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THE ACTION OF 2-AMINO-4-(METHYLPHOSPHINYL)-BUTANOIC ACID (PHOSPHINOTHRICIN) AND ITS 2-OXO-DERIVATIVE ON THE METABOLISM OF CYANOBACTERIA AND HIGHER PLANTS

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Key Word Index—*Anabaena*, *Anacystis*, Cyanobacteria, *Triticum*, *Zea*, Gramineae, *Pisum*, Leguminosae, *Helianthus*, Compositae, 2-amino-4-(methylphosphinyl)-butanoic acid, phosphinothricin, ammonia evolution, herbicide action

Abstract—When adequate concentrations of phosphinothricin (a potent inhibitor of glutamine synthetase) are added to *Anacystis nidulans* cells suspended in nitrate medium, ammonia excretion into the medium takes place Similarly, when phosphinothricin is added to nitrogen fixing cultures of *Anabaena* ATCC 33047, ammonia is also released at high rates Methionine sulphoximine, phosphinothricin and its 2-oxo-derivative (1 mM) stimulate ammonia production and cause a sharp drop in glutamine and asparagine concentrations, when fed to leaves of *Triticum*, *Pisum* and *Helianthus* Less pronounced effects were detected with the leaves of a C_4 plant *Zea*

INTRODUCTION

L-Methionine-*S*-sulphoximine (MSO) has been examined extensively by Meister and his colleagues [1] as an inhibitor of mammalian glutamine synthetase (GS) [L-glutamate ammonia ligase (ADP-forming), EC 6 3 1 2], and was later shown to be a potent inhibitor of bacterial [2, 3] and plant [3, 4] GS MSO has been used as an inhibitor of ammonia assimilation in cyanobacteria [5, 6], *Chlamydomonas reinhardii* [7], *Lemna minor* [8], rice [9], *Datura* [10] and spinach [11] Such observations have been taken as one piece of evidence to show that the GS/glutamate synthase pathway [12] operates in green plants [13, 14]

The addition of micromolar concentrations of MSO to illuminated cell suspensions of nitrate-reducing *Anacystis nidulans* [15] and nitrogen-fixing *Anabaena* ATCC 33047 [16] caused the excretion of ammonia into the surrounding medium at a high rate The levels of GS activity in the two types of cyanobacterial cells fell rapidly, but there was no evidence of any detrimental effect on the cells [17], thus suggesting that a biological system for the continuous production of ammonia using solely light energy could be devised [18]

Phosphinothricin (PPT) [2-amino-4-(methylphosphinyl)-butanoic acid] was first isolated as a dialanyl derivative from *Streptomyces viridochromogenes* and was shown to be a potent inhibitor of GS from *E coli* [19] Subsequently the compound was shown to have strong herbicidal properties [20, 21] and was also active as an inhibitor of plant GS at lower concentrations than that required for MSO [4] The 2-oxo-analogue of phosphinothricin (PPO) [2-oxo-4-(methylphosphinyl)-butanoic acid] also acts as a herbicide [22], but no reports have been published as to its ability to inhibit GS

The aim of this work was (a) to identify the mode of action of the potential herbicides PPT and PPO by examining their effect on amino acid metabolism in a number of different higher plant species, and (b) to ascertain whether PPT and PPO are able to act in a similar manner to MSO on nitrate-reducing or nitrogen-fixing cyanobacteria

RESULTS

Anacystis nidulans

The effect of varying concentrations of PPT on nitrate-grown cells of *A nidulans* can be readily seen in Table 1 For a cell density value of 5 μg chlorophyll per ml, PPT concentrations above 100 μM strongly inhibited cell growth and decreased the levels of both nitrate reductase and GS Growth was also inhibited, although only moderately, by PPT concentrations between 25 and 100 μM, without significant effect on nitrate reductase or GS Ammonia production was observed at concentrations above 25 μM, with a maximum at 50–100 μM The duration of effective ammonia production was, however, dependent on the concentration of PPT employed (Fig 1), being shorter at the high concentration of 1 mM After 24 hr in 50 μM PPT, the carbohydrate content of the cells increased over 3-fold, but the levels of chlorophyll and protein decreased slightly (Table 2) Ultimately the C/N ratio of the cells changed from 4 2 to 6 5 after 24 hr

Anabaena *ATCC* 33047

For this filamentous cyanobacterium, PPT behaved as a very potent inhibitor of cell growth, with 2 5 μM preventing growth almost completely and promoting maximum ammonia production (Table 3) Nitrogenase levels were increased almost 3-fold at this PPT concentration PPT above 5 μM provoked a decrease in nitrogenase activity Nevertheless, GS activity apparently was not affected at all by PPT in the concentration range assayed The time course of the appearance of nitrogenase activity can be seen in Fig 2 with maximum ammonia production

Table 1 Effect of different concentrations of phosphinothricin (PPT) on cell growth, nitrate reductase and glutamine synthetase activities and ammonia production by *Anacystis nidulans* cells

[PPT] (μM)	Cell growth (% of control)	Nitrate reductase (μmol/mg Chl per min)	Glutamine synthetase (μmol/mg Chl per min)	NH_3 produced (mM)
0	100	3 3	21 6	0
5	100	3 4	20 8	0
25	59	3 5	22 1	0 2
50	22	4 0	18 1	1 9
100	17	3 8	16 2	2 3
250	2	1 5	9 5	1 2
500	0	1 3	8 2	0 8
1000	0	0 9	6 5	0 6

Suspensions of 4 *nidulans* containing 5 μg chlorophyll per ml culture medium were treated for 16 hr with PPT at the indicated concentration Cell growth in the control suspension was from 5 to 18 6 μg chlorophyll per ml culture medium

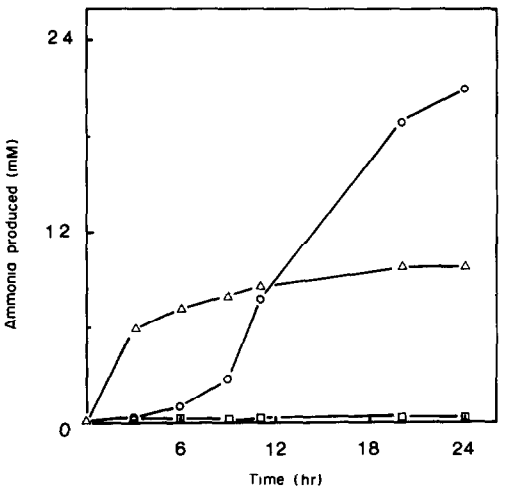

Fig 1 Time course of phosphinothricin-promoted ammonia production by *Anacystis nidulans A nidulans* cell suspensions containing 6 μg chlorophyll per ml culture medium were incubated under standard culture conditions in the absence (□) or presence of 50 μM (○) or 1 mM (△) phosphinothricin Ammonia in medium was estimated at the times indicated

Table 2 Effect of phosphinothricin (PPT) on the levels of different cell components in *Anacystis nidulans*

		t = 24 hr	
	$t = 0$	No addition	50 μM PPT added
A_{628}/A_{680}	1 1	1 2	1 1
Chlorophyll*	6 0	5 9	5 2
Protein*	120 4	124 1	105 4
Carbohydrates*	6 2	6 0	21 5
Nitrogen†	9 9	10 2	7 7
Carbon†	42 2	41 0	50 1

A suspension of *A nidulans* cells containing 1 μl cells per ml culture medium was divided into two halves, to one of which PPT was added at $t = 0$ to reach a final concentration of 50 μM After 24 hr of incubation under standard culture conditions the cell density values reached were 4 0 and 1 4 μl cells per ml for the PPT-free and PPT-containing suspensions, respectively

* μg/μl cells

† % of dry wt

Table 3 Effect of different concentrations of phosphinothricin (PPT) on cell growth, nitrogenase activity and ammonia production by *Anabaena* ATCC 33047 cells

[PPT] (μM)	Cell growth (% of control)	Nitrogenase (μmol C_2H_4/mg Chl per hr)	NH_3 produced (mM)
0	100	51 4	0
1	63	90 0	0 3
2 5	5	144 5	2 2
5	5	56 5	1 8
7 5	2	13 4	1 0
10	0	7 5	0 7

Suspensions of *Anabaena* ATCC 33047 containing 7 1 μg chlorophyll per ml culture medium were treated for 8 hr with PPT at the indicated concentrations Cell growth in the control suspension was from 7 1 to 13 9 μg chlorophyll per ml culture medium

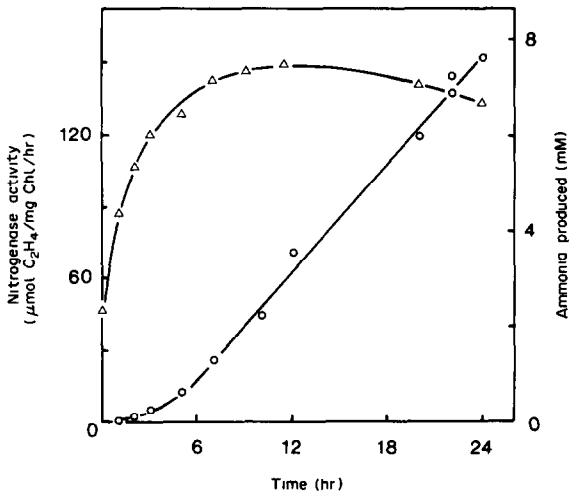

Fig 2 Effect of phosphinothricin on the activity level of nitrogenase and on the production of ammonia by *Anabaena* ATCC 33047 cells At t = 0, PPT (2 5 μM final concentration) was added to a cell suspension of *Anabaena* ATCC 33047 containing 8 μg chlorophyll per ml culture medium Aliquots were withdrawn at the times indicated and nitrogenase in the cells (△) and ammonia (○) in the medium were estimated

after 24 hr After this time the nitrogenase activity decreased and ammonia production ceased Re-addition of 2 5 μM PPT, however, restored ammonia production and increased the nitrogenase level Similar changes in carbohydrate, protein and chlorophyll levels and in nitrogen and carbon content of the cells after incubation for 20 hr in 2 5 μM PPT to that determined for *Anacystis* were also observed The C/N ratio increased from 4 4 to 6 8 after 20 hr in the presence of PPT Contrary to the situation in *Anacystis*, a strong decrease (90%) in the phycocyanin level was observed for *Anabaena* PPO exerted no effect on either *Anabaena* or *Anacystis* at a concentration of 1 mM

Higher plants

Table 4 shows the changes in the amounts of amino acids and ammonia caused by MSO, PPT and PPO, when fed to shoots via the xylem stream at a concentration of 1 mM There was a considerable increase in ammonia in all plants tested, although the increase in *Zea* was not readily apparent after 1 5 hr feeding. There was no major difference between the three compounds tested in their effect Levels of glutamine and asparagine tended to fall in *Pisum*, *Triticum* and *Helianthus* but not in *Zea* Similarly levels of glycine, serine and alanine all decreased in *Pisum*, *Triticum* and *Helianthus* but tended to increase in *Zea*

DISCUSSION

The addition of PPT to nitrate-reducing cells of *A nidulans* causes ammonia evolution in a similar manner to MSO, although concentrations of 50–100 μM are required for optimal ammonia production as compared to 5–10 μM MSO [15] The higher the PPT concentration the shorter the lag period to start the ammonia evolution, but also the shorter the period of effective production (Fig 1) It can be seen in Table 1 that there is little decrease in GS activity at the time of maximum ammonia evolution However, the assay employed involved toluene permeabilized cells that had been washed twice in buffer and any free PPT would have been washed out of the cells Studies with GS isolated from *Pisum sativum* indicate that at low concentrations PPT acts as a competitive inhibitor with glutamate, but at high concentrations as a non-competitive inhibitor [4] and it then becomes irreversibly bound to the enzyme Similar studies on GS isolated from a cyanobacterium have not been carried out but it must be assumed from Table 1 that external concentrations of 25–100 μM inhibit GS reversibly, and above 100 μM irreversibly

PPT concentrations above 100 μM also inhibited nitrate uptake in *A nidulans* [Ramos, J L and Guerrero, M G, unpublished results], which probably accounts for the drop in nitrate reductase activity and ammonia production The slight decrease in protein level but large increase in the carbohydrate level caused by 50 μM PPT (Table 2) was also seen in MSO-treated *A nidulans* [15], and suggests that whilst amino acid synthesis is blocked due to unavailability of ammonia, CO_2 fixation can still continue

The optimum level of PPT required to stimulate maximum ammonia production in *Anabaena* ATCC 33047 was 2 5–5 μM as compared to a concentration of 35 μM required for MSO Once again when GS was assayed in permeabilized cells of *Anabaena* ATCC 33047, there was little evidence of inhibition even at 10 μM PPT, suggesting that PPT is readily removed from the active site of the enzyme There was an almost 3-fold increase in the nitrogenase level at 2 5 μM PPT (Table 3), confirming the previously reported data that ammonia needs to be incorporated into an organic form before it is able to repress nitrogenase synthesis [5, 16] The reason for the marked decrease in nitrogenase activity at concentrations of PPT above 2 5 μM is not clear, but may suggest that either the cells do not have sufficient amino acids to synthesize the nitrogenase protein, or that PPT is exerting a second inhibitory effect The evolution of ammonia caused by 2 5 μM PPT was only maintained for 24 hr (Fig 2), but could be restored by the re-addition of 2 5 μM PPT, suggesting that the inhibitor was being slowly metabolized in the cells

Perhaps the most striking feature about the data shown in Table 4 is that the changes in ammonia and amino acid levels for each plant species were very similar for all three compounds tested The only major difference was the particularly sharp decline of the glutamine level in *Helianthus* caused by PPT

In *P sativum*, *T vulgare* and *H annuus* the levels of glutamine, asparagine and glutamate fell markedly after treatment with the three compounds This would be predicted if the compounds were acting as inhibitors of glutamine synthesis, as asparagine and glutamate receive nitrogen directly from glutamine [12] and are rapidly turned over in the leaf in the light [23] The levels of serine, glycine and alanine decreased to a lesser extent suggesting that transamination reactions could still proceed and equilibration of amino groups could take place In *Z mays* there was a decrease in glutamine levels caused by MSO and PPO but the concentration of all the other amino acids tended to rise, suggesting that there was no block in their rate of synthesis

Levels of ammonia rose in all the plants following treatment with the three compounds, although in *Z mays* there was little effect until after 6 hr The highest levels of

Table 4 The effect of methionine sulphoximine (MSO), phosphinothricin (PPT) and its 2-oxo derivative (PPO) on the levels of amino acids and ammonia in the leaves of four plant species

Compound	None		MSO		PPT		PPO	
Time (hr)	1 5	6 0	1 5	6 0	1 5	6 0	1 5	6 0
(a) *Pisum sativum*								
Asp + βIA	2 98	2 89	3 16	2 49	3 72	3 30	3 63	3 16
Thr	0 25	0 34	0 31	0 43	0 38	0 33	0 35	0 33
Ser	1 03	1 07	0 55	0 51	0 67	0 46	0 67	0 67
Asn	4 14	3 62	7 52	3 20	4 45	1 51	3 63	1 13
Glu	2 35	1 41	1 60	0 75	1 16	0 47	1 01	0 47
Gln*	1 35	0 93	0 94	0 60	0 60	0 46	0 60	0 45
Hse*	0 98	2 65	1 31	1 16	1 72	1 39	2 32	1 50
Gly	0 07	0 19	0 13	0 08	0 12	0 04	0 10	0 07
Ala	0 19	0 27	0 22	0 15	0 14	0 08	0 14	0 07
Ammonia	1 22	1 33	2 3	9 08	3 60	8 75	3 00	7 30
(b) *Triticum vulgare*								
Asp	1 14	1 28	0 51	0 69	0 99	0 72	0 53	0 89
Thr	0 57	0 47	0 48	0 73	0 57	0 75	0 43	0 83
Ser	0 93	0 72	0 52	0 50	0 79	0 56	0 48	0 74
Asn	3 24	2 45	1 46	1 10	2 68	1 36	1 15	1 93
Glu	2 10	2 16	1 01	1 18	1 84	1 22	1 02	1 41
Gln	1 04	0 87	0 18	0 07	0 57	0 03	0 12	0 21
Gly	0 21	0 10	0 16	0 09	0 18	0 09	0 12	0 13
Ala	0 91	0 94	0 41	0 41	0 69	0 46	0 40	0 64
Val	0 99	0 61	0 67	1 05	0 89	1 08	0 50	1 29
Ammonia	1 03	0 55	2 83	5 41	2 03	5 53	2 08	5 24
(c) *Helianthus annuus*								
Asp	0 70	0 89	0 40	0 24	0 71	0 18	0 23	0 32
Thr	0 48	0 48	0 25	0 27	0 44	0 19	0 32	0 44
Ser	1 08	0 85	0 49	0 23	0 80	0 13	0 55	0 64
Asn	3 36	2 91	2 30	1 10	5 33	0 69	1 03	1 46
Glu	0 73	0 73	0 49	0 25	0 48	0 23	0 38	0 29
Gln	6 41	5 44	2 71	1 47	2 81	0 065	1 96	2 34
Gly	0 40	0 34	0 39	0 09	0 69	< 0 05	0 18	0 18
Ala	0 61	0 35	0 20	0 08	0 41	< 0 05	0 32	0 34
Val	0 60	0 44	0 20	0 23	0 44	0 20	0 34	0 53
Ammonia	0 65	0 60	1 50	3 01	2 96	3 30	1 54	3 10
(d) *Zea mays*								
Asp	0 71	0 65	0 93	0 58	1 02	0 98	1 02	0 57
Thr	0 41	0 52	0 59	1 15	1 21	0 95	0 89	1 01
Ser	2 94	2 85	4 36	5 03	5 63	5 04	4 31	4 19
Asn	8 72	6 88	13 28	12 15	20 9	11 80	13 06	10 15
Glu	1 08	1 57	1 39	1 14	1 74	2 03	1 38	0 98
Gln	3 07	2 47	3 89	1 52	4 06	6 18	3 80	1 65
Gly	3 66	3 94	5 16	6 05	5 47	5 40	5 98	4 55
Ala	3 23	2 73	6 46	5 23	7 18	5 74	6 40	4 19
Val	0 30	0 37	0 66	0 93	0 97	0 75	0 85	0 92
Ammonia	1 63	2 10	1 85	5 04	2 08	5 51	2 46	4 96

βIA, β-(Isoxazolin-5-on-2-yl) alanine, Hse, homoserine

*Approximate values due to poorly resolved peaks of Gln and Hse

All values quoted as μmol/g fresh wt

ammonia were produced in *Pisum* with a maximal rate of 1 6 μmol ammonia/hr per g fresh wt being obtained, rates in the other plants varied between 0 4 and 1 5 μmol/hr per g fresh wt Platt and Anthon [11] examined the effect of 8 mM MSO on isolated spinach leaf discs and showed that ammonia was produced at a rate of 4 8 μmol/hr per g fresh wt for a period of 150 min

The major source of ammonia produced in the leaf of a C_3-plant is thought to be that released during the conversion of glycine to serine in photorespiration [12] In C_4-plants levels of photorespiration are much lower [24] and it would be predicted that the rate of ammonia production in *Z mays* would be less than in the other three plants The experiments reported here were not

designed to yield an accurate time course of ammonia evolution, but it can be seen in Table 4 that after 1 5 hr there is little ammonia evolution in *Z mays* compared with the other three plants However, after 6 hr the ammonia concentration in *Z mays* is in the same range as that found in *H annuus* and *T vulgare*

It is clear that the rates of ammonia production measured here and by Platt and Anthon [11] are not of the same order as the magnitude of normal CO_2 fixation (200 μmol/hr per g fresh wt) The reason for this is that the addition of MSO severely inhibits CO_2 fixation [11, Lea, P J, unpublished results] and hence the photorespiratory source of ammonia is removed It must be assumed that if ammonia is not assimilated by GS it diffuses into the chloroplast where it inhibits electron transport [25–28] and thus the production of ATP and NADPH required for CO_2 fixation The levels of ammonia detected in the four plants do not reflect the rates of photorespiration, but the ability of the chloroplast to tolerate high ammonia levels within the cell before photosynthetic reactions are curtailed A paper published during the production of this manuscript [29] indicates that concentrations of ammonia produced by 8 mM MSO in *Zea*, *Sorghum* and *Datura* were 13 4, 17 2 and 10 0 mM, respectively

In the present paper we have demonstrated two uses of the glutamine synthetase inhibitors MSO and PPT (a) to produce ammonia photosynthetically from either nitrate or nitrogen gas using cyanobacteria, and (b) to inhibit the growth of plants by increasing the level of ammonia inside the cell to a point where normal metabolism is prevented

The precise role of PPO is not clear as it does not inhibit GS by itself [Lea, P J, unpublished results] In the cyanobacteria the compound had no effect, suggesting that either it does not enter the cell or it is not converted to the active form of the inhibitor In the higher plant PPO exerts a very similar effect to PPT suggesting that it is converted enzymically after entering The precise mechanism of this conversion is presently being studied

EXPERIMENTAL

Cyanobacteria Anacystis nidulans and *Anabaena* ATCC 33047 were cultured as described in refs [15] and [16], respectively Expts involving PPT were carried out in the same manner as those originally described for MSO Glutamine synthetase (GS), nitrate reductase and nitrogenase were assayed again as described in refs [15] and [16] Care was taken to wash out PPT prior to the *in situ* assay of GS C and N were determined with a Carlo Erba 1106/R elemental analyser

Higher plants All seedlings were grown at 22° with 16 hr light Shoots of the following ages were used for the expts *Pisum* (15 days), *Helianthus* (18 days), *Triticum* (9 days), and *Zea* (9 days) Roots were cut under H_2O and placed in tubes containing 3 ml of the compound under test at 1 mM, and left at 20° and a light intensity of 1–1 2 mEinsteins/m^2 per sec for the periods stated

Approximately 2 g of tissue was used for each feeding, shoots were ground in a pestle and mortar with sand and initially 3 ml H_2O per g tissue, followed by an additional 3 ml 5-sulphosalicylic acid to yield a final concn of 50 mg/ml 5-sulphosalicylic acid Extracts were centrifuged and the supernatants brought to pH 2 with NaOH

Aliquots of the extracts were analysed for NH_3 and amino acids on a Beckman Model 119 BL automatic analyser as described in ref [30] Glutamine was not denatured during the extraction and analysis procedure

PPT was synthesized as described in ref [19] and PPO by the method of ref [22]

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NOTE ADDED IN PROOF

Similar data has been recently published on the increases in ammonia levels in wheat, barley, corn and sorghum caused by MSO Martin, F, Winspear, J, MacFarlane, J D and Oaks, A (1983) *Plant Physiol* **71**, 177