The Effect of Phosphinothricin on the Assimilation of Ammonia in Plants

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The effects of DL-phosphinothricin and L-methionine sulfoximine on the enzymes of nitrogen assimilation were studied. Furthermore we investigated the accumulation of ammonia and the photosynthetic activity after the treatment of mustard plants with phosphinothricin. Phosphinothricin was a specific and very strong inhibitor of glutamine synthetase. Major differences, however, were found between the phosphinothricin affinity of the leaf enzyme and that of the root of mustard plants. The leaf enzyme was 50% inhibited at a concentration of 10^{-4} M phosphinothricin ($pI_{50} = 4$), whereas the root enzyme already showed the same effect at a concentration of 2×10^{-5} M ($pI_{50} = 4.7$). In addition K_i values of about 0.03 mM for the leaf enzyme and 0.002 mM for the root enzyme respectively were determined. Phosphinothricin treatment of plants caused an ammonia accumulation in tissues. The accumulation was light dependent. At the beginning of the light period the major sources of ammonia accumulation could be the nitrogen assimilation as well as catabolic processes of nitrogen compounds. A clear contribution of photorespiration was only found when higher concentrations of ammonia were reached. The application of phosphinothricin induced a strong reduction of CO_2 assimilation.

Introduction

In this project we tested the inhibitory effects of DL-phosphinothricin on the enzymes of the assimilatory nitrogen metabolism, *i.e.* nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase (GOGAT, glutamine: 2-oxoglutarate aminotransferase), and glutamate dehydrogenase. For comparison, experiments were performed with L-methionine sulfoximine, too. After the treatment of plants with PPT, we further investigated the photosynthetic rate and the accumulation of ammonia in the leaves.

PPT was initially isolated from a tripeptide antibiotic [1], which is produced by a species of *Streptomyces* (*S. viridochromogenes*). The compound is a potent competitive inhibitor of glutamine synthetase of *Escherichia coli* and *Pisum sativum* [2]. For our tests Höchst Inc. (Frankfurt/M., West Germany) supplied us with the free acid (Hoe 35956) and the ammonium salt (Hoe 39866) of PPT. Ammonium-(3-amino-3-carboxypropyl)-methylphosphinate, coded Hoe 39866, is a new non-selective foliar-applied herbicide for the control of undesirable mono- and dicotyledonous plants in orchards, vineyards and plantations [3].

Materials and Methods

Growth conditions

Plants of *Sinapis alba* (mustard plants) were grown in expanded (burnt) clay with Hoagland solution. They were illuminated in a 16:8 h light-dark regime by fluorescent tubes (Osram L, 115 W/25 Sa) with photosynthetically active radiation of about $11 \text{ W} \times \text{m}^{-2}$. The temperature was 21 °C and the relative humidity about 75%.

Chemicals

Chemical structure of PTT:

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-CH_2-CH_2-\overset{\overset{\displaystyle NH_2}{|}}{CH}-COOH,$$
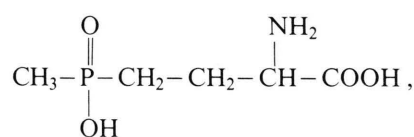
code no. Hoe 35956 (acid); Hoe 39866 (NH_4-salt), nomenclature of IUPAC: ammonium-(3-amino-3-carboxy-propyl)-methylphosphinate. MSO: Serva Corp. (Heidelberg).

Isolation and assay of glutamine synthetase: Leaves or roots of mustard plants were ground in a

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mortar in 50 mM Tris-HCl buffer pH 7.5 with 5 mM dithiothreitol and 0.5 mM EDTA at 4 °C. The homogenate was centrifuged at $20\,000 \times g$ for 10 min, and the supernatant desalted in a Sephadex G 25 column. Enzyme activity was measured at 37 °C by the formation of γ-glutamyl hydroxamate in the synthetase reaction [4]. The concentration (mM) of the reactants in the 2 ml assay mixture was: imidazol-HCl buffer pH 7.2, 100; $MgSO_4$, 50; EDTA, 10; NH_2OH, 10; ATP, 15; glutamate, 50 with the root enzyme and 160 with the leaf enzyme.

Ammonia was determined with the method described by Weatherburn [5]. The determination of the CO_2 fixation in single leaves was performed with an infra-red gas analyzer under controlled conditions while the leaves were still attached to the intact plant [6, 7].

Results and Discussion

The activity of nitrate reductase, nitrite reductase, the ferredoxin-dependent glutamate synthase and the NAD-linked glutamate dehydrogenase was not influenced by PPT and MSO. To some extent MSO inhibited the pyridine-nucleotide dependent glutamate synthase in root tissue (Table I). PPT and MSO, however, were potent inhibitors of glutamine synthetase. The activity of the leaf enzyme was reduced by 50% at 10^{-4} M PPT ($pI_{50} = 4$) and 6.5×10^{-3} M MSO ($pI_{50} = 2.2$), whereas the 50% inhibition of the root enzyme was performed at 2×10^{-5} M PPT ($pI_{50} = 4.7$) and 8×10^{-4} M MSO ($pI_{50} = 3.1$), respectively (Fig. 1).

The kinetics of the inhibition of glutamine synthetase by PPT and MSO were determined. At low concentrations both compounds were competitive inhibitors with respect to glutamate. A K_i
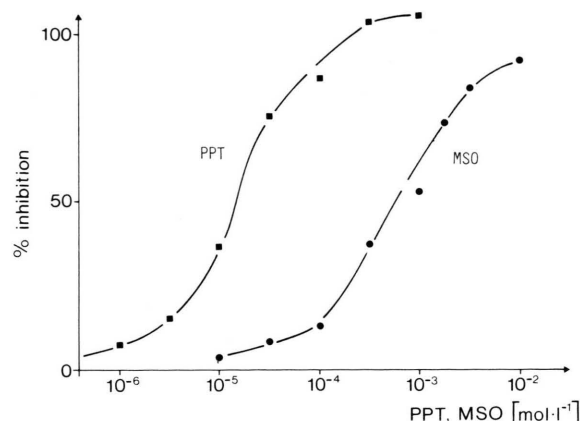

Fig. 1. Inhibition of glutamine synthetase from mustard roots by PPT (■—■) and MSO (●—●). Enzyme activity of control: 200 µmol γ-glutamyl hydroxamate/h $\times g$ fresh weight.
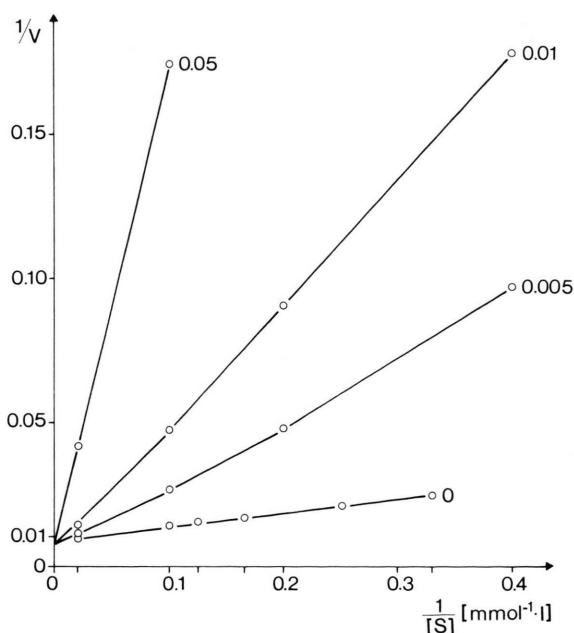

Fig. 2. Inhibitory action of PPT on glutamine synthetase of mustard roots. Lineweaver-Burk plot with varying inhibitor concentrations: O (control), 0.005, 0.01, 0.05 mM, S = glutamate; V = GS activity (in % of the activity of the control). From the slope of the curves it is possible to calculate the K_i value for PPT to 2 µM.

Table I. Effect of PPT and MSO on NADH-dependent GOGAT (enzyme activity in %).

Assay with PPT or MSO, mM	PPT	MSO	H_2O, control
1	99 ± 5	100 ± 9	100 ± 5
10	102 ± 7	91 ± 6	100 ± 5
20	97 ± 2	77 ± 9	100 ± 4
40	99 ± 1	58 ± 6	100 ± 5

Rate of control: 0.6 µmol NADH oxidized/h $\times g$ fresh weight (= 100%).

value of about 0.03 mM for PPT was determined for the leaf enzyme. The K_i value for MSO was about ten times higher. The K_i values obtained for the root enzyme, however, were 2 µM for PPT and 70 µM for MSO (Fig. 2).

The K_m value for glutamate of the root enzyme was 5.2 mM, whereas the leaf enzyme did not show normal kinetics. Therefore, we are now looking whether there are two isoforms of glutamine synthetase in the leaves of mustard plants. Furthermore, we found evidence that the glutamine synthetase in the root and in the leaf tissues was irreversibly inhibited by PPT and MSO when the enzyme was not fully saturated with its substrates. That might cause the upward slope at low glutamine concentrations in the Lineweaver-Burk plots.

Plants make use of inorganically bound nitrogen; that is, they are autotrophic in respect to nitrogen as well as to carbon. Nitrogen is taken up from the soil as nitrate or ammonia. The nitrate taken up is reduced by the nitrate reductase and nitrite reductase to ammonia before the nitrogen is incoporated into organic matter. But ammonia also arises from internal sources of metabolism, *e.g.* asparagine, arginine, glycine or phenylalanine. Thus, ammonia assimilation takes place in different tissues often at higher rates than that of N reduction. For example ammonia release from glycine during photorespiration may reach values which are several times higher than the calculated rate of net ammonia assimilation [8]. Ammonia is an important link between catabolic and anabolic processes and released and reassimilated in large amounts at different processes in the plant metabolism. Regardless of the origin, however, it is essential that the ammonia is rapidly converted into a form which is not toxic for the organism.

Until 1970 glutamate dehydrogenase was considered to be the major enzyme involved in ammonia assimilation in all organisms. Nowadays it is generally accepted that ammonia incorporation into organic compounds occurs *via* the glutamine synthetase-glutamate synthase pathway. This may be characteristic of all plant groups with the exception of most fungi [9]. Glutamine synthetase and glutamate synthase are able to cooperate to form the glutamate synthase cycle. The crucial point of this pathway is that glutamate is both acceptor and product of ammonia assimilation. Glutamine and glutamate are the primary products of inorganic nitrogen assimilation as well as of reassimilation of ammonia and as such occupy central positions in the intermediary nitrogen metabolism. Glutamine synthetase catalyzes the initial step of both primary ammonia incorporation and ammonia recycling. The ubiquious distribution of high levels of glutamine synthetase in plant tissues is necessary to prevent the building up of toxic ammonia levels.

MSO has been used in a number of studies to demonstrate the formation of ammonia when it is applied to plant tissues. MSO treatment brought about an increase in the ammonia content of all species studied [10]. The release of ammonia has been taken as one evidence that glutamine synthetase is the first enzyme operating in ammonia assimilation. In the presence of ATP the MSO becomes tightly bound to the enzyme in the form of MSO-phosphate and can only be removed by heating it up to 100 °C [11, 12].

This investigation with mustard glutamine synthetase however, clearly shows that PPT is an exceptionally specific and much better inhibitor than MSO, particularly as a mixture of the two isomers was applied in the tests. Furthermore, the root glutamine synthetase differs markedly from the enzyme in the leaves with regard to its activity. The K_m value for glutamate as well as the K_i value for PPT were essentially lower for the root enzyme. Studies on the temperature dependence also exhibited a clear difference. The leaf enzyme showed a temperature optimum at 37 °C, compared to 48 °C for the root enzyme.

Concerning glutamine synthetase, recent studies confirmed the existence of two different isoforms in the leaf cells of most higher plants [13, 14]. The first isoform, named GS_1, was detected in the cytosol. The second isoform, called GS_2, is located in the chloroplasts and is specific for ammonia assimilation inside the plastids. The two forms of the enzyme, however, have not been separated from mustard leaves.

Leason *et al.* [2] obtained a K_i value for PPT of 0.073 mM for pea leaf glutamine synthetase, whereas for the *Escherichia coli* enzyme a value of 0.0059 mM was determined [1]. Studies with phosphorylated derivatives of glutamate detected major differences between the affinity of the prokaryotic and eukaryotic enzyme for various inhibitors suggesting a possible difference in the action of the active site of the two groups of enzymes [15].
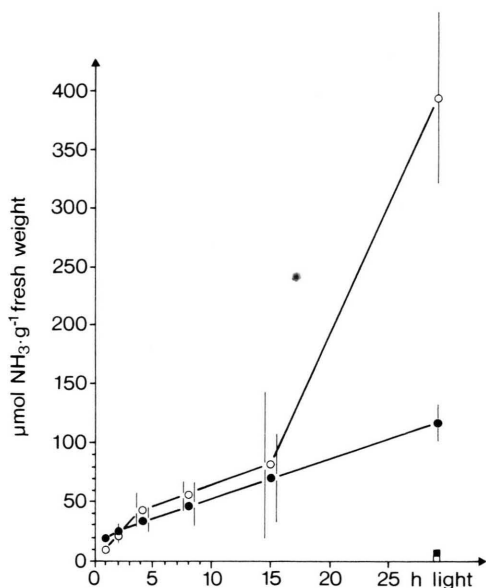
Fig. 3. NH_4^+ accumulation of mustard primary leaves with and without photorespiration after the application with 0.4 kg/ha PPT. The plants were 14 days old. PPT was sprayed 15 h before exposing to light. During the light period the leaf (still attached to the plant) was in an assimilation chamber at 25 °C, 56% relative humidity, 160 W × m^{-2} photosynthetically active radiation (phar), with different composition of the air flowing through the leaf curvette: ○—○ = 0.04% CO_2 + 21% O_2; ●—● = 0.1% CO_2 + 2% O_2 (v/v), □, ■ controls without PPT. | standard deviation of the sample.
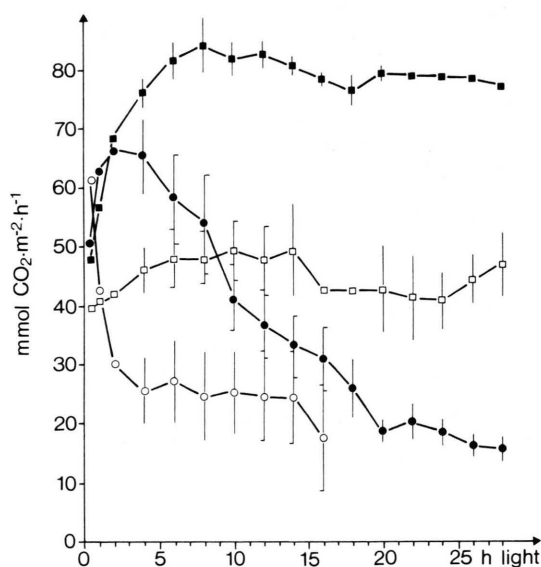
Fig. 4. Photosynthetic activity of mustard primary leaves with and without photorespiration after the application with 0.4 kg/ha PPT. PPT was sprayed 15 h before exposing to light. During the light period the leaf (still attached to the plant) was in an assimilation chamber at 25 °C, 56% relative humidity, 160 W × m^{-2} phar, with different composition of the air flowing through the leaf cuvette: ○—○ = 0.04% CO_2 + 21% O_2; ●—● = 0.1% CO_2 + 2% O_2; □ = control without PPT, 0.04% CO_2 + 21% O_2; ■ = control without PPT, 0.1% CO_2 + 2% O_2. % expressed as v/v.

PPT treatment of plants caused a high ammonia accumulation in the tissues (Fig. 3). Some of the ammonia produced was released into the air. The accumulation was light dependent. The ammonia content did not continue to increase during a dark period. There may be three major potential sources for the ammonia: nitrogen assimilation, catabolic processes of nitrogen compounds and the conversion of glycine to serine occurring during photorespiration. In the C_2 pathway of photorespiration, the glycine decarboxylase reaction produces not only CO_2, but also an equivalent quantity of ammonia. Photorespiration is promoted by high concentrations of O_2, and low concentrations of CO_2, and inhibited by the reciprocal conditions. In order to study the role of photorespiration we compared the production of ammonia under two different conditions of air: 400 ppm CO_2/21% O_2 (v/v) and 1000 ppm CO_2/2% O_2 v/v, respectively. During a light period of 15 h the ammonia concentration increased similarly under both air conditions. After having reached concentrations of over 60 µmol · g^{-1}

fresh weight the accumulation of ammonia was strongly accelerated in leaves performing photorespiration. Therefore we may conclude that the recycling of ammonia *via* the glutamine synthetase-glutamate pathway was inhibited by the PTT treatment. Then with increasing concentrations of ammonia it seems to be likely that the regeneration of glutamate as amino donor for glycine synthesis could be performed by another pathway.

PPT induced a strong reduction of light saturated CO_2 assimilation, too (Fig. 4). Under conditions of suppressed photorespiration – high CO_2 and low O_2 concentrations – the PPT induced inhibition seems to be retarded. These observations suggest that the ammonia accumulation in the cells causes the decline of the photosynthetic rate. The ammonia levels attained exceed those known to depress photosynthesis by uncoupling of photophosphorylation. The killing of the plants by the application of PPT, however, does not appear to be caused by the inhibition of photosynthesis.

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