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THE HERBICIDE GLYPHOSATE IS A POTENT INHIBITOR OF 5-ENOLPYRUVYL-SHIKIMIC ACID-3-PHOSPHATE SYNTHASE

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SUMMARY

The broadspectrum herbicide glyphosate (N-[phosphonomethyl]-glycine), which causes the accumulation of shikimic acid in plant tissues, inhibits the enzymatic conversion of shikimic acid to anthranilic acid in a cell-free extract of Aerobacter aerogenes 5o% at 5 to 7 μM concentrations. Of the four enzymes involved in the transformation, only 5-enolpyruvylshikimic acid-3-phosphate synthase is inhibited by the herbicide.

INTRODUCTION

Essentially all major classes of pesticides have been found via random synthesis and screening programs (1). Baillie et al. (2) proposed the biochemical design de novo of inhibitors of shikimate pathway enzymes as potential herbicides, because this pathway operates only in plants and microorganisms (3) and because it was hoped that an inhibitor of a key enzyme of plant metabolism might be herbicidal without being toxic to animals. The authors failed, however, in their search for potential irreversible inhibitors of shikimate dehydrogenase.

It was proposed by Jaworski (4) that the herbicide glyphosate (N-[phosphonomethyl]-glycine) (5) inhibits the biosynthesis of aromatic amino acids, presumably at the site of chorismate mutase and/or prephenate dehydratase. It was rather consistently found that phenylalanine and tyrosine acted synergistically to reverse the growth inhibition caused by glyphosate in

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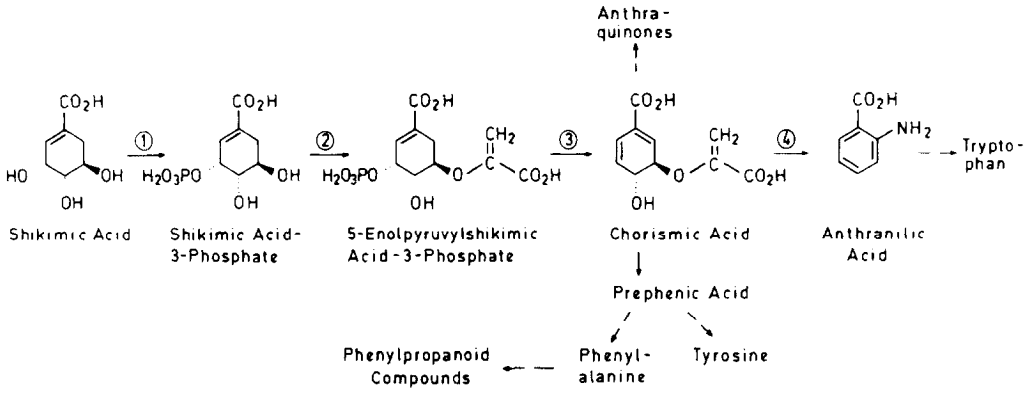

Figure 1. Scheme of metabolic reactions that were investigated with regard to glyphosate action. (1) = shikimate kinase; (2) = 5-enolpyruvylshikimate-3-phosphate synthase; (3) = chorismate synthase; (4) = anthranilate synthase.

microorganisms as well as in higher plants (cf. 6), but neither chorismate mutase nor prephenate dehydratase were found to be subject to inhibition by the herbicide (7). We have recently shown that glyphosate inhibits the formation of phenylalanine-derived phenylpropanoid compounds and reduces phenylalanine concentrations in buckwheat (8,9). Furthermore, the herbicide inhibits the incorporation of $[^{14}C]$shikimic acid into all three aromatic amino acids (9), causes an up to several hundredfold accumulation of shikimic acid in plant tissues (1o) and blocks the formation of anthraquinoid pigments, which are biosynthetically derived from chorismic acid, in cultured plant cells (1o). These results are best explained by the assumption that glyphosate inhibits an enzymatic step en route from shikimic acid to chorismic acid (Fig. 1). This assumption was confirmed by the finding that glyphosate inhibits the formation of anthranilic acid from shikimic acid in a cell-free system from Aerobacter aerogenes, strain 62-1 (1o). We now report that of the four enzymes involved in the transformation (Fig. 1) 5-enolpyruvylshikimic acid-3-phosphate synthase is the target of glyphosate action.

MATERIALS AND METHODS

Aerobacter aerogenes (=Klebsiella pneumoniae, ATCC 25306) was grown and cell-free extracts prepared according to Morgan et al. (11). The reaction mixture for the conversion of shikimic acid to anthranilic acid contained in a total volume of 1 ml: 10 mM Tris/HCl, pH 8.2; 5 mM $MgCl_2$; 1 mM NAD; 1 mM ribose-5-phosphate; 5 mM glutamine; 1 mM shikimic acid, and 2.5 mg protein. After 40 min at 37°C anthranilic acid was measured spectrophotometrically (11). In some experiments [^{14}C]shikimic acid (The Radiochemical Centre Amersham) was used (sp. Act. = 37 kBq/µMol), and the volume of the incubation mixture was reduced tenfold. After termination of the reaction by heating for 1 min in a boiling waterbath and subsequent centrifugation 50 µl of the supernatant were spotted on precoated cellulose thin-layer plates, which were developed in 2-butanol: methanol: NH_4OH: H_2O (50:35:5:10, v/v). Radioactivity on the chromatograms was detected with a Berthold-Frieseke Scanner LB 2760. For treatment with alkaline phosphatase (calf intestinal, Boehringer, Mannheim) incubation mixtures were adjusted to 0.1 mM glycine/NaOH, pH 10.5, and 0.1 mM $ZnCl_2$, and then incubated for 30 min at room temperature. The assay mixture for 5-enolpyruvylshikimic acid-3-phosphate synthase contained 10 mM Tris/HCl, pH 8.2; 5 mM KF; 2 mM phosphoenolpyruvate, 1 mM shikimic acid-3-phosphate (12) and 0.25 mg protein of the dialyzed extract in a total volume of 100 µl. The reaction was followed by measuring the shikimic acid-3-phosphate-dependent disappearance of phosphoenolpyruvate (13). Neither pyruvate kinase nor lactate dehydrogenase, which were used in the assay of phosphoenolpyruvate, were inhibited by glyphosate. Shikimate kinase was assayed as described elsewhere (10), and anthranilate synthase according to Egan and Gibson (14). The latter procedure was also used for the assay of chorismate synthase, except that chorismate (15) was replaced by 5-enolpyruvylshikimic acid-3-phosphate (16) and that 2 mM $NADH_2$ was added to the incubation mixture.

RESULTS

At 0.1 mM concentration glyphosate inhibited the cell-free formation of anthranilic acid from shikimic acid almost completely, and 50% inhibition of the overall reaction was achieved with 5 to 7 µM glyphosate (Fig. 2). When radioactive shikimic acid was employed as the substrate, thin-layer chormatography established that, in the absence of glyphosate, it was converted to anthranilic acid (R_F = 0.62) when glutamine was present, and to chorismic acid (R_F = 0.30), when glutamine was absent. In the presence of 0.5 mM glyphosate a polar metabolite of shikimic acid accumulated, which hardly migrated from the

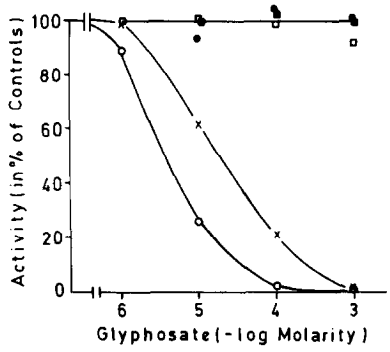

Figure 2. Effect of glyphosate on the conversion of shikimic acid to anthranilic acid (-o-o-) and on the activities of shikimate kinase (-●-●-), 5-enolpyruvylshikimate-3-phosphate synthase (-x-x-), chorismate synthase (-□-ü-), and anthranilate synthase (-■-■-) in a cell-free extract of Aerobacter aerogenes 62-1.

origin (R_F = o.o2). Treatment of the metabolite with alkaline phosphatase released phosphate, and the dephosphorylated product was identified as shikimic acid (R_F = o.26) rather than 5-enolpyruvylshikimic acid (R_F = o.14). High voltage paper electrophoresis at pH 8.6 confirmed the identification. Therefore, the metabolite accumulating in the presence of glyphosate was shikimic acid-3-phosphate, and the target of glyphosate was consequently identified as 5-enolpyruvylshikimic acid-3-phosphate synthase (step 2 in Fig. 1). When the enzymes catalyzing the 4 numbered steps in Fig. 1 were assayed individually with their appropriate substrates in the absence and presence of glyphosate it was confirmed that the herbicide inhibited exclusively 5-enolpyruvylshikimic acid-3-phosphate synthase (Fig. 2).

DISCUSSION

We have shown here that glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase from Aerobacter aerogenes, and we have preliminary evidence that glyphosate

also inhibits the 5-enolpyruvylshikimic acid-3-phosphate synthases of E. coli and of the mung bean, Vigna radiata Wilczek. These findings lend solid support to our previous conclusion (9,1o) that glyphosate blocks the formation of chorismic acid in vivo. The fact that shikimic acid, rather than shikimic acid-3-phosphate, accumulates in glyphosate-treated plant tissues (1o) is best explained by the assumption that shikimic acid-3-phosphate is dephosphorylated by a phosphatase prior to its deposition (in the vacuole?).

It is apparent from Fig. 2 that the inhibition of 5-enolpyruvylshikimic acid-3-phosphate synthase by glyphosate is less efficient than inhibition of the overall reaction from shikimic acid to anthranilic acid. This difference is likely to be due to the lower stationary concentrations of 5-enolpyruvylshikimic acid-3-phosphate in the latter system. We are currently investigating the nature of the interaction of glyphosate with 5-enolpyruvylshikimic acid-3-phosphate synthase. It appears that both the sensitivity and the specificity of the inhibition by glyphosate and structurally related compounds correlate well with the effects of these compounds in vivo.

In retrospect, our results confirm the suggestion of Baillie et al. (2) that an inhibitor of a shikimate pathway enzyme might be a potential herbicide.

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