Ammonia Accumulation and Inhibition of Photosynthesis in Methionine Sulfoximine Treated Spinach

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ABSTRACT

Ammonia accumulation and photosynthetic rate inhibition took place when spinach leaf tissue was supplied with methionine sulfoximine (MSO), an inhibitor of glutamine synthetase. This effect was observed in the absence of significant inorganic nitrogen reduction or an exogenous source of ammonia. Both the time lag prior to the initial photosynthetic rate decrease and the rate of that decrease depend on the O2 and MSO concentrations supplied to the leaf tissue. However, the total rate of ammonia accumulation was similar at 20% and 2.2% O2. The decline in photosynthetic rate was not caused by stomatal closure but may be a result of ammonia toxicity. The data point out the importance of glutamine synthetase in preventing the poisoning of leaf metabolism by ammonia generated internally through processes not involved in net nitrogen assimilation. The rapidity of the action of MSO in suppressing photosynthesis was unexpected and should not be overlooked in interpreting data from other experiments involving that inhibitor. MSO shows promise as a tool for investigating C-N flow, particularly during photorespiration.

A thorough understanding of plant carbon-nitrogen metabolic flows is essential to efforts to control plant protein production (18). MSO, an irreversible inhibitor of GS (27), has been widely used in such studies, partly because of evidence that GS catalyzes the primary incorporation of ammonia (14). Investigations of plant ammonia assimilation (1, 3, 25), leaf amino acid metabolism (11, 26), blue-green algal growth and heterocyst formation (23), photorespiration (12), and attempts to develop mutants producing elevated levels of methionine (6, 24) have involved use of MSO. MSO inhibits the growth of Chlorella (16), blue-green algae (23), and cowpea (6), corn, wheat, and soybean plants (24). Such experiments have involved observation of parameters related to long-term growth, and little attempt was made to explain the mechanism of growth inhibition. Sinden and Durbin (22), however, did observe an elevated ammonia level in tobacco leaves several days after MSO treatment. They suggested that inhibition of GS and a buildup of the products of nitrate metabolism might be involved in MSO toxicity.

Here, we demonstrate that MSO rapidly inhibits leaf PS and causes a rapid increase in ammonia levels. We discuss our results with respect to photorespiration and photorespiratory N-recycling in C3 plant leaves (12).

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2 Abbreviations: MSO, methionine sulfoximine; C3 plants, plants which initially fix CO2 primarily by the photosynthetic carbon reduction or C3 cycle; GS, glutamine synthetase [L-glutamate:ammonia ligase (ADP), EC 6.3.1.2]; PR, photorespiration; PS, photosynthesis.

MATERIALS AND METHODS

Plant Material. Spinach (Spinacia oleracea L., cv. Burpee Hybrid 7) was grown in a sand-soil-peat mixture under artificial light with a 9-h light period (260 µE m-2 s-1, 21 C) and a 15-h dark period (16 C). Plants were fertilized with modified Hoagland solution. Leaves used were about 0.35 dm² in area and were rinsed with deionized H2O. When necessary, the leaves were placed on a wet rubber sheet and 1-cm diameter discs were cut out with a cork borer.

Photorespiration Experiments with Whole Leaves. A closed steady-state gas circulation system was employed (17). It provides for circulation of a gas stream of monitored and controlled 14CO2 and O2 content. 14CO2 concentration was measured by means of a Beckman 865 IR analyzer (Beckman Instruments, Fullerton, CA). A 348-ml Lucite chamber (water-jacketed, 25 ± 1 C) illuminated with two opposed banks of high output cool-white fluorescent lamps (General Electric, Nela Park, OH) was utilized. The photosynthetic photon flux density (400–700 nm) was adjusted by means of white-paper diffusing panels and was measured by means of an LI-190S quantum sensor fitted to an LI-185A quantum/radiometer/photometer (Lambda Instruments, Lincoln, NE). The photosynthetic photon flux density values at the upper and lower leaf surfaces (kept equal), were summed to give the reported value of 375 µE m-2 s-1. Each experiment utilized two leaves with their petioles initially immersed in water.

Experiments were begun by exposing the leaves to CO2-free air and allowing them to respire in the light to about 40 µl/CO2 for 15 min. The CO2 level then was adjusted to 325 µl/1 and the leaves were allowed to photosynthesize for 20 to 30 min. CO2 concentration was maintained in the range 265 to 325 µl/1 throughout the remainder of the experiment by manually adding 21% CO2 in N2 as necessary. Following the first period of PS, the petiole solution was replaced with 1, 5, or 10 mM MSO solution (l-methionine sulfoximine, Aldrich Chemical Co., Milwaukee, WI), and CO2 uptake was recorded (treatment); alternatively, the PS rate was followed without replacing the petiole solution (controls). PS rates were calculated from the slope of the 14CO2 recorder trace at 295 µl/L, system gas volume, and leaf area.

In several experiments, the stomatal resistances of the abaxial (lower) leaf surfaces were determined with an LI-65 diffusive resistance porometer and a calibrated LI-205 sensor (Lambda Instruments). In these experiments, an 8 mM MSO solution was utilized as the treatment condition.

Photorespiration Experiments with Leaf Discs. Leaf-disc experiments, also using the closed gas circulation system, were conducted...
essentially as described previously (19). For each experiment, several randomly selected discs were weighed to determine the average disc fresh weight. Each sample consisted of five 1-cm diameter discs floated upside down on water in an exposure flask. For a single experiment (Figs. 2–4; Tables I and II), eight flasks were placed in a shaking device (24 ± 1°C and illuminated (550 μE m⁻² s⁻¹) from below by means of a bank of high output Criticole fluorescent lamps (Verd-a-Ray Corp., Toledo, OH). Gas flow was commenced with CO₂-free air followed by 325 μl/l CO₂ in air as described above for whole leaves. Following 15 to 30 min with air level CO₂ and O₂, the O₂ concentration was adjusted to either 2.2% or 20%, and CO₂ was adjusted to either 190 or 295 μl/l. CO₂ concentration was maintained within ±10% of those values throughout the remainder of the experiment by manual addition of NaCO₃ as necessary. After the first 15 to 25 min with the final CO₂-O₂ gas composition, MSO solution was injected into the flasks so as to bring the solution upon which the discs were floating to 8 mM MSO. PS rates were determined from the recorder slope at 190 or 295 μl/l, the system gas volume, and the leaf-disc area. Exposure flasks were removed as a function of time, and the discs contained were immediately plunged into hot water (80°C) and ground by means of a Duall tissue grinder (Kontes Glass Co., Vineland, NJ). The homogenized discs were extracted in hot water for 10 min and then for an additional 45 min (with stirring) at room temperature. The supernatant fraction from each sample was collected following centrifugation (20 min at 1900 × g). The solution upon which each leaf-disc sample was floating was also collected during several experiments. Experiments were repeated several times.

Ammonia, Nitrate, and Nitrite Analyses. The leaf-disk extract supernatant solutions and float solutions collected above were analyzed for inorganic nitrogen. Ammonia was transferred by diffusion from buffered (pH 11) aliquots of extracts solution into 2% boric acid by means of a Conway cell. The ammonia was determined using the method of Weatherford (28) with standards prepared in boric acid solution. Float solutions were analyzed directly by the same method with standards prepared in water (control samples) or 8 mM MSO (treated samples). Both float solutions and disc extracts were analyzed directly for nitrite by adding a 1:1 mixture of the color reagents (20). Nitrate was analyzed by means of a spectrophotometric procedure based on that of Heuer and Plaut (10). Although the assay also detects NO₃⁻, correction for A due to that material was obviated by its virtual absence in our samples (Table III). Nitrate indicator solution was prepared as follows. Equal volumes of concentrated H₂SO₄ and H₃PO₄ were mixed. After 1 week, 0.5 g diphenylamine sulfonic acid, sodium salt (Mallinkrodt, Paris, KY), was dissolved per 100 ml mixture. This gave an initially dark blue solution which, after about 10 days, had lost much of its color (blanks prepared as described below have an A of <0.1 relative to water). Aliquots (0.25 ml) of float solution or leaf extract were mixed with 2.5 cc aged indicator solution and the A at 570 nm (1-cm cells) was determined after 30 min. Standards containing 0.00 to 0.15 μg NO₃⁻/N/0.25 cc H₂O were similarly prepared. Added internal standards equivalent to 6.5 μg NO₃⁻/N/g fresh weight could be detected with 90% recovery.

Conversion Factors. The individual leaf discs used in an experiment had an average fresh weight of 22.6 ± 2.0 mg (mean ± SD, 20 determinations) and an area of 0.00785 dm². Hence, ammonia accumulation rates expressed as μg NH₃-N/g fresh weight-h can be divided by 6 to yield approximate rates in units of μmol NH₃/disk-h. Expressed disc NH₃ contents in mm units were calculated by taking 1 g leaf fresh weight to be equivalent to 1 cm² leaf volume (excluding air spaces).

RESULTS

Whole Leaf Photosynthesis Experiments. MSO rapidly inhibited PS in air (295 μl/l CO₂, 20% O₂) by whole excised spinach leaves (Fig. 1). With 10 mM MSO, the spinach leaf PS rate declined to 50% of its initial value after 55 min. Virtually complete inhibition occurred after 120 min. The time lag prior to the initial rate decrease and the slope of that decrease were dependent upon the concentration of MSO. Control spinach leaves exhibited an unchanged PS rate for 350 min.

Stomatal resistance of leaves prior to insertion in the Lucite experimental chamber was 8.6 ± 2.4 s cm⁻¹. Following the low CO₂ pretreatment and 15 min PS (295 μl/l CO₂, 20% O₂, 375 μE m⁻² s⁻¹, 24°C), stomatal resistance had declined to 2.9 ± 0.4 s cm⁻¹. After treatment with 8 mM MSO, PS-rate inhibition occurred. When the PS rate was 87 ± 5% inhibited, stomatal resistance was 2.8 ± 0.6 s cm⁻¹. Hence, MSO did not bring about stomatal closing. Resistance values reported are mean ± standard deviation (three experiments, two leaves each).

Leaf Disc Photosynthesis Experiments. The PS rate (295 μl/l CO₂, 20% O₂) of spinach leaf discs floating on MSO behaved similarly to that shown for whole leaves. The results of a typical experiment are shown in Figure 2. After a short lag period, the PS rate dropped rapidly. As in the case of whole leaves, the rate of decrease and the length of the lag period were dependent upon the concentration of MSO (data not shown). Leaf disc ammonia levels rose rapidly following addition of MSO, and some ammonia was released to the float solution (Fig. 2). Based on an estimated leaf disc volume, excluding air spaces, the maximum intraleaf disc ammonia level of 150 μg NH₃-N/g fresh weight is equivalent to 11 mM NH₃. The PS rate and ammonia content of control discs were unchanged (Fig. 2).

Experiments under low O₂ concentration (2.2% O₂), were conducted at both air level (295 μl/l) and decreased (190 μl/l) CO₂ concentration. With 190 μl/l CO₂ and 2.2% O₂, the initial PS rate was identical (99 ± 3%, four determinations) to that with 295 μl/l CO₂ and 20% O₂. Therefore, the low-CO₂-low-O₂ experiments allow comparison of ammonia accumulation rates at high and low O₂ levels but with equal initial PS rates. With 295 μl/l CO₂ and 2.2% O₂, the initial PS rate of the discs was 145 ± 3% (mean ± SD, six determinations) of that obtained with air level CO₂ and O₂. This increase in PS rate at 2.2% O₂ is diagnostic of the occurrence of rapid photorespiration in C₃ plants at 20% O₂ (4).

As with the disc experiments under 20% O₂, the disc-PS rate under 2.2% O₂ declined and ammonia concentration increased following addition of MSO. Control discs had unchanged rates of PS and levels of ammonia over an extended time period (Figs. 3 and 4).

Fig. 1. Spinach leaf photosynthesis in the presence of MSO at 295 μl/l CO₂, 20% O₂, 375 μE m⁻² s⁻¹, and 24°C. (C), control. MSO concentration after time zero: (△), 1 mM; (×), 5 mM; (○), 10 mM.
The total rate of ammonia accumulation was not appreciably different at high and low O₂, but the rate of PS decreased more gradually under low O₂ (Figs. 2–4; Table I). Discs exposed to MSO under low O₂ started with lower initial ammonia levels than those exposed to air level O₂ (Table II). Higher ammonia levels were associated with a given degree of PS inhibition in the experiments conducted at low O₂ (Table II). The lag phase (here taken to be the time period between MSO addition and 10% inhibited PS rate) was greatly increased at low O₂ concentration, as was the time period required to reach 75% PS rate inhibition (Table I). The discs seemed to release a greater fraction of the accumulated ammonia to the solution when maintained at low O₂ (Table I). The accumulation rate of 67 μg NH₃-N/g fresh weight...
Table I. Photosynthetic Rate Inhibition and Ammonia Accumulation in 8 mM MSO-treated Leaf Discs

Discs were exposed to a light level of 550 µE m$^{-2}$ s$^{-1}$ at 25 C. Experiments were conducted with conditions described in Figures 2 to 4. Each experiment utilized eight sample flasks, prepared as given under "Materials and Methods." Values are mean ± SD. The number of experiments is given in parentheses.

<table>
<thead>
<tr>
<th>Gas Composition</th>
<th>Ammonia Accumulation Rate</th>
<th>Time after MSO Addition for Photosynthetic Rate Inhibition</th>
<th>Time Between 10 and 60% Photosynthetic Rate Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total*</td>
<td>In discs</td>
<td>10% Inhibition</td>
</tr>
<tr>
<td></td>
<td>µg NH$_3$-N/g fresh wt-h</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>295 µl/1 CO$_2$, 20% O$_2$</td>
<td>67 ± 4 (2) 55 ± 5 (4)</td>
<td>32 ± 10 (5) 90 ± 19 (5)</td>
<td>40 ± 10 (5)</td>
</tr>
<tr>
<td>295 µl/1 CO$_2$, 2.2% O$_2$</td>
<td>62 (1) 33 ± 12 (2)</td>
<td>90 ± 14 (2) 294 ± 36 (2)</td>
<td>131 ± 16 (2)</td>
</tr>
<tr>
<td>190 µl/1 CO$_2$, 2.2% O$_2$</td>
<td>57 ± 14 (2) 35 ± 8 (3)</td>
<td>105 ± 15 (3) 258 ± 26 (3)</td>
<td>111 ± 8 (3)</td>
</tr>
</tbody>
</table>

* Includes ammonia released to float solution.

Table II. Ammonia Accumulation in Leaf Discs as a Function of MSO (8 mM) Inhibition of PS

Experiments were conducted with conditions described in Figures 2 to 4 and Table I. Values are mean ± SD and do not include ammonia found in the float solution. The number of experiments is given in parentheses.

<table>
<thead>
<tr>
<th>Gas Composition</th>
<th>NH$_3$-N Accumulation at the Following Extents of PS-rate Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>Amount</td>
</tr>
<tr>
<td></td>
<td>µg NH$_3$-N/g fresh wt</td>
</tr>
<tr>
<td>295 µl/1 CO$_2$, 20% O$_2$</td>
<td>44 ± 7 (4) 3.1</td>
</tr>
<tr>
<td>295 µl/1 CO$_2$, 2.2% O$_2$</td>
<td>34 ± 2 (2) 2.4</td>
</tr>
<tr>
<td>190 µl/1 CO$_2$, 2.2% O$_2$</td>
<td>26 ± 10 (3) 1.9</td>
</tr>
</tbody>
</table>

* Concentration was calculated using leaf-disc volume estimated as described under "Materials and Methods."

Table III. Effect of MSO Solution on Total Leaf Disc NO$_3^-$, NO$_2^-$, and NH$_3$ Contents during PS

Four experiments were conducted at each O$_2$ level. Each experiment utilized one leaf from which discs were selected to prepare two sample flasks. Discs from one of the flasks were analyzed after PS under the conditions given and prior to MSO addition; the discs from the second flask were analyzed after an additional 140 min in the presence of 8 mM MSO. Photosynthetic conditions were: 550 µE m$^{-2}$ s$^{-1}$; 295 µl/1 CO$_2$, 20% O$_2$, and 2.2% O$_2$, 25 C. Values shown include material in float solution as well as in discs.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inorganic N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level Prior to MSO Addition</td>
</tr>
<tr>
<td></td>
<td>µg inorganic-N/g fresh wt</td>
</tr>
<tr>
<td>Air level O$_2$ (20%)</td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>&lt;2.0*</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>33.9 ± 2.2</td>
</tr>
<tr>
<td>Decreased O$_2$ (2.2%)</td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>&lt;2.0*</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>23.5 ± 5.1</td>
</tr>
</tbody>
</table>

* NO$_3^-$-N content was near the lower limit of the assay's sensitivity.

Equivalent to estimated rates of PR. That work involved the use of MSO, as a specific inhibitor of ammonia reabsorption, but at levels which seemingly would also inhibit PS. In summary, Keys et al. proposed that glycolate produced during PR is converted to glycine and then to serine + CO$_2$ + NH$_3$. The ammonia released in this conversion initially came from glutamate; the cycle is closed through ammonia reabsorption catalyzed by leaf GS and glutamate synthase. The CO$_2$ released contributes to the lowering of the net rate of PS caused by PR.

We considered that the NH$_3$ observed in our leaf discs following MSO treatment might originate from PR with MSO blocking its reabsorption as catalyzed by GS. Our low O$_2$ concentration experiments were conducted to investigate that possibility; low O$_2$ concentration is accepted to decrease PR in C$_3$ plants (4) and would, therefore, be expected to decrease NH$_3$ release by the glycine → serine conversion. We found that, even under low O$_2$ conditions, ammonia accumulated in our discs and PS was inhibited. While at low O$_2$, the lag period was increased and a greater concentration of ammonia was associated with a given degree of PS inhibition, rates of ammonia production were surprisingly similar to those found at elevated O$_2$ (Tables I and II).

To the extent that our ammonia accumulation rates approximate glycine → serine activity, the rate similarity at high and low O$_2$ is evidence against greatly differing carbon flows through that reaction under the two conditions. However, several factors prevent our drawing that conclusion with certainty at this time: ammonia may be produced from other sources, such as amino acid degradation during protein turnover; PS is declining (Figs. 2–4) as NH$_3$ accumulates and this could affect regeneration of the ribulose 1,5-bisP necessary as a glycylate precursor. Furthermore, prior to MSO addition, a high rate of PR N recycling could occur, utilizing only a small pool of pre-existing glutamate or glutamine. Once MSO is added, significant NH$_3$ is produced and not reoxidized (Figs. 2–4). The rate of the glycine-serine conversion then might be limited by the availability of amino donors to glyoxylate (the precursor of glycine). The difference in initial ammonia levels at 2.2 and 20% O$_2$ (Table II) may indicate unequal ammonia production prior to MSO addition.

Interpretations of carbon-labeling data (2, 8) as evidencing a high rate of glycine → serine flow in air are of limited value given...

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multiple sources and uses of those amino acids (8, 18, 21). The high degree of PS inhibition by O2 in C3 plants need not imply a high rate of glycine → serine flow and CO2 + NH3 release. Direct competitive inhibition of ribulose bisP carboxylase by O2 (4), along with CO2 release directly from glyoxylate by pathways which do not release ammonia (15), may bring about much of the PS rate inhibition.

Additional experimentation is necessary to establish the biochemical source of the accumulating ammonia. If it in fact originates from the glycine → serine reaction, MSO treatment of isolated leaf cells supplied with a proper exogenous source of amino nitrogen, combined with rapid NH3 analysis prior to a significant PS rate decline, may provide a new means of measuring both glycine → serine flow and PR N recycling in leaves. Such measurements would supplement the usual estimates of PR based upon carbon tracer studies or physiological parameters. Given the promise of MSO as a tool for studying C-N flow during PR, these matters are being further investigated.

Ammonia, although a plant nutrient and metabolite, is toxic in excess, affecting chloroplast morphology and a wide range of metabolic functions, including patterns of carbon flow, enzyme activity, and the coupling of photosynthetic phosphorylation to electron transport (7, 9, 13, 19). However, it has proven difficult to determine the specific cause of ammonia toxicity in higher plant tissue (7). The increase in disc ammonia levels (Tables 1 and 2) following MSO treatment strongly suggests that ammonia toxicity is partly responsible for the observed PS decline. For example, the levels attained exceed those found to cause uncoupling in cell-free chloroplast systems (9, 13). Involvement of mechanisms other than ammonia toxicity in PS inhibition by MSO cannot be ruled out. TaToxin, an active principle in wild fire disease, has many biological effects similar to those of MSO (although the pure toxin has in one case been reported to not inhibit GS) (16, 22). TaToxin has recently been found to inhibit ribulose bisP carboxylase (5); MSO should be examined for similar activity.

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