Mode of Action of *N*-Phosphonomethylglycine: Inhibition of Aromatic Amino Acid Biosynthesis

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N-Phosphonomethylglycine, a unique postemergence herbicide, appears to inhibit the aromatic amino acid biosynthetic pathway. The growth inhibition of *Lemna gibba* in the presence of this herbicide can be alleviated by the addition of L-phenylalanine to the nutrient medium. The growth inhibition of *Rhizobium japonicum* caused by *N*-phosphonomethylglycine can only be alleviated by the addition of both L-phenylalanine and L-tyrosine. The data suggest that *N*-phosphonomethylglycine may inhibit or repress chorismate mutase and/or prephenate dehydratase.

N-Phosphonomethylglycine represents a unique product with postemergence herbicidal properties (Baird *et al.*, 1971). While it is known that phosphonic acids possess metal-chelating properties (Carter *et al.*, 1967), essentially nothing is known about the interaction of such acids with biological systems.

The objective of this investigation was to identify the possible sites of action. The results in this article suggest that the *N*-phosphonomethylglycine interferes with the biosynthesis of phenylalanine and, more specifically, with the metabolism of chorismic acid in the aromatic amino acid biosynthetic pathway.

EXPERIMENTAL SECTION

Plant Material. The aquatic flowering plant *Lemna gibba* L. strain G_3 was grown axenically in 125-ml Erlenmeyer flasks with 25 ml of E medium as described previously (Cleland and Gibbs, 1967). This medium contained the major and minor elements, EDTA, iron, tartaric acid, and sucrose. Flasks were inoculated with two four-frond clusters and incubated at 28° under continuous illumination (500 ft-candles fluorescent white light). Growth studies were run for 8-day periods. Plants were harvested on a fishnet, rinsed with tap water, and gently blotted with paper toweling. After obtaining the fresh weight, the plants were dried *in vacuo* for 24 hr at 50°. Fresh and dry weights paralleled each other closely, and only fresh weights are reported.

Chemical Treatment. All chemicals were dissolved in water at appropriate concentrations and neutralized where necessary to pH 5.6. The solutions were sterile filtered through Millipore filters (0.45 μ) and introduced aseptically into nutrient media.

Culture of Bacteria. *Rhizobium japonicum*, USDA Strain 71, was grown in a mineral salts, mannitol, and glutamic acid medium (Elkan, 1969). Liquid cultures were grown in 250-ml flasks on a gyratory shaker (150 rpm) at 30°. Growth was assessed turbidimetrically at an optical density of 660 nm. The initial inoculum level was adjusted in all experiments to give an od_{660} of 0.03 per milliliter of medium.

Amino Acid Extraction. Fresh *Lemna* tissue was ground in liquid nitrogen and homogenized with hot 80% ethanol (5 ml/g fresh weight). The homogenate was filtered through two layers of Miracloth, and the residue was reextracted with 80% ethanol. The ethanol in the filtrate was removed *in vacuo*, and the remaining aqueous solution was adjusted in volume to make 1 ml equivalent to 1 g of fresh weight of tissue. This solution was extracted five times with equal volumes of chloroform to remove pigments and other lipid-soluble materials. The aqueous solution was lyophilized and the residues were analyzed in the Beckman amino acid analyzer.

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RESULTS AND DISCUSSION

Initial studies with *Lemna* illustrated that *N*-phosphonomethylglycine inhibited growth completely at 10^{-3} *M* and significantly at 10^{-4} *M*, as shown in Table I. The characteristic visual effects of this chemical are unusual. *N*-Phosphonomethylglycine induced some chlorosis and caused abnormal frond development characterized by elongate stipes and irregular daughter-frond margins.

Attempts to reverse the toxicity of the above compound with essential metal ions on the basis of its potential chelating properties were unsuccessful. However, the possibility existed that *N*-phosphonomethylglycine could bind to metal-containing enzymes and an examination of the literature suggested that dehydratase enzymes should be considered. One key dehydratase involved in the aromatic amino acid pathway (shikimic acid pathway) is prephenate dehydratase. This enzyme converts prephenic acid to phenylpyruvic acid, which is then aminated to form phenylalanine. On this basis, attempts were made to reverse the *N*-phosphonomethylglycine growth inhibition of *Lemna* by using phenylalanine as well as tyrosine and tryptophan. Table II summarizes these results, and it is apparent that only L-phenylalanine reversed the inhibition. It was of particular interest to note that L-tyrosine alone at 2×10^{-4} *M* was as inhibitory to *Lemna* as *N*-phosphonomethylglycine. Furthermore, tyrosine elicited the same abnormal development of stipes and fronds as was observed with *Lemna* grown in the presence of the herbicide.

The effects of combinations of the three aromatic amino acids with *N*-phosphonomethylglycine were also examined (Table III). The best reversal of growth inhibition was achieved with a mixture of the three aromatic amino acids. It should also be noted that phenylalanine combined with tyrosine eliminated the toxic effects of tyrosine.

The question of whether phenylalanine was unique in reversing the toxicity of *N*-phosphonomethylglycine was examined by evaluation of other amino acids, cofactors, and products in or derived from the aromatic amino acid pathway. The results (Table IV) were largely negative with certain exceptions. The exceptions were precursors of phenylalanine

Table I. Effect of *N*-Phosphonomethylglycine on *Lemna* Growth

Treatment[a]	Chemical concn, *M*	Total fresh weight, mg[b]	Percent inhibition
Control		560	00
$HOOCCH_2N(—H)CH_2P(=O)(OH)_2$	10^{-3}	20	100
PMG	10^{-4}	140	75
	10^{-5}	490	12

[a] PMG: *N*-phosphonomethylglycine. [b] Eight days growth, weight of initial inoculum was approximately 25 mg. The coefficient of variation for *Lemna* fresh weights was less than 15% in all studies.

Table II. Reversal of *Lemna* Growth Inhibition; Use of Single Amino Acids

Treatment[a]	Chemical concn, *M*	Percent inhibition
Control	...	0
Phe	2×10^{-4}	0
Tyr	2×10^{-4}	64
Tryp	2×10^{-4}	0
PMG	10^{-4}	56
Phe + PMG	$2 \times 10^{-4} + 10^{-4}$	28
Tyr + PMG	$2 \times 10^{-4} + 10^{-4}$	61
Tryp + PMG	$2 \times 10^{-4} + 10^{-4}$	56

[a] Preliminary titration indicated that phenylalanine was inhibitory at levels above 4×10^{-4} *M*, and hence most reversal studies were conducted at levels twice the molar concentration of the inhibitor. Phe = L-phenylalanine, Tyr = L-tyrosine, Tryp = tryptophan.

and L-proline. In no instances were these compounds as effective as phenylalanine itself.

Since many microorganisms possess the aromatic amino acid pathway, it was of interest to determine whether *N*-phosphonomethylglycine would inhibit microbial growth and whether this inhibition could be reversed as with *Lemna*. As shown in Table V, *Rhizobium japonicum* was inhibited by the herbicide, even at 10^{-5} *M*. While this inhibition was not reversed by phenylalanine alone, the combination of phenylalanine and tyrosine was quite effective (Table VI). Tyrosine alone was not inhibitory to the bacterial growth in contrast to the findings with *Lemna*. Definite, though far from complete, reversals were obtained with a combination of phenylalanine and tyrosine precursors and with chorismic and prephenic acids. Chorismic acid was particularly interesting, since

Table III. Reversal of *Lemna* Growth Inhibition; Use of Amino Acid Combinations

Treatment	Chemical concn, *M*	Percent inhibition
Control		0
Phe + Tyr	10^{-4} each	0
Phe + Tryp	10^{-4} each	0
Tyr + Tryp	10^{-4} each	35
Phe + Tyr + Tryp	10^{-4} each	0
PMG	10^{-4}	65
PMG + Phe + Tyr	10^{-4} each	14
PMG + Phe + Tryp	10^{-4} each	28
PMG + Tyr + Tryp	10^{-4} each	64
PMG + Phe + Tyr + Tryp	10^{-4} each	0

reversal was relatively good even at a level where chorismic acid by itself was somewhat inhibitory to the growth of the bacteria.

The data in Tables II–VI supported the hypothesis that *N*-phosphonomethylglycine could interfere with the aromatic amino acid pathway. If the pathway were blocked in *Lemna* at the bifurcation leading to phenylalanine and tyrosine synthesis, it should be possible in a short term inhibition experiment to see a shift in the cell concentration of these two amino acids, namely a decrease in the phenylalanine concentration and possibly an increase in tyrosine. Additionally, one would expect the total amino acid pool to increase, since a specific block in phenylalanine biosynthesis should slow protein synthesis without necessarily having an immediate effect on the biosynthesis of other amino acids. The data in Table VII show that these expectations were upheld and that the ratio of tyrosine–phenylalanine doubled in inhibited plants. Almost all amino acids detected in the Beckman amino acid analyzer increased in concentration per unit plant weight. Decreases were noted only for phenylalanine and threonine.

The growth inhibition of *L. gibba* caused by *N*-phosphonomethylglycine was reversed to a large degree by L-phenylalanine. This was the only single amino acid capable of producing significant reversals of growth inhibition. The growth inhibition of *R. japonicum* was reversed well only by a combination of L-phenylalanine and L-tyrosine or by known precursors in the aromatic amino acid biosynthetic pathway (Lingens, 1968) leading to the formation of these two amino acids.

Table IV. Compounds Tested as Reversing Agents for PMG in *Lemna*

Inactive compounds

L-Amino acids		Cofactors	Others
Glycine	Threonine	*p*-Aminobenzoic acid	*trans*-Cinnamic acid
Alanine	Aspartic acid	Folic acid	*o*-Coumaric acid
Valine	Glutamic acid	Dihydrofolic acid	Caffeic acid
Leucine	Lysine	Tetrahydrofolic acid	Shikimic acid
Isoleucine	Histidine	Pyridoxal	Phosphoenol pyruvic acid
Serine	Arginine	Pyridoxine	Erythrose-4-phosphate
Cystine	Methionine	Pyridoxamine	
Cysteine	Hydroxyproline	Thiamine	
Tyrosine	Tryptophan	Riboflavin	
		p-Hydroxybenzoic acid	

Active compounds

L-Amino acids	Others
Phenylalanine	Phenylpyruvic acid
Phenylalanine amide	Phenyllactic acid
Phenylalanine, methyl ester	Chorismic acid
Phenylalanyltyrosine	
Proline	

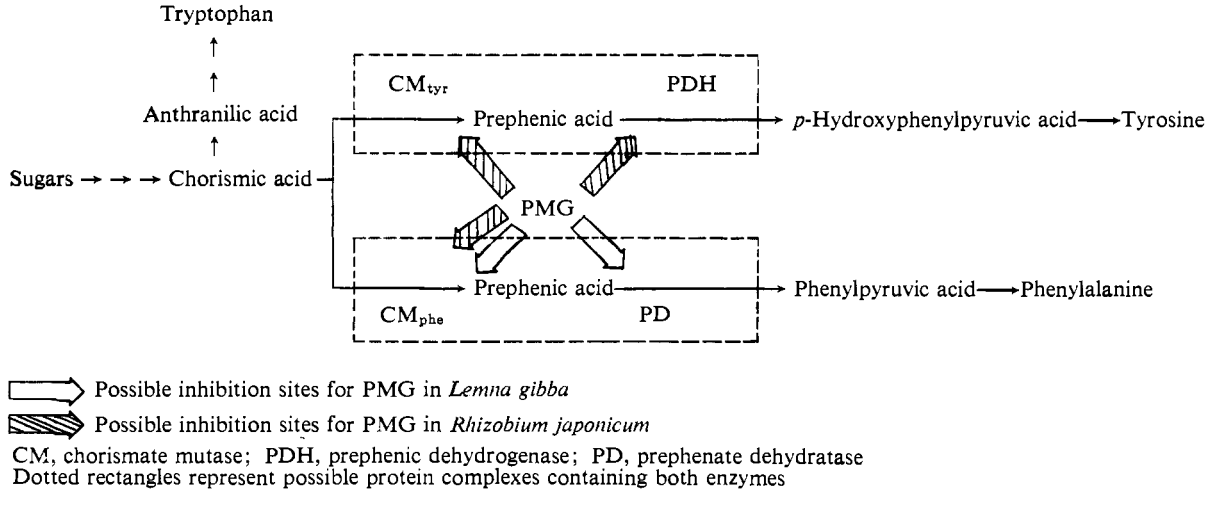

Figure 1. Abbreviated schematic for the aromatic amino acid biosynthetic pathway showing postulated control sites for *N*-phosphonomethylglycine

Table V. Effects of PMG on Bacterial Growth (*R. japonicum*)

Treatment	od_{660}[a]
Control	0.857
PMG, 10^{-3} *M*	0.073
10^{-4} *M*	0.090
10^{-5} *M*	0.263

[a] od_{660}/ml used as measure of growth after a 5-day incubation.

Table VI. Reversal of PMG Growth Inhibition in Bacteria (*R. japonicum*)

Treatment	Chemical concn, *M*	Percent inhibition
Control	...	0
PMG	10^{-4}	88
Phe	2×10^{-4}	0
Tyr	2×10^{-4}	0
PMG + Phe	$10^{-4} + 2 \times 10^{-4}$	81
PMG + Tyr	$10^{-4} + 2 \times 10^{-4}$	81
Phe + Tyr	2×10^{-4} each	0
PMG + Phe + Tyr	$10^{-4} + 2 \times 10^{-4} + 2 \times 10^{-4}$	39
Phenylpyruvic acid (PP)	2×10^{-4}	0
PMG + PP	$10^{-4} + 2 \times 10^{-4}$	69
p-Hydroxyphenylpyruvic acid (HPP)	2×10^{-4}	0
PMG + HPP	$10^{-4} + 2 \times 10^{-4}$	68
PP + HPP	2×10^{-4} each	0
PMG + PP + HPP	$10^{-4} + 2 \times 10^{-4} + 2 \times 10^{-4}$	43
Prephenic acid	1.7×10^{-4}	18
PMG + prephenic acid	$10^{-4} \times 1.7 \times 10^{-4}$	70
Chorismic acid	10^{-4}	25
PMG + chorismic acid	10^{-4} each	48

Table VII. Effect of PMG on Free Amino Acid Levels in *Lemna*[a]

Treatment	Fresh weight of plants, g	80% EtOH-soluble residues, mg	Concn of amino acids[b] Total amino acids	Phenylalanine	Tyrosine	Tyrosine-to-phenylalanine ratio
Control	10.9	152	277	2.3	2.8	1.2
PMG (10^{-4} *M*)	8.1	139	452	1.8	4.3	2.4

[a] Five grams fresh weight of *Lemna* were introduced into control and treated medium. Flasks were incubated for 48 hr (approximately one doubling time. [b] Picomoles/g of fresh weight.

Chorismic acid is a key intermediate in the biosynthesis of phenylalanine, tyrosine, and tryptophan. Chorismic mutase converts chorismic acid to prephenic acid, the precursor of phenylalanine and tyrosine, and phenylalanine dehydratase converts prephenic acid to phenylpyruvic acid which can then be ammoniated to phenylalanine. There is little information about prephenate dehydratase in higher plants and only a limited amount of data on chorismate mutase (Cotton and Gibson, 1968; Gilchrist *et al.*, 1972). Much of our understanding of these enzymes is based on studies with bacteria, fungi, and algae (Lingens, 1968; Weber and Boeck, 1969; Yoshida, 1969). Two references regarding prephenate dehydratase (Schmidt and Zalkin, 1971) and chorismate mutase (Gilchrist *et al.*, 1972) are of interest. These present evidence that the two enzyme activities may reside in the same protein molecule and that chorismate mutase exists in two forms. At least three forms of the latter enzyme have been found in *Bacillus subtilis* (Lorence and Nester, 1967). While unequivocal proof as to the precise site(s) of action of *N*-phosphonomethylglycine is not yet available, the following schematic representation (Figure 1) of possible sites is consistent with the data reported and with the information cited above.

In the case of *Lemna*, an interference with the conversion of chorismic acid to prephenic acid or the conversion of prephenic acid to phenylpyruvic acid either by inhibition or repression of the enzymes for which they serve as substrates could reduce the formation of phenylalanine. If the block were highly specific, chorismic acid could be diverted to form an excess of tyrosine, resulting in the feedback control of chorismate mutase, prephenic dehydratase, and/or some other portion of the aromatic pathway. The striking similarities in the inhibitory properties of L-tyrosine and *N*-phosphonomethylglycine, especially with reference to morphological changes, appear particularly relevant. The elevation in tyrosine levels in *Lemna* inhibited by *N*-phosphonomethylglycine are also noteworthy.

These results suggest a possible control mechanism in *Lemna* similar to that found in *Saccharomyces cerevisiae* (Lingens *et al.*, 1966), where chorismate mutase is inhibited by tyrosine, activated by tryptophan, and unaffected by phenylalanine.

The studies with *Rhizobium* suggest that different control mechanisms are involved relative to *Lemna*. Since both phenylalanine and tyrosine were required to achieve significant reversal of growth inhibition, it could be postulated that *Rhizobium* has a common chorismate mutase that is inhibited or repressed by *N*-phosphonomethylglycine. Alternatively,

if there are two or more isozymes of chorismic mutase, one containing prephenate dehydratase and another prephenate dehydrogenase, both could be inhibited. The reversals with a combination of phenylpyruvic and *p*-hydroxyphenylpyruvic acids would suggest that the block is not at the level of the transaminase reactions. This was also supported by comparable reversals with aryllactic acids which were assumed to be oxidized *in vivo* to their corresponding arylpyruvic acids (data not reported). Similar reversals were noted for *Lemna* but to a lesser degree.

Tryptophan was not a reversing agent in *Lemna*, and combinations of tryptophan with phenylalanine or phenylalanine and tyrosine were not absolutely essential for significant reversals. Thus, it is not likely that the block occurs prior to chorismic acid synthesis. It should be noted, however, that the most complete reversals in *Lemna* were usually achieved with a combination of all three aromatic amino acids. Some blockage of any reaction where chorismic acid serves as the substrate could therefore be invoked, including anthranilate synthetase. Another possible explanation for the benefits of tryptophan in conjunction with phenylalanine and tyrosine may reside in its activation of chorismate mutase, as shown in bacteria (Lingens, 1968), fungi (Baker, 1966), yeast (Lingens *et al.*, 1966), green algae (Weber and Boeck, 1969), and higher plants (Cotton and Gibson, 1968; Gilchrist *et al.*, 1972). This type of activation could lead to a conformational change in chorismate mutase and alter the affinity of the inhibitor for the enzyme.

The reversal studies with chorismic and prephenic acids, while encouraging, present some problems in that they or metabolites derived from them are quite inhibitory to growth. Kinetic studies with these substrates and *N*-phosphonomethylglycine may be particularly appropriate at the subcellular level.

It is apparent that further investigations will be required to sort out the specific site(s) and type of interaction for *N*-phosphonomethylglycine. However, based on these studies and current work with *Salmonella typhimurium* auxotrophs for phenylalanine and tyrosine, the model proposing a localization of the effects of *N*-phosphonomethylglycine at chorismate mutase and/or prephenate dehydratase appears to be reasonable.

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Dieldrin and *p,p'*-DDT Effects on Some Microsomal Enzymes of Livers of Chickens and Mallard Ducks

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Two experiments were conducted to determine effects of dieldrin and *p,p'*-DDT on some mixed-function oxidases of hepatic microsomes of White Leghorn chickens and mallard ducks. Aniline hydroxylase and aminopyrine *N*-demethylase activities in control birds were lower in ducks than in chickens, whereas cytochrome P_{450} concentration and estradiol metabolism in microsomes were about equal in both species. Dieldrin at levels of 10 and 20 μg/g of diet and DDT at 100 and 200 μg/g of diet increased cytochrome P_{450} concentration and estradiol metabolism in microsomes of both species, but they increased these parameters more in ducks than in chickens. DDT decreased aniline hydroxylase activity in chicken microsomes and increased aniline hydroxylase activity in duck microsomes, but did not affect aminopyrine *N*-demethylase activity of either species. Dieldrin did not affect aniline hydroxylase activity in chickens or ducks and did not affect aminopyrine *N*-demethylase activity in ducks, but it probably increased *N*-demethylase activity in chickens.

Trace amounts of chlorinated hydrocarbon insecticides are commonly found in our environment and are regularly found in the body fat of domestic and wild animals. Although it is virtually impossible to determine the effects that very low levels of these residues may have on the physiology of any species of animals, or to study these effects by feeding the chemicals causing these residues to all species of animals, it is important to test these chemicals by feeding them to several species. Data so obtained may be used as guidelines for evaluating research where different species of animals are used and for predicting whether or not effects may occur in nonexperimental animals. Also, biological

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