The Effect of Bialaphos on Ammonium-Assimilation and Photosynthesis
I. Effect on the Enzymes of Ammonium-Assimilation

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In this investigation, the effect of bialaphos (phosphinothricyl-alanyl-alanine) on the enzymes involved in NH_4^+-assimilation − glutamine synthetase, glutamine-2-oxoglutarate aminotransferase, glutamate dehydrogenase − is examined and compared to the effect of phosphinothricin (glufosinate) on the same enzymes. Bialaphos was given to whole plants *(in vivo)* and to leaf homogenate *(in vitro)*.

The investigation showed that bialaphos has an inhibiting effect on glutamine synthetase *in vivo*, but not *in vitro*. In contrast to this, phosphinothricin inhibits glutamine synthetase *in vitro* as well as *in vivo*. It was found that bialaphos, similar to phosphinothricin, does not inhibit glutamine-2-oxoglutarate aminotransferase and glutamate dehydrogenase *in vivo* or *in vitro*. Only at bialaphos concentrations exceeding 10 mM, there is an inhibition of glutamate dehydrogenase *in vitro*.

Using radioactive [³H]bialaphos (phosphinothricyl-³H-alanyl-alanine) it could be demonstrated that in the plant, bialaphos is split into phosphinothricin and alanine. The phosphinothricin released is probably the active herbicide component. Consequently, the herbicidal effects of phosphinothricin and bialaphos are the same.

Introduction

The tripeptide bialaphos (phosphinothricyl-alanyl-alanine) is produced naturally by *Streptomyces viridochromogenes* and acts as tripeptide antibiotic [1]. Bialaphos was introduced as a non-selective herbicide by Meiji Seika Kaisha Ltd. (Tokyo, Japan) [2]. Bialaphos is easily metabolized and biodegraded.

The mechanism of action of bialaphos could be the result of the inhibition of GS [3].

The inhibition of GS leads to a toxic accumulation of NH_4^+ in plants. GS catalyses the NH_4^+-assimilation in the GS/GOGAT pathway [4] which is the main path of NH_4^+-assimilation in higher plants [5, 6]. The primary products of NH_4^+-assimilation are either glutamine or glutamate. These products are involved as amino donors in many important syntheses in cells.

Phosphinothricin (glufosinate), a well-known GS inhibitor, was introduced as a non-selective herbicide by Hoechst AG (Frankfurt/Main, West Germany). PPT inhibits GS in intact plants *(in vivo)* as well as in plant extracts *(in vitro)* [7]. As a consequence of this inhibition, there is a heavy NH_4^+-accumulation [8, 9]. PPT shows no inhibiting effect on GOGAT and GDH [7].

The enzyme activity of GS, GOGAT and GDH were examined in this investigation. Whole plants were treated with bialaphos, and in addition, the homogenate from untreated plants was subjected to bialaphos. The question was whether the effect of PPT and bialaphos was related.

Materials and Methods

Plant material

Sinapis alba plants were cultured as described by [7]; for the experiments 16 to 19-day-old plants were used.

Chemicals

The Na^+-salt of bialaphos (phosphinothricyl-alanyl-alanine, code No. Hoe 070094), the [³H]bialaphos and the [¹⁴C]PPT were supplied by Hoechst AG (Frankfurt/Main, West Germany). In addition bialaphos was used in the form of the formulated commercial product. Chemical structure of bialaphos:

$$\begin{array}{ccccccc} O && NH_2 && CH_3 && CH_3 \\ \parallel && | && | && | \\ H_3C-P-CH_2-CH_2-CH-CONH-CH-CONH-CH-COOH \\ | \\ OH \end{array}$$

Nomenclature corresponding to IUPAC:
L-2-amino-4-[(hydroxy)(methyl)phosphinoyl]-butyryl-L-alanyl-alanine

Measurement of GS activity

GS activity was determined in primary leaves. The plant material was ground in a mortar in the following homogenization mixture: 0.1 M Tris-HCl (pH 7.8), 0.5 mM EDTA (Titriplex III), 1 mM MgSO$_4$, 10 mM DTE. The resulting pulp was centrifuged for 10 min at $20,000 \times g$ and the supernatant was used for further measurements. These steps of the procedure were carried out at 4 °C.

GS activity was measured at 37 °C by the formation of γ-glutamylhydroxamate in the synthetase reaction [10]. Final composition of the reaction medium (total 2 ml): 100 mM imidazol (pH 7.2), 10 mM EDTA, 50 mM MgSO$_4$, 10 mM NH$_2$OH, 15 mM ATP, 150 mM sodium glutamate, 0.3 ml extract. The reaction proceeded for 15 min at 37 °C. It was started by the addition of the extract and stopped by addition of 1.5 ml of an acid FeCl$_3$ solution (0.37 M FeCl$_3$, 0.67 M HCl, 0.2 M CCl$_3$COOH). The brown colored complex of γ-glutamylhydroxamate with ferric chloride was determined spectrophotometrically at a wavelength of 540 nm.

Measurement of GDH activity

The reaction was measured in direction of reductive amination. All preparation steps were carried out at 4 °C. 0.5 g plant material was ground in a mortar in 50 mM Tris-HCl, pH 7.8, 5 mM DTE. The resulting pulp was centrifuged for 20 min at $15,000 \times g$ and the supernatant was used to study the enzyme. The crude extract was desalted on a Sephadex G-25 column (1.5×30 cm) in 10 mM Tris-HCl, pH 7.8, 1 mM DTE. Fractions with GDH activity were pooled. Final composition of the reaction medium (total 2.5 ml): 50 mM Tris-HCl, pH 7.8, 10 mM 2-oxoglutarate, pH 7.8, 60 mM NH$_4$Cl, 2 mM NADH in 100 mM Tris-HCl, pH 7.8, 0.75 ml extract.

The temperature of the reaction medium without NADH was brought to 25 °C. The reaction was started by the addition of NADH and allowed to proceed for 10 min. The NADH-oxidation by enzymatic reaction was determined as absorbance decrease per time by 340 nm (ε_{NADH} = 6.22 cm$^2 \cdot \mu$mol^{-1}).

Measurement of Fd-GOGAT activity

2 g plant material was ground in a mortar in 50 mM KH$_2$PO$_4$, pH 7.5, 25 mM ME, 5 mM EDTA and the mixture was centrifuged for 15 min at $10,000 \times g$. The supernatant was used for the enzyme reaction. The reaction mixture contained 50 mM KH$_2$PO$_4$, pH 8.0, 10 mM glutamine, pH 7.6, 50 mM freshly prepared methylviologen and 0.5 ml of enzyme extract in a total volume of 1.5 ml. The reaction was started by the addition of 0.1 ml 0.27 M freshly prepared Na$_2$S$_2$O$_4$ and 0.6 M NaHCO$_3$, incubated at 37 °C for 15 min and stopped by boiling for 2 min. The denaturated proteins were centrifuged for 25 min at $3,000 \times g$. The supernatant included the product glutamate and the substrate glutamine. These amino acids were separated with a DOWEX AG 1×8 column. 2 ml of ninhydrin were added to 1 ml of the glutamate fraction before boiling for 10 min. The absorbance was determined at 506 nm.

Gel filtration of leave homogenate after treatment of leaves with [^{3}H]bialaphos and [^{14}C]PPT

Primary leaves were treated with 1 mM [^{3}H]bialaphos (PPT-^{3}H-ala-ala) and 1 mM [^{14}C]PPT. After 2 h the crude extract was produced as described for measurement of GS activity. 1 ml of this extract was put on a Sephadex G-50 (1.5×30 cm) column. Fractions with proteins and radioactivity were pooled. GS activity in protein fraction was pooled by untreated plant material.

Protein determination

Protein concentrations were determined with the BIO-RAD protein assay.

Results

Effect of bialaphos on GS activity

Treatment of leaf homogenate with bialaphos (in vitro). After addition of high bialaphos concentrations (1 mM, 10 mM, 30 mM, 50 mM) to leaf homogenate of untreated *Sinapis alba* plants, it could be observed that the enzyme activity decreased with increasing bialaphos concentration (Fig. 1).

The bialaphos which was used contained about 0.15% (w/w) of PPT, *i.e.* in single bialaphos concentrations there were the following PPT concentrations: 1 mM bialaphos (3 μM PPT), 10 mM bialaphos (30 μM PPT), 30 mM bialaphos (90 μM PPT), 50 mM bialaphos (150 μM PPT). According to Manderscheid and Wild [11] these minimal PPT concentrations suffice in order to inhibit totally GS *in vitro*. Bialaphos does not inhibit the GS activity *in vitro* in such low concentrations, in contrast to PPT (Fig. 2).

Treatment of leaves with bialaphos (in vivo). After treatment of entire *Sinapis alba* plants with a 0.05% (0.2 kg/ha) herbicide solution, it became evident that, with increasing time of light exposure, the GS activity decreased significantly (Fig. 3). After 3 h of exposure, the GS activity was totally inhibited.

Similar, plants were treated with the Japanese product "Meiji Herbiace" which contains undiluted 32% bialaphos and less than 0.1% (w/v) PPT. By the
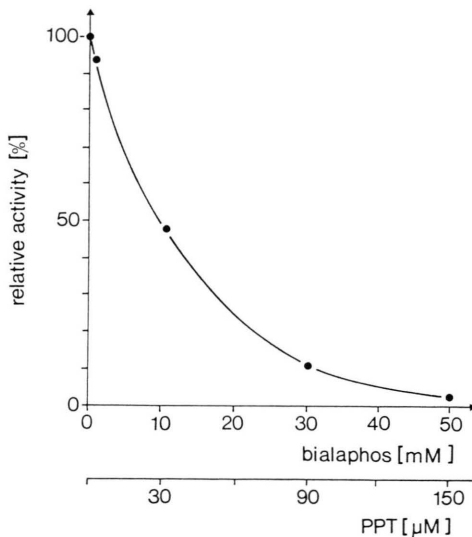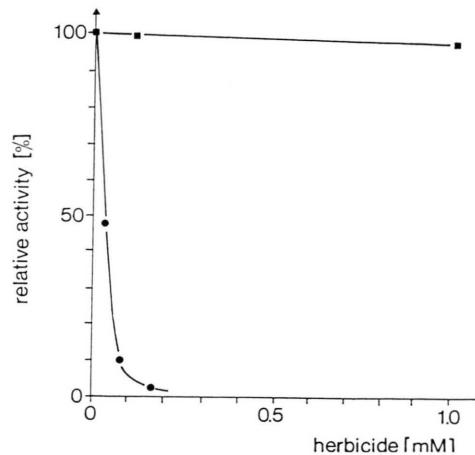

Fig. 2. GS activity of bialaphos and PPT treated homogenate of mustard primary leaves (concentrations: 0.05−1.0 mM). ■ = Bialaphos treated; ● = PPT treated.
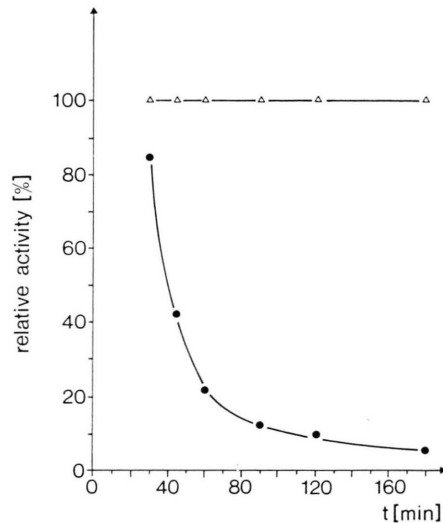

Fig. 3. GS activity of bialaphos treated mustard primary leaves. △ = control (untreated primary leaves); ● = bialaphos treated (0.05%; 0.2 kg/ha) primary leaves.

Fig. 1. GS activity of bialaphos treated homogenate of mustard primary leaves. Bialaphos was contaminated with 0.15% (w/v) PPT.

dilution of the solution, PPT lay in such low concentrations (8 μM) that the results could not have been influenced by PPT (see also Fig. 1).

Effect of bialaphos on Fd-GOGAT activity

Treatment of leaf homogenate with bialaphos (in vitro). The enzyme activity was determined after the addition of high bialaphos concentrations (1 mM,

10 mm, 30 mm) to untreated *Sinapis alba* plant leaf extracts. It was clearly demonstrated (data not shown) that bialaphos did not exert an inhibiting effect on the Fd-GOGAT activity.

Treatment of leaves with bialaphos (in vivo). *Sinapis alba* plants were sprayed with a 2.5% (10 kg/ha) herbicide solution and exposed to light for either 60 min or 120 min. The herbicide concentration applied was much higher than needed for GS inhibition. Nevertheless, an inhibition of Fd-GOGAT activity was not observed *in vivo* (data not shown).

Effect of bialaphos on GDH activity

Treatment of leaf homogenate with bialaphos (in vitro). *In vitro*, low concentrations of bialaphos cause a slight increase in GDH activity. However, high concentrations of bialaphos cause notable inhibition in GDH activity (Table I).

Table I. Effect of bialaphos on GDH activity *in vitro* (enzyme activity in %; control with H_2O = 100%).

bialaphos (0.1 mm)	111.0 ± 5.1
bialaphos (1 mm)	108.4 ± 5.6
bialaphos (10 mm)	90.0 ± 10.6
bialaphos (30 mm)	60.9 ± 3.4
bialaphos (50 mm)	45.9 ± 2.6

Treatment of leaves with bialaphos (in vivo). The plants were sprayed with a 0.25% (1 kg/ha) bialaphos solution. Using a constant dosage, the exposure time was varied. After 60 min and 120 min a slight inhibition of GDH activity was registered. However, after 240 min inhibition was no longer recognizable (Table II).

Table II. Effect of bialaphos on GDH activity *in vivo* (enzyme activity in %; control with H_2O = 100%).

Exposure time [min]	Bialaphos [1 kg/ha]
60	87.0 ± 3.0
120	76.8 ± 0.9
240	109.2 ± 11.4

Splitting of bialaphos in leaves

Primary leaves of the *Sinapis alba* plants were treated with either 1 mm [³H]bialaphos or 1 mm [¹⁴C]PPT.

After gel filtration of extract of [³H]bialaphos treated *Sinapis alba* plants, peaks in protein and in radioactivity were clearly discriminated. The exposure time of [³H]bialaphos lasted about 3 h. In the protein peak, which included GS, no radioactivity could be found (Fig. 4).

In comparison with this, an experiment using [¹⁴C]PPT was carried out. Radioactivity could be clearly observed in the protein peak (Fig. 5).

Discussion

PPT does not inhibit the Fd-GOGAT enzyme and the GDH, either *in vitro* or *in vivo*. However, PPT inhibits irreversibly GS *in vitro* and *in vivo* [11, 12]. Our comparison showed that bialaphos does also not inhibit the Fd-GOGAT enzyme *in vitro* or *in vivo*. Bialaphos does not affect GDH *in vivo* although high concentrations were found to be inhibitory *in vitro*. Only in high concentrations the GS is inhibited *in vitro*. However, it could be proved that this GS inhibition is caused by the PPT, present in small quantities in the herbicide product (Fig. 1, 2). Therefore
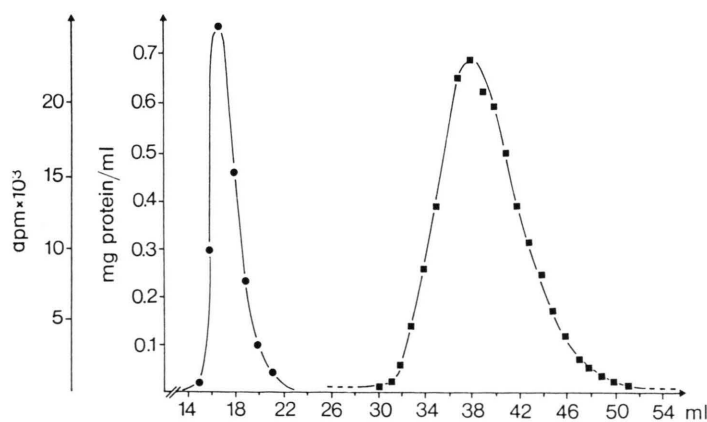

Fig. 4. Gel filtration profiles of crude extract with [³H]bialaphos treated mustard primary leaves. Distribution of protein (●——●) and radioactivity (■——■).
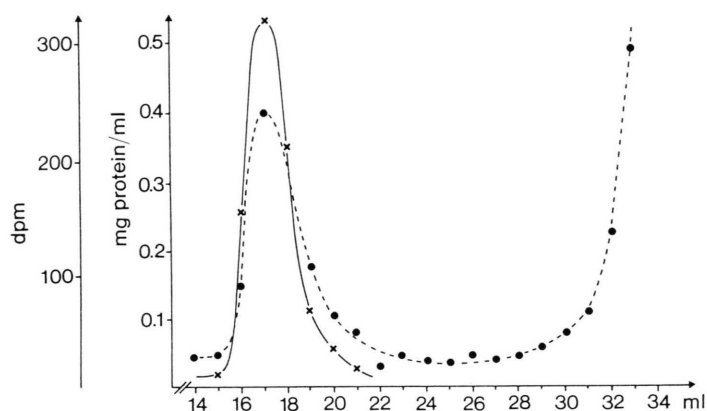
Fig. 5. Gel filtration profiles of crude extract with [^{14}C]PPT treated mustard primary leaves. Distribution of protein (x——x) and radioactivity (●– – –●).

bialaphos, in contrast to PPT, does not inhibit GS *in vitro*.

Nevertheless, the investigations on whole plants gave evidence that bialaphos causes a distinct inhibition of GS (Fig. 3).

This result indicates that bialaphos may be split into PPT and alanine in plants, and through this splitting PPT becomes the only actual active ingredient.

This assumption could be tested through measurements with [^{3}H]bialaphos and [^{14}C]PPT. [^{3}H]-bialaphos was marked on the first alanine residue and the C-atoms 3 and 4 were marked by [^{14}C]PPT.

After a 3 h period, a gel filtration was made with the homogenate of the plants treated with either [^{3}H]bialaphos or [^{14}C]PPT. GS was inhibited by PPT as well as by bialaphos after the acting period.

A protein peak without radioactivity and a separate activity peak could be found in the gel filtration with the homogenate of [^{3}H]bialaphos treated plants (Fig. 4). GS is found in the protein peak. If bialaphos would inhibit GS as an intact molecule, then radioactivity should be observed in the protein peak. It can therefore be argued that the GS inhibition is caused by PPT split from the bialaphos. Since the PPT residue in the [^{3}H]bialaphos was not marked, no radioactivity could be detected in the protein peak. The alanine or its decomposition products remain in the radioactivity peak. As the chromatographical investigations have shown, alanine seems to be converted into other products very quickly in plants.

However, the gel filtration investigation with homogenate of [^{14}C]PPT treated plants show radioactivity in the protein peak (Fig. 5). [^{14}C]PPT binds irreversibly at the active centre of GS. This has been shown in earlier investigations [12].

The results of this investigations confirm that bialaphos is very quickly split into PPT and alanine in plants. Thus PPT is freed to be the actual active herbicidal component.

Investigations on GS from *Escherichia coli* also indicated that the tripeptide PPT-ala-ala, in contrast to PPT, did not exercise any inhibitive effects on GS activity *in vitro*. *In vivo*, however, the tripeptide acted as the inhibitor of GS [1].

Furthermore, there is a strong NH_4^+-accumulation through the inhibition of GS *in vivo*, which is evident after PPT treatment [8, 9] as well as after bialaphos treatment [13].

Interestingly, bialaphos and PPT cause an immediate inhibition of photosynthesis in plants. This bialaphos effect will be further investigated [13].

Acknowledgements

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