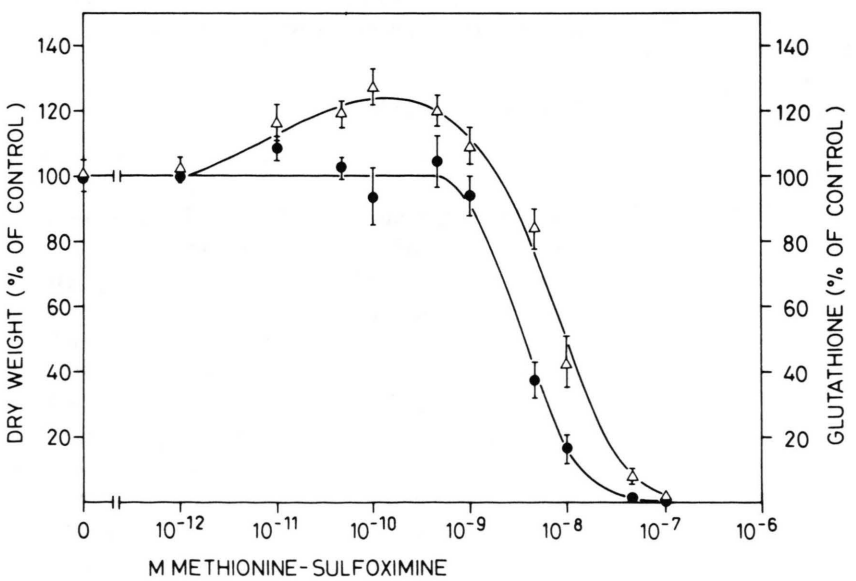
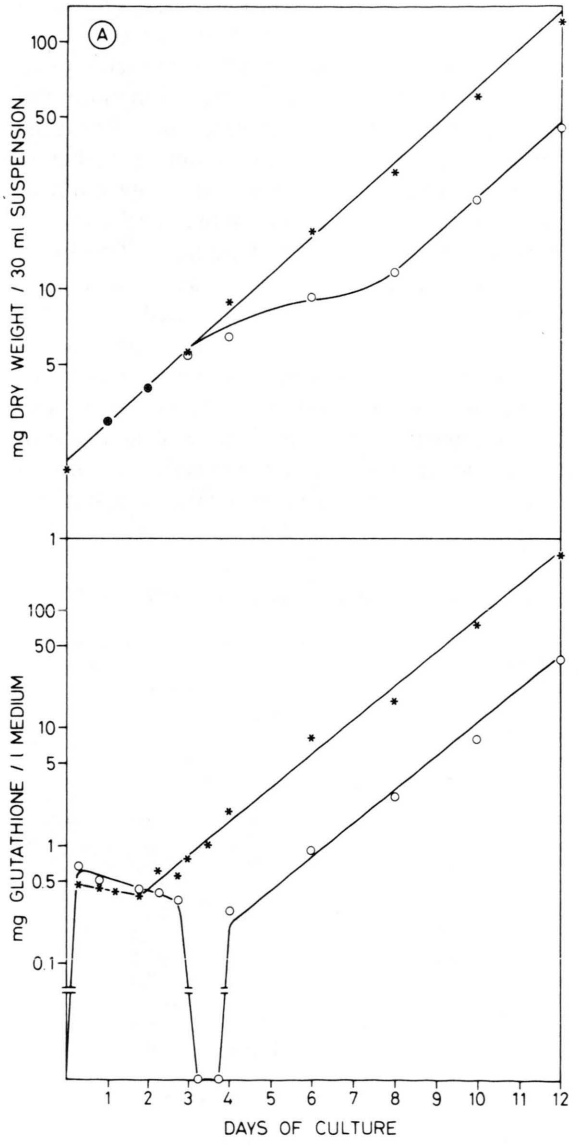


Fig. 1. Effects of different methionine-sulfoximine concentrations on efflux of glutathione and growth of tobacco suspension cultures. 1.7–2 mg d.w. exponential phase tobacco cells were inoculated in 30 ml portions of M+S media containing different amounts of methionine-sulfoximine. After 10 days of photoheterotrophical growth, cells were filtered and the d.w. yield of the cells as well as the glutathione content of the growth media were measured. ○–○ glutathione; △–△ dry weight yield.

onine-sulfoximine concentration. To reduce dry weight yield to 50% addition of 10^{-8} M methionine-sulfoximine to the growth medium is necessary. Methionine-sulfoximine concentrations of 10^{-11} M to 10^{-9} M showed a slight stimulation of growth, but did not influence efflux of glutathione.

The decreased dry weight yield in methionine-sulfoximine treated tobacco suspensions is not caused by a reduction of the growth rate of the cells, but by an inhibition of growth during a definite time of culture. Growth of tobacco cells, inoculated into a



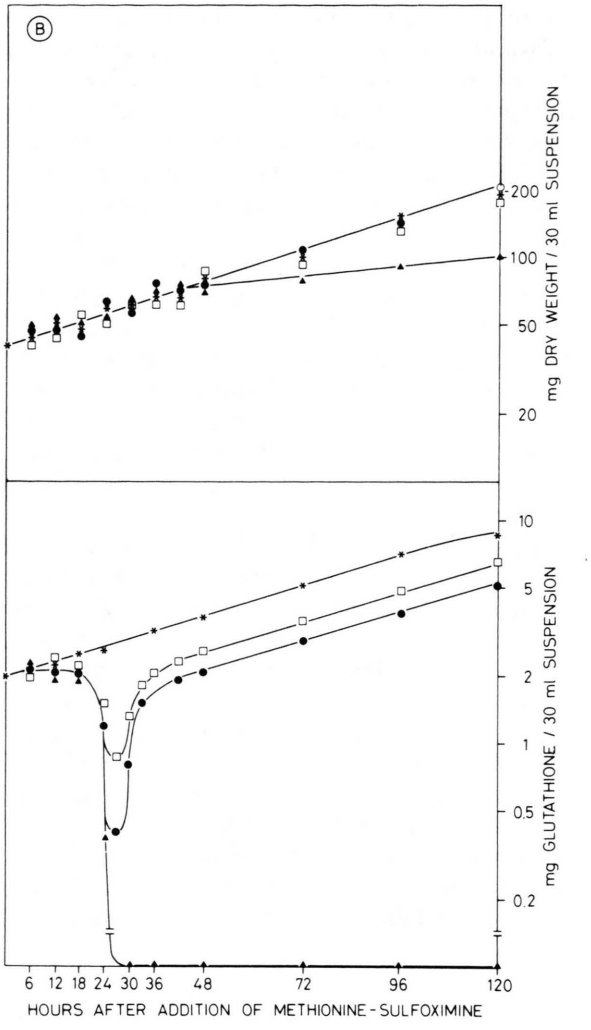


Fig. 2. Influence of methionine-sulfoximine on duration of growth and efflux of glutathione in tobacco suspension cultures. A 1.7–2 mg d.w. exponential phase tobacco cells were inoculated into 30 ml portions of M+S media containing 10^{-8} M methionine-sulfoximine. In controls methionine-sulfoximine was omitted. Suspensions were grown photoheterotrophically for the times shown, filtered, and the dry weight yield of the cells as well as the glutathione content of the culture media were measured. ×–× controls without methionine-sulfoximine; ○–○ 10^{-8} M methionine-sulfoximine. B 1.7–2 mg d.w. exponential phase tobacco cells were inoculated into 30 ml portions of methionine-sulfoximine free M+S media. After 10 days of photoheterotrophically growth methionine-sulfoximine was added to the suspensions. In controls bidestilled water was added instead of methionine-sulfoximine. Dry weight yield of the cells and the glutathione content of the growth media were measured at the culture times shown. ×–× controls without addition of methionine-sulfoximine; ○–○ addition of 10^{-8} M methionine-sulfoximine; ●–● addition of 5×10^{-7} M methionine-sulfoximine; ▲–▲ addition of 10^{-6} M methionine-sulfoximine.

culture medium that contained 10^{-8} M methionine-sulfoximine, is unaffected by this compound during the first three days, inhibited during the following five days, but continued with the rate of controls without methionine-sulfoximine after the eighth day of culture (Fig. 2 A). A similar picture was observed for the influence of methionine-sulfoximine on the duration of efflux of glutathione. After inoculation of tobacco cells into a culture medium with 10^{-8} M methionine-sulfoximine, glutathione is released like in controls without methionine-sulfoximine for about two days, but taken up again between the third and forth day of culture, so that hardly any glutathione could be detected in the medium for about 12 hours; one day later, efflux of glutathione takes place again with the same rate as observed in controls without methionine-sulfoximine (Fig. 2 A).

Addition of $10^{-8} - 5 \times 10^{-7}$ M methionine-sulfoximine to tobacco suspensions, precultured without methionine-sulfoximine for 10 days, caused an uptake of glutathione from the culture medium, too, but did not affect the growth of the cells; about two days after addition of methionine-sulfoximine, accumulation of glutathione in the medium continued with the rate of controls, again (Fig. 2 B). Addition of higher amounts of methionine-sulfoximine reduced the growth rate of tobacco cells and caused the uptake of all the glutathione accumulated in the culture medium; under these conditions no further efflux of glutathione or recovery of the growth rate was observed in the suspensions (Fig. 2 B). These data indicate that tobacco cells in suspension cultures are able to use extracellular glutathione, when synthesis of glutathione inside the cells is inhibited; furthermore they show evidence that small amounts of methionine-sulfoximine can be detoxicated by tobacco cells.

Influence of methionine-sulfoximine on intracellular glutamine content

Tobacco suspensions, inoculated in methionine-sulfoximine containing media, need less time to recover from the methionine-sulfoximine treatment with respect to the accumulation of glutathione in the media than with respect to growth (Fig. 2 A). This observation indicates an action of methionine-sulfoximine in tobacco cells different from inhibition of glutathione synthesis. As methionine-sulfoximine is known to be an inhibitor of glutamine synthetase in higher plants [7, 8], amino acid composition of methionine-sulfoximine treated tobacco cells was investigated. As shown in Table I, addition of 5×10^{-7} M methionine-sulfoximine to tobacco suspensions, precultured in NH_4NO_3-containing media without methionine-sulfoximine for 10 days, caused a decrease of the glutamine concentration to about 13% of controls, accompanied with an enhanced ammonium content of the cells. The concentrations of other amino acids in the tobacco cells are reduced by addition of methionine-sulfoximine, too, but to a

Table I. Influence of methionine-sulfoximine on the amino acid content of tobacco cells grown with NH_4NO_3 in suspension culture.

Amino acid [µM/g d. w.]	Days of culture after a 10 days preculture without MSO									
	−methionine-sulfoximine					+ methionine-sulfoximine				
	0	1	2	3	4	0	1	2	3	4
ASP	3.3	2.9	3.5	–	–	3.3	–	–	–	–
THR	5.6	5.4	5.5	4.5	4.8	5.6	5.7	5.2	3.4	–
SER	18.0	15.7	15.6	12.6	15.2	18.0	16.3	15.3	6.3	5.6
ASN	20.6	24.0	23.4	27.0	27.1	20.6	16.0	8.6	6.4	7.2
GLU	11.2	10.7	10.4	15.4	11.3	11.2	15.2	5.5	11.8	9.3
GLN	398.9	391.4	387.3	374.6	416.0	398.9	112.8	50.2	83.8	108.2
GLY	4.2	3.6	3.2	3.7	4.3	4.2	3.1	–	3.4	1.2
ALA	34.4	54.4	46.1	40.4	36.3	34.4	35.4	23.9	33.1	31.1
CIT	5.5	4.2	3.2	–	–	5.5	9.2	7.9	6.4	4.1
VAL	3.0	3.1	–	–	–	3.0	3.2	–	–	–
γ-ABS	34.3	46.6	31.6	29.3	28.8	34.3	28.8	18.9	25.8	25.5
NH_4^+	176.2	167.8	146.7	132.9	82.0	176.2	191.8	189.3	200.4	158.4
LYS	3.5	3.4	2.9	3.1	3.0	3.5	–	–	–	–
HIS	3.3	3.4	–	–	5.0	3.3	–	–	–	–
ARG	4.9	5.3	4.4	5.7	8.5	4.9	3.4	–	–	–

much lesser extent than glutamine (Table I). Similar to the glutathione content of the culture medium (Fig. 2 B), the glutamine concentration inside the cells increased again, two days after addition of methionine-sulfoximine. From these results it is concluded, that tobacco cells are able to cope with certain amounts of methionine-sulfoximine because of an intracellular glutamine and an extracellular glutathione reservoir, that can be used during the time glutamine and glutathione synthesis are inhibited by methionine-sulfoximine.

Prevention of methionine-sulfoximine mediated growth inhibition by extracellular glutathione and glutamine supply

To confirm this assumption, methionine-sulfoximine was added to tobacco cells grown with nitrate as sole nitrogen source. In suspensions supplied with this nitrogen source only small amounts of glutathione were released into the culture medium and the intracellular glutamine concentration was maintained at a low level [3, 11]. Dry weight yield of tobacco cells treated with methionine-sulfoximine, four days after inoculation into a culture medium with nitrate as sole nitrogen source, is not influenced by 10^{-9} M methionine-sulfoximine, but reduced by higher methionine-sulfoximine concentrations (Fig. 3). Growth inhibition caused by 10^{-7} M methionine-sulfoximine could be prevented by addition of both 0.5 mM glutathione and 0.5 mM glutamine; addition of these amounts of glutathione and glutamine was not appropriate to prevent growth inhibition caused by 10^{-6} M methionine-sulfoximine completely (Fig. 3). In cultures grown with 10^{-7} M methionine-sulfoximine the whole amount of glutamine and glutathione added to the medium was taken up by the cells. These data show, that a sufficient supply with glutamine and glutathione counteracts the methionine-sulfoximine mediated decrease of growth by an uptake of these substances from the culture medium, indicating that the action of methionine-sulfoximine *in vivo* is due to an inhibition of the synthesis of these two compounds.

Influence of methionine-sulfoximine on enzymes of glutathione synthesis

Inhibition of glutathione synthesis by methionine-sulfoximine could be demonstrated *in vitro*, too, using crude homogenates of tobacco cells grown photoheterotrophically with high ammonium and

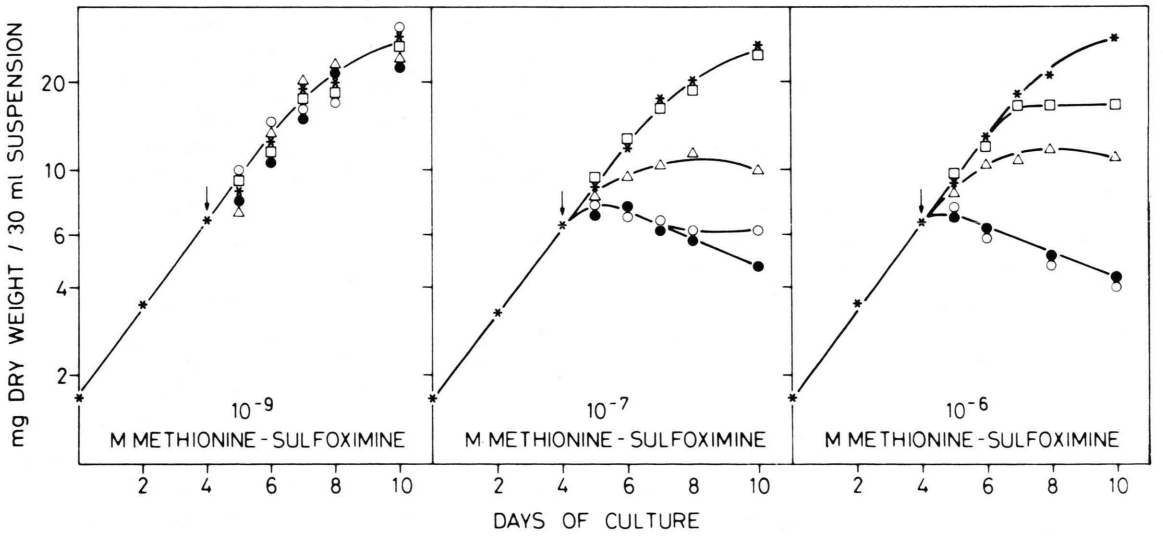


Fig. 3. Influence of extracellular glutathione and/or glutamine on growth of methionine-sulfoximine treated tobacco cells, cultured with nitrate as sole nitrogen source. 1.7–2 mg d. w. exponential phase tobacco cells were inoculated in 30 ml portions of M + S media, containing equimolar amounts of $Ca(NO_3)_2$ instead of NH_4NO_3. After 4 days of photoheterotrophical growth methionine-sulfoximine, glutamine and glutathione were added. At the culture times shown, suspension were filtered and dry weight yields of the cells as well as glutathione and glutamine content of the growth media were measured. ×—× Dry weight: controls without addition; ●—● dry weight: addition of methionine-sulfoximine (10^{-6}–10^{-9} M); ○—○ dry weight: addition of methionine-sulfoximine (10^{-6}–10^{-9} M) + GSH (0.5 mM); △—△ dry weight: addition of methionine-sulfoximine (10^{-6}–10^{-9} M) + Gln (0.5 mM); □—□ dry weight: addition of methionine-sulfoximine (10^{-6}–10^{-9} M) + GSH (0.5 mM) + Gln (0.5 mM).

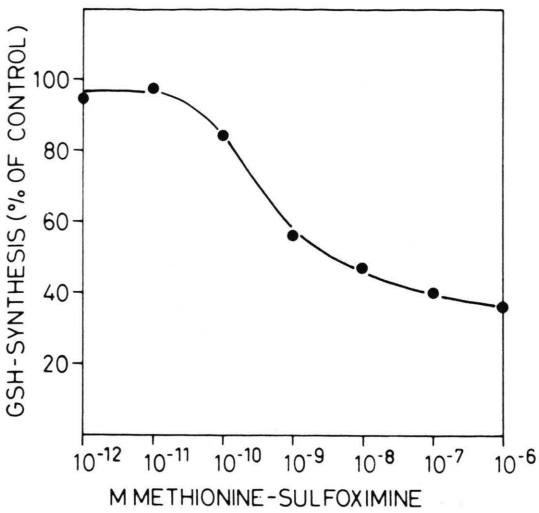
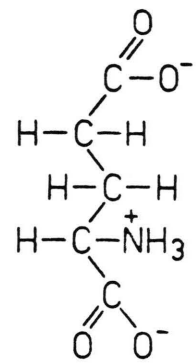
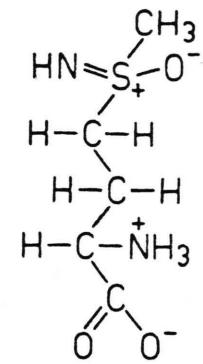


Fig. 4. Inhibition of glutathione synthesis by methionine-sulfoximine *in vitro*. Homogenates of tobacco cells, grown photoheterotrophically in an M+S medium, were inoculated in a reaction mixture, that contained [^{14}C-U]glycine, glutamic acid, cysteine, ATP, and different concentrations of methionine-sulfoximine; in controls methionine-sulfoximine was omitted. Labeled substrate and product were separated by TLC after alkylation of SH-compounds and measured with a TLC-Scanner.



Glutamic acid



Methionine-sulfoximine

sulfate concentrations. In the test tube system, glutathione synthesis from the consisting amino acids decreased to 50% in the presence of 3×10^{-8} M methionine-sulfoximine (Fig. 4). This result fits well with the observation that the accumulation of glutathione in the medium of tobacco suspensions, cultured for 10 days with the same amount of methionine-sulfoximine, is reduced to 50%, too (Fig. 1). Furthermore the inhibition *in vitro* indicates that the activity, and not the amount of enzymes of glutathione synthesis is affected by methionine-sulfoximine.

Discussion

Methionine-sulfoximine is a metabolic inhibitor that has found numerous applications in studies of the nitrogen metabolism in plants (*cf.* [15]). The mode of action of this growth inhibitor in plant cells, however, has not completely been elucidated. In animal cells methionine-sulfoximine is known to be a potent inhibitor of glutamine synthetase (GS), acting as a competitive inhibitor with respect to glutamate and as an irreversible inhibitor, simultaneously [6].

About the action of methionine-sulfoximine on GS in higher plants only few data are available: Addition of methionine-sulfoximine to Lemna minor brings about a rapid loss of GS activity and a decrease in the glutamine content of the plants [7]; such a decrease was confirmed by the present experiments in methionine-sulfoximine treated tobacco cells. Furthermore, incorporation of labeled ammonium and nitrate into glutamine was shown to be inhibited by methionine-sulfoximine in tobacco suspension cultures [8]. These data indicate, that GS of higher plants is inhibited by methionine-sulfoximine, although studies with purified GS are lacking.

Activation of the γ-carboxyl-group of glutamate by formation of an enzyme bound acyl-phosphate is not restricted to the synthesis of glutamine, but also takes place during enzymatic synthesis of other γ-glutamyl compounds. Orlowski and Meister [16] have shown that – despite a number of significant differences – in animal cells the reactions catalyzed by GS and γ-glutamylcysteine-synthetase (GCS) both involve intermediate formation of enzyme bound γ-glutamylphosphate. So it is not surprising that GCS is inhibited in animal cells by methionine-sulfoximine in a similar way than GS [17]. As GCS catalyzes the first step in glutathione synthesis, methionine-sulfoximine is a potent inhibitor of the synthesis of this peptide in animal cells [6].

The current experiments present evidence that synthesis of glutathione in tobacco suspension cultures is also inhibited by methionine-sulfoximine. In contrary to animal cells, where mmolar amounts of methionine-sulfoximine are necessary for inhibition of glutathione synthesis *in vivo* [18] and *in vitro* [17], glutathione synthesis in tobacco cells is affected by much smaller methionine-sulfoximine concentra-

tions. *In vivo,* treatment with 5×10^{-7} M methionine-sulfoximine caused an uptake of glutathione from the culture medium accompanied with a considerable decrease of the glutamine content of the cells. Growth of tobacco suspensions is affected by methionine-sulfoximine to a much lesser extent. Methionine-sulfoximine mediated growth inhibition in suspensions that did not contain high amounts of extracellular glutathione and intracellular glutamine can be prevented by addition of these substances to the growth medium. These observations can be explained by an inhibition of glutathione and glutamine synthesis or by an enhanced turnover of these compounds caused by methionine-sulfoximine. As, *in vitro,* enzymes of glutathione synthesis are inhibited by treatment with methionine-sulfoximine, the effect of methionine-sulfoximine on extracellular glutathione in tobacco suspensions *in vivo* is probably due to such an inhibition, too.

Tobacco suspensions with a sufficient glutathione and glutamine supply are able to cope even with high amounts of methionine-sulfoximine (Fig. 3). No inhibition of growth was observed under these conditions. These data indicate, that glutathione and glutamine reservoirs can be used by tobacco cells during the time, synthesis of these compounds inside the cells is inhibited by methionine-sulfoximine. The demonstration of an uptake of glutathione from the culture medium in methionine-sulfoximine treated suspensions confirms this conclusion. The amount of glutathione taken up by methionine-sulfoximine treated tobacco cells, grown with nitrate as sole nitrogen source, is calculated to be sufficient to maintain a glutathione concentration of 40 μM inside the cells, provided that degradation of glutathione can be neglected. This calculated glutathione concentration agrees with the glutathione content of tobacco cells measured in former experiments [2]. In addition, the recovery of growth and the recurrance of the initial rate of growth and efflux of glutathione in suspensions inoculated into methionine-sulfoximine containing media shows, that tobacco cells are able to detoxicate certain amounts of this inhibitor (Fig. 2 A). The ability of tobacco cells to use extracellular glutathione to counteract inhibition of glutathione synthesis by methionine-sulfoximine inside the cells, indicates an uptake of the complete glutathione molecule. As it is known from animal cells that extracellular glutathione is taken up after degradation, only [19], further experiments using labeled glutathione will show, whether such a difference in the uptake of glutathione can be confirmed between plant and animal cells.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. We thank Prof. Dr. L. Bergmann for valuable suggestions and critical reading of the manuscript, Dr. W. Grosse for carrying out amino acid analysis, and Mrs. B. Schaer for cooperation in measuring enzymes of glutathione synthesis. The skilful technical assistance of Mrs. A. Napieraj is greatfully acknowledged.

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