Enzymological Basis for Herbicidal Action of Glyphosate[1]

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ABSTRACT

The effects of 1 millimolar glyphosate (*N*-[phosphonomethyl]glycine) upon the activities of enzymes of aromatic amino acid biosynthesis, partially purified by ion-exchange chromatography from mung bean seedings (*Vigna radiata* [L.] Wilczek), were examined. Multiple isozyme species of shikimate dehydrogenase, chorismate mutase, and aromatic aminotransferase were separated, and these were all insensitive to inhibition by glyphosate. The activities of prephenate dehydrogenase and arogenate dehydrogenase were also not sensitive to inhibition. Two molecular species of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase were resolved, one stimulated several-fold by Mn^{2+} (DAHP synthase-Mn), and the other absolutely dependent upon the presence of Co^{2+} for activity (DAHP synthase-Co). Whereas DAHP synthase-Mn was invulnerable to glyphosate, greater than 95% inhibition of DAHP synthase-Co was found in the presence of glyphosate. Since Co^{2+} is a V_{max} activator with respect to both substrates, glyphosate cannot act simply by Co^{2+} chelation because inhibition is competitive with respect to erythrose-4-phosphate. The accumulation of shikimate found in glyphosate-treated seedlings is consistent with *in vivo* inhibition of both 5-enolpyruvylshikimic acid 3-phosphate synthase and one of the two DAHP synthase isozymes. Aromatic amino acids, singly or in combination, only showed a trend towards reversal of growth inhibition in 7-day seedlings of mung bean. The possibilities are raised that glyphosate may act at multiple enzyme targets in a given organism or that different plants may vary in the identity of the prime enzyme target.

Glyphosate (*N*-[phosphonomethyl]glycine) is an exceedingly effective broad-spectrum herbicide which is readily translocated between plant tissues (29). Residual glyphosate also biodegrades very satisfactorily in soil environments (32). Beyond its herbicidal properties, glyphosate is often a potent inhibitor of microbial growth. The general finding that glyphosate inhibition is antagonized by one or more of the aromatic amino acids extends to a wide range of organisms, including prokaryotes, eukaryotic algae, and plants (15).

In buckwheat and cultured cells of *Galium mollugo* L. (*Rubiaceae*), physiological experiments have shown that glyphosate causes shikimate accumulation and blocks the entry of radiolabeled shikimate into aromatic amino acids (1). Evidently, the accumulation of shikimate in response to glyphosate treatment generally occurs in plants, inasmuch as such data have now been obtained from dozens of diverse species (2, 3). These data are consistent with the possibility that EPSP[2] synthase might be the enzyme target of herbicide action, a possibility indirectly supported by the great sensitivity of EPSP synthase from *Klebsiella pneumoniae* to glyphosate inhibition (31).

Whether the highly effective herbicidal action of glyphosate can be related convincingly to a specific enzyme blockage at the metabolic pathway level requires that enzymologic data be directly obtained from plant sources. To date, the only nonmicrobial studies relating enzyme data to glyphosate effects were those (9–11, 17), testing a hypothesis that glyphosate-mediated induction of PAL in higher plants might account for growth inhibition. However, it now seems likely that PAL induction is an effect, rather than the cause, of metabolic events initiated by glyphosate (1).

Although the literature overall seems to reflect an assumption that the antimicrobial and herbicidal actions of glyphosate in nature will prove to operate in some universal way, there are reasons to consider the possibilities of different enzymic targets in different organisms or even of multiple enzymic targets in the same organism. First, the details of aromatic biosynthesis and regulation have proven to be surprisingly diverse in microorganisms (4). Data suggesting diversity in plant material as well (e.g. corn [6], mung bean [27], and tobacco [unpublished data]) are now coming to light. Second, the particular details of reversal of glyphosate inhibition by aromatic acids are variable from organism to organism. In *E. coli*, all three aromatic amino acids are required for complete reversal (25). In *Rhizobium japonicum*, the combination of phenylalanine plus tyrosine reversed glyphosate inhibition (18) in contrast to *Lemna gibba* where phenylalanine alone was adequate for reversal (18). In *Euglena gracilis*, aromatic amino acids only partially reverse inhibition (40%) unless the minor pathway products: 4-aminobenzoate, 4-hydroxybenzoate, 2,3-dihydroxybenzoate, and 3,4-dihydroxybenzaldehyde are also present (5).

Where partial reversal of inhibition is obtained with either L-phenylalanine alone or L-tyrosine alone, the greatest reversal is usually accomplished by L-phenylalanine. For example, the antagonism of inhibition by L-phenylalanine and L-tyrosine was 41% and 12% in *Escherichia coli*, and 27% and 3% in *Chlamydomonas reinhardii* (15). Qualitatively, it was found that L-phenylalanine was a better partial antagonist of glyphosate than L-tyrosine in suspension cultures of both carrot and soybean (15), although reversal of inhibition by any individual amino acid was still minor. Most plant systems (tobacco, carrot, soybean, *Arabidopsis thaliana*) thus far characterized (15, 16) display a glyphosate sensitivity that is synergistically reversed by the combination of L-tyrosine and L-phenylalanine.

Recently, it was shown (22) that tricarboxylic acid cycle intermediates (such as α-ketoglutarate, succinate, and malate) are as effective as aromatic amino acids in reversing glyphosate inhibition in suspension cultures of carrot (*Daucus carota* L.). Aspartate and glutamate were also effective antagonists of glyphosate, probably via α-ketoglutarate and oxaloacetate formation. The latter authors suggested that the prime herbicidal event may be depletion of respiratory substrates owing to the loss of erythrose-4-P and

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[2] Abbreviations: EPSP, 5-enolpyruvylshikimic acid-3-phosphate; PAL, phenylalanine ammonia-lyase; E-4-P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; PMSF, phenylmethylsulfonyl fluoride; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; EPPS, *N*-2-hydroxy-ethylpiperazine propane sulfonic acid.

PEP into the glyphosate-blocked (presumably at EPSP synthase) pathway of aromatic biosynthesis. An alternative possibility is that addition of exogenous tricarboxylic acid cycle intermediates elevates the availability of PEP and E-4-P for aromatic biosynthesis, thereby overwhelming competitive inhibition at the enzyme target(s) of glyphosate. It has been shown (19) that the availability of substrate rather than level of endproduct is a key aspect of regulation in aromatic biosynthesis of photosynthetic prokaryotes (cyanobacteria).

A systematic enzymologic study of aromatic pathway enzymes as potential targets for glyphosate is desirable in a sensitive plant system. Detailed baseline studies can then serve as a foundation for comparative studies required for conclusions about the universality or the diversity of glyphosate action in nature. We have optimized assay conditions for many of the shikimate pathway enzymes in mung bean, and have examined these partially purified plant enzymes for possible sensitivity to inhibition by glyphosate.

MATERIALS AND METHODS

Plant Materials. Seeds of *Vigna radiata* [L.] Wilczek, obtained in bulk from a local food co-op, were grown as previously described (27) in a growth chamber (16-h photoperiod at 24°C with 18°C nights), 140 cm from the light source (about 5,000 lux). Mung bean seedlings were frozen intact in liquid N_2 and ground to a fine powder in a Waring Blendor, prior to the preparation of enzyme extracts.

Preparation of Crude Extract. Cold extraction buffer, containing 10 mM piperazine-*N,N'-bis*[2-ethane-sulfonic acid] (Pipes, pH 7.2), 1 mM DL-DTT, 0.1 mM PMSF and 50 mM Na-ascorbate, was combined with freshly prepared frozen mung bean powder, hydrated polyvinylpolypyrrolidone and acid-washed Amberlite nonionic polymeric adsorbent XAD-4 (23) [1:1:1:1, v/w/w/w], and stirred until melted. All further procedures were carried out as quickly as possible at 4°C. The slurry was filtered through two layers of cheesecloth and one layer of Miracloth (Chicopee Mills, Inc.), centrifuged for 10 min at 75,000*g*, and the resulting supernatant was desalted on a Sephadex G-25 column (2.5 × 54.5 cm) equilibrated with 10 mM Pipes buffer (pH 7.2) containing 1 mM DTT and 0.1 mM PMSF. Fractions containing protein were designated as crude extract and pooled; protein concentration was estimated spectrophotometrically (21).

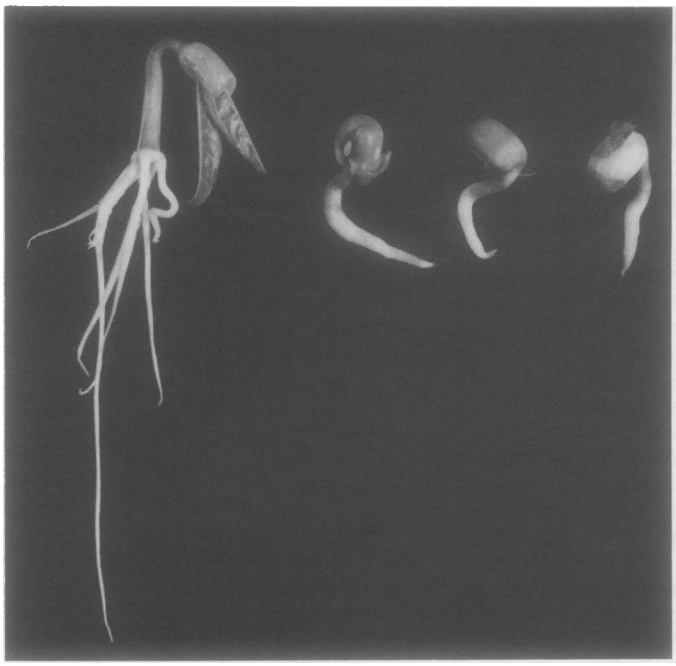

FIG. 1. Comparison of untreated germinated seedling (left) with three glyphosate-treated (0.05 mM) seedlings after 7 d (right). Treated seedlings were short and stubby, and the roots had few or no secondary roots.

Column Chromatography. Crude extract (400 mg protein) was chromatographed on a DEAE-cellulose (Whatman DE-52) column (2.5 × 20 cm) equilibrated with 10 mM Pipes buffer (pH 7.2) containing 1 mM DTT and 0.1 mM PMSF. The column was washed with 450 ml of the equilibration buffer prior to the application of a 600-ml linear gradient (0-0.5 M KCl in the same buffer). Fractions of 4.2 ml were collected.

DAHP synthase-Co was purified further as follows. The peak of DAHP synthase-Co eluted from DEAE-cellulose was concentrated by ultrafiltration (Amicon PM-10 membrane) and then desalted on a Sephadex G-25 column (2.5 × 54 cm) that was equilibrated and eluted with 5 mM K-phosphate buffer (pH 7.2) containing 1 mM DTT and 0.1 mM PMSF. Eluate protein was applied to a hydroxylapatite (Bio-Gel HTP) column (2 × 7 cm) that was washed with the latter-described buffer. DAHP synthase-Co was eluted with a linear gradient (150 ml), ranging from 5 mM to 250 mM K-phosphate buffer (pH 7.2). Each reservoir buffer contained 1 mM DTT and 0.1 mM PMSF. Eluate fractions of 1.8 ml were collected. DAHP synthase-Co fractions were pooled, desalted on a Sephadex G-25 column (2.5 × 54 cm) that was equilibrated with 10 mM Pipes buffer (pH 7.2) containing 0.1 mM PMSF. A 2.5-fold concentration was obtained by ultrafiltration. This preparation was free of phosphatase and chorismate mutase activities, originally present in DEAE-cellulose eluates, and had a specific activity of 99.1 nmol/min^{-1} mg^{-1} protein at 37°C.

Enzyme Assays. Chorismate mutase was assayed by the method of Cotton and Gibson (8). Reaction mixtures (200 μl) contained enzyme, 1 mM chorismate and 25 mM Pipes (pH 7.5). Aromatic aminotransferases were assayed at pH 8.0 as previously described (27), using a mixture of keto acid substrates (prephenate, 4-hydroxyphenylpyruvate, and phenylpyruvate) combined with a mixture of radioactive (^{14}C) amino acids (L-glutamate, L-aspartate, and L-alanine). Arogenate dehydrogenase, prephenate dehydrogenase, and shikimate dehydrogenase were assayed as previously described (27). Reaction mixtures (200 μl) contained 25 mM Pipes buffer (pH 7.5), 0.5 mM NADP and either 1 mM shikimate, 1 mM prephenate or 0.94 mM arogenate, as appropriate. DAHP synthase was assayed by the method of Srinivasan and Sprinson (30).

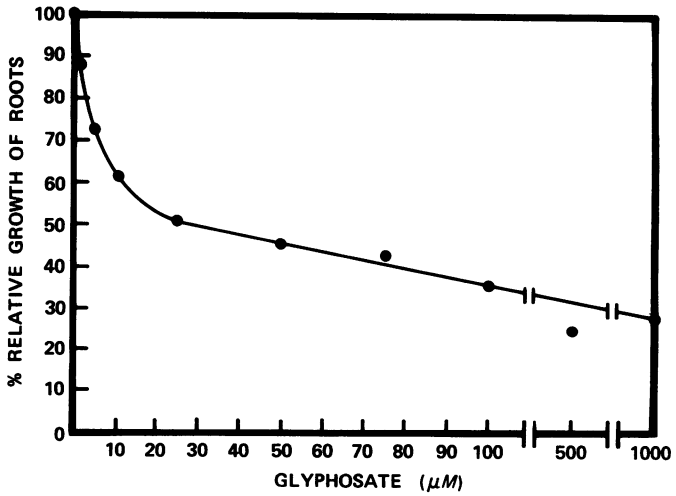

FIG. 2. Inhibition of mung bean seedling development by glyphosate. The fresh weight of 35 roots/sample was determined. Roots were excised along the boundary formed by the white base of the root and the burgundy-colored hypocotyl (Fig. 1). Data are expressed as the fraction of fresh weight in the presence of glyphosate to fresh weight in the absence of glyphosate. The fresh weight of 35 roots grown in the absence of glyphosate was 1.4527 g.

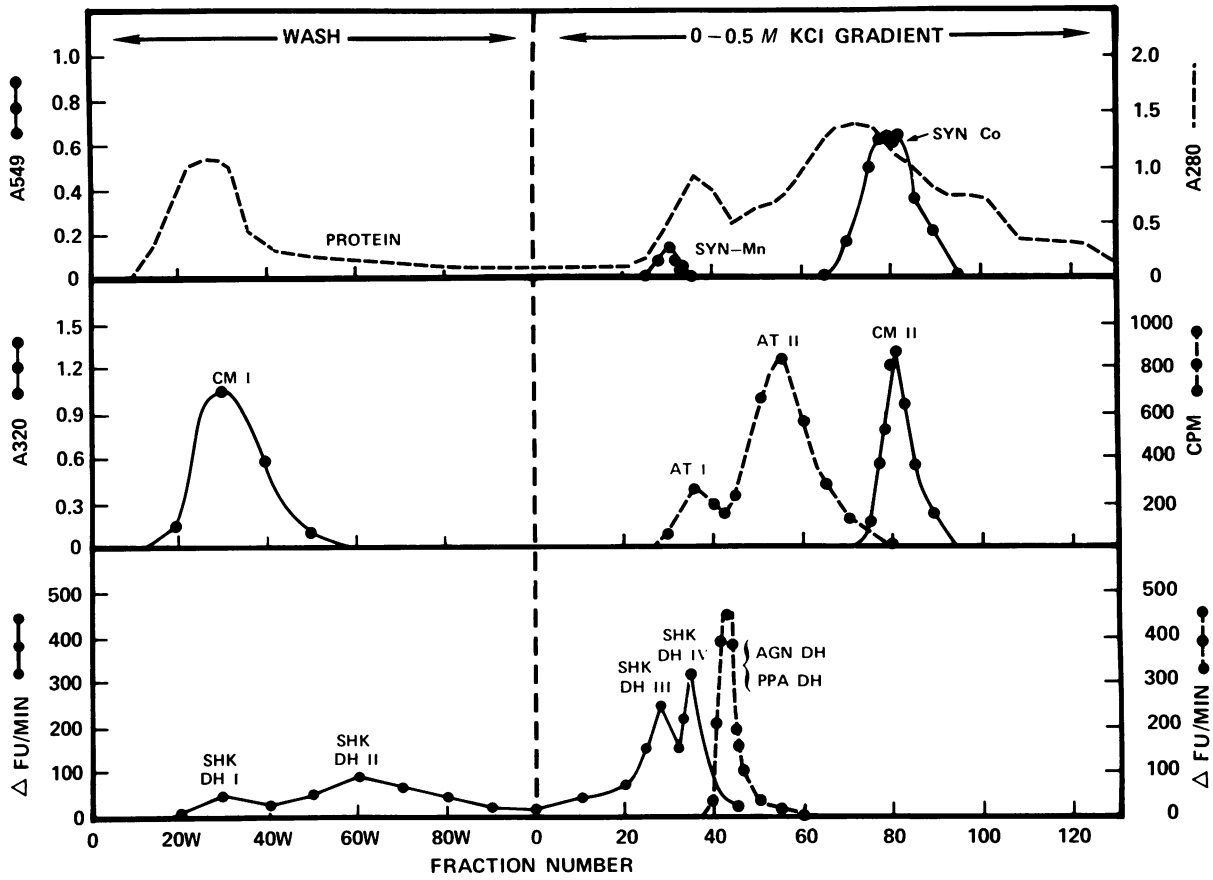

FIG. 3. Partial purification of mung bean seedling crude extract by DEAE-cellulose column chromatography as described in "Materials and Methods." Enzyme assays were conducted as described in "Materials and Methods" for 15 min at 37°C using 100 μl of fraction, except for the assays of aromatic aminotransferases (25 μl of fractions used) and dehydrogenases (continuous assay measuring increase in NADPH product). Abbreviations: SYN-Mn, Mn^{2+}-stimulated isozyme of DAHP synthease; SYN-Co, Co^{2+}-dependent isozyme of DAHP synthase; CM, chorismate mutase; AT, aromatic aminotransferase; SHK DH, shikimate dehydrogenase; PPA DH, prephenate dehydrogenase; AGN DH, arogenate dehydrogenase; FU, fluorescent unit.

Reaction mixtures contained 2 mM PEP, enzyme, and either 0.5 mM $CoCl_2 \cdot 6H_2O$, 4 mM E-4-P, and 80 mM EPPS (pH 8.8) or 0.4 mM $MnCl_2 \cdot 4H_2O$, 0.5 mM E-4-P, and 10 mM Pipes buffer (pH 7.2). Reaction times were 30 min at 37°C unless noted otherwise.

Phosphatase activity was assayed by measuring enzymic formation of *p*-nitrophenol formed from *p*-nitrophenylphosphate. Reaction mixtures (1 ml) contained enzyme, 1 mM substrate, and 10 mM Pipes buffer (pH 7.2). After incubation for 15 min at 37°C the reaction was stopped by addition of 200 μl of 2.5 N NaOH. Chromophore absorption was read at 410 nm in a Gilford spectrophotometer.

Assay of Shikimic Acid Accumulation. Five-g samples of seedling material were extracted by a modification of a procedure described by Nagels *et al.* (24). Extraction was carried out by emulsification in 20 ml 80% ethanol, refluxing with three changes of ethanol. The ethanol from each 15-min reflux period was removed by filtration through a Whatman GF-C glass filter disc. The combined ethanol extracts were concentrated to 10 ml by vacuum distillation and passed through a polyvinylpolypyrollidone column (1 × 4 cm) which was then eluted with water. Eluate was made up to 10% (w/v) with TCA and then centrifuged. TCA was removed from the supernatant by four ether extractions, followed by vacuum distillation at room temperature to remove residual ether. The sample was passed through a Sigma SP-Sephadex (H^+) column (1 × 4 cm), eluted with water, and concentrated as before by vacuum distillation. The method of Gaitonde and Gordon (13) was used to assay for shikimate concentration.

Chemicals. Chorismate was prepared from culture supernatants of *Klebsiella pneumoniae* 62-1 according to the methods of Gibson (14). Barium prephenate was prepared from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (12) and was converted to the potassium salt with excess K_2SO_4 prior to use. L-Arogenate was prepared from culture supernatants of a triple auxotroph of *Neurospora crassa* ATCC 36373 (20) and was prepared according to the modified method of Zamir *et al.* (33).

Liquifluor and radioactive amino acids were obtained from New England Nuclear, thiobarbituric acid was from Eastman Kodak Co., and paraperiodic acid was from G. Frederick Smith Chemical Co. DEAE-cellulose (DE 52), hydroxylapatite (Bio-Gel HTP), and both Sephadex G-25 and XAD-4 were obtained from Whatman Ltd., Bio-Rad Laboratories and Sigma, respectively. Analytical-grade glyphosate (99.88% pure) was a gift from Monsanto Co. All other biochemicals were obtained from Sigma, and were of the highest grade available.

RESULTS

Glyphosate Inhibition of Growth. Documentation of the sensitivity of mung bean to the herbicidal action of glyphosate is shown in Figure 1. Mung beans in 3-g amounts were imbibed for 24 h in the presence of various concentrations of glyphosate (pH 6.0), and were then transferred to 600-ml beakers containing the same concentration of glyphosate in 1% (w/v) agar. Each beaker was

Table I. *The Effect of Glyphosate on the Activity of Shikimate Pathway Enzymes in Mung Bean Seedlings*

Mung bean crude extract was partially purified as described in "Materials and Methods," and peak fractions of enzyme activity were assayed as described in "Materials and Methods" in the presence and absence of 1 mM glyphosate.

Enzyme	Fraction Numbers[a]	Original Activity in Presence of Glyphosate[b]
		%
Shikimate dehydrogenase I	30W	106.4
Shikimate dehydrogenase II	60W	103.0
Shikimate dehydrogenase III & IV	32	95.5
Aromatic aminotransferase I[c]	35	96.5
Aromatic aminotransferase II[c]	55	101.5
Chorismate mutase I	30W	97.3
Chorismate mutase II	81	93.6
DAHP synthase-Mn[d]	31	109.4
DAHP synthase-Co[e]	80	15.0
Arogenate dehydrogenase	45	103.1
Prephenate dehydrogenase	45	100.0

[a] See Figure 3.

[b] Data presented in terms of percent activity measured in the presence of 1 mM glyphosate compared to a control containing no glyphosate.

[c] AT I and AT II were identified (27) as 4-hydroxyphenylpyruvate aminotransferase and prephenate aminotransferase, respectively.

[d] Assayed in the presence of 0.4 mM $MnCl_2 \cdot 4H_2O$, 0.5 mM E-4-P, 2 mM PEP, and 40 mM Pipes (pH 7.2).

[e] Assayed in the presence of 0.5 mM $CoCl_2 \cdot 6H_2O$, 4 mM E-4-P, 2 mM PEP and 80 mM EPPS (pH 8.8).

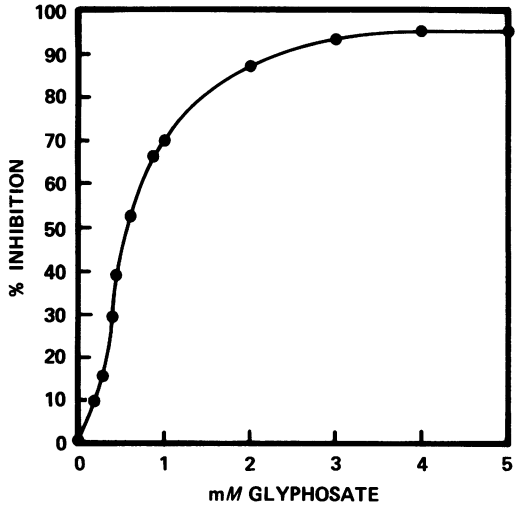

FIG. 4. Glyphosate inhibition of DAHP synthase-Co activity. Partially purified enzyme preparation recovered from hydroxylapatite column chromatography was utilized, and was assayed as described in "Materials and Methods."

covered with plastic and placed in a growth chamber as described in "Materials and Methods. After 6 d, the fresh weight of seedling roots was determined. The results (Fig. 2) show the quantitative effects of glyphosate in the form of an inhibition curve.

Neither individual aromatic amino acids nor a combination of total aromatic pathway endproducts produced more than slight reversal of glyphosate inhibition. Mung bean seedlings were sacrificed after 7 d of germination in the presence or absence of 0.05 mM glyphosate as described in "Materials and Methods." Individual aromatic amino acids were tested as glyphosate antagonists at

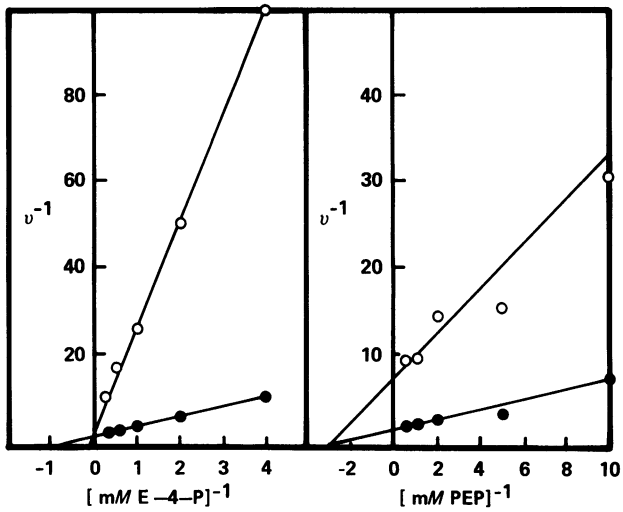

FIG. 5. Double reciprocal plots of DAHP synthase-Co with E-4-P as the variable substrate (left panel) or PEP as the variable substrate (right panel). Partially purified enzyme recovered from hydroxylapatite column chromatography was utilized and was assayed as described in "Materials and Methods" in the absence (●) or presence (○) of 1 mM glyphosate. A v^{-1} value of 10 corresponds to a specific activity of 18.8 nmol/min^{-1} mg^{-1} protein at 37°C.

1 mM initial concentration. Various combination of aromatic pathway endproducts including minor metabolites (4-hydroxybenzoate, 4-aminobenzoate, 2,3-dihydroxybenzoate, and 3,4-dihydroxybenzaldehyde) were also tested. The fresh weight of 35 roots was determined under each condition. Under all reversal conditions tested, glyphosate inhibition was always greater than 50%.

Fractionation of Enzymes. Unlike most microbial preparations, the assay of many aromatic pathway enzymes is difficult or impossible with crude extracts from mung bean. A satisfactory regimen has been worked out (as given in "Materials and Methods") to protect enzymes from proteases and phenolics, as well as from oxidation prior to partial purification on DEAE-cellulose. With enzymes such as DAHP synthase, multiple species of phosphatase exist which degrade both of the reaction substrates as well as the reaction product. Proper kinetics and characterization of DAHP synthase require its resolution from these phosphatases and probably from dehydroquinate synthase, an enzyme which usually is capable of utilizing DAHP in the second pathway reaction without addition of other reactant molecules. A detailed enzymologic study of the two DAHP synthase isozymes will be described elsewhere (in preparation).

Crude extract prepared from mung bean seedlings was passed through a chromatography column of DEAE-cellulose as specified in "Materials and Methods." The enzymes studied are shown in the elution profiles of Figure 3. The activities of prephenate dehydrogenase and arogenate dehydrogenase coeluted. This prephenate-arogenate dehydrogenase (formerly known as prephenate-pretyrosine dehydrogenase) was previously shown (27) to exist as co-purifying activities, even following additional fractionation steps. Although chorismate mutase and one isozyme of DAHP synthase coeluted, these were readily separated by hydroxylapatite column chromatography (see "Materials and Methods"). Aromatic aminotransferases II and I correspond to the previously described (27) prephenate aminotransferase and 4-hydroxyphenylpyruvate aminotransferase, respectively. Two isozymes of DAHP synthase were resolved. DAHP synthase-Co exhibits an absolute requirement for cobalt, whereas DAHP synthase-Mn is stimulated several-fold by manganese. The different conditions specified in the legend of Table I for assay of the two isozymes

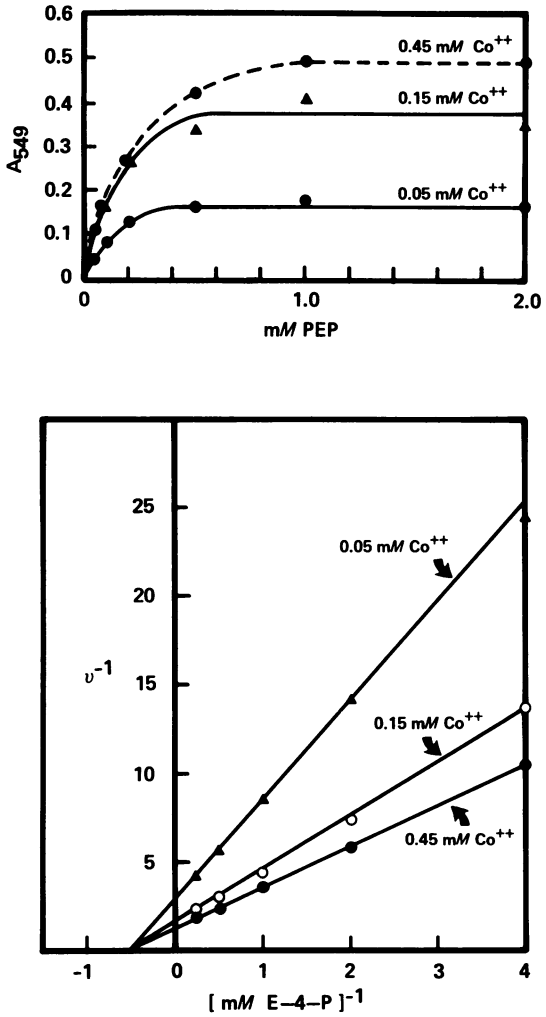

FIG. 6. Stimulation of DAHP synthase-Co by Co^{2+}. Substrate saturation curves obtained with PEP as the variable substrate are shown in the top panel. The concentration of fixed substrate, E-4-P, was 4 mM. The bottom panel shows a double reciprocal plot obtained with E-4-P as the variable substrate and PEP concentration fixed at 2 mM. Enzyme was obtained from hydroxylapatite column chromatography and was assayed as described in "Materials and Methods." A v^{-1} value of 10 corresponds to a specific activity of 18.8 nmol/min^{-1} mg^{-1} protein at 37°C.

Table II. *Accumulation of Shikimic Acid in Mung Bean Seedlings Grown in the Presence of Glyphosate*

Mung bean seedlings were imbibed and grown in the presence of the indicated concentrations of glyphosate, and shikimic acid was determined as described in "Materials and Methods."

Glyphosate Added	Shikimate Content
mM	*nmol g^{-1} fresh wt*
0	72
1	120
5	200
10	290

reflect the differing optimal conditions determined. Multiple enzyme species of chorismate mutase and of shikimate dehydrogenase were also separated (Fig. 3).

No activity for prephenate dehydratase was found. It is interesting that the presence of arogenate dehydratase in *Euglena gracilis* (7) is currently the sole report of a phenylalanine branchlet dehydratase in a photosynthetic eukaryote. We are working to stabilize a preparation of arogenate dehydratase in mung bean.

Enzyme Sensitivity to Glyphosate. Glyphosate at 1 mM concentration was tested as a potential inhibitor of the 12 enzymes specified in Table I. Of these enzymes, only DAHP synthase-Co was vulnerable to inhibition by glyphosate. We have also observed that tobacco (*Nicotiana sylvestris*) possesses a similar glyphosate-sensitive DAHP synthase (unpublished). Using the regimen described in "Materials and Methods" through the DEAE-cellulose step, a partially purified DAHP synthase-Co was obtained from diploid tobacco leaves that was inhibited 86% by 1 mM glyphosate in the presence of 0.5 mM cobalt.

Inhibition Kinetics of DAHP Synthase-Co. DAHP synthase-Co recovered from DEAE-cellulose as shown in Figure 3 was not suitable for rigorous, quantitative studies because of highly active phosphatase activity capable of reaction with either of the DAHP synthase substrates. This problem was completely resolved by use of a hydroxylapatite chromatography step (see "Materials and Methods"), which yielded a stable preparation of DAHP synthase-Co. An inhibition curve obtained with this preparation is shown in Figure 4. A slightly sigmoid inhibition curve was found in which inhibition leveled off at about 95% under the assay conditions specified.

Conventional enzyme kinetics were done (Fig. 5). The results of Lineweaver-Burk analysis show glyphosate inhibition to be competitive with respect to E-4-P and noncompetitive against PEP.

Metal activation of DAHP synthase-Co by cobalt was characterized by detailed kinetic studies (Fig. 6). At saturating substrate concentrations, cobalt activation is maximal at about 0.45 mM. Higher cobalt concentrations produced a rather dramatic degree of inhibition relative to the optimal metal concentration. The top panel of Figure 6 shows a series of substrate saturation curves which clearly illustrate the enhancing effect of increasing cobalt concentration upon the apparent V_{max} with respect to PEP. Activity is not observed at all in the absence of cobalt. The double reciprocal plot given in the bottom panel shows that cobalt also functions as a V_{max} activator with respect to E-4-P. Thus, with either substrate cobalt affects the kinetic parameter of velocity rather than substrate affinity.

Glyphosate-Promoted Accumulation of Shikimic Acid. If glyphosate treatment leads to shikimate accumulation in mung bean seedlings as appears to be characteristic of many glyphosate-treated plants (2, 3), then this would reflect the *in vivo* contribution of DAHP synthase-Mn as a bypass of the glyphosate-sensitive DAHP synthase-Co. Table II shows that shikimic acid does indeed accumulate in response to increasing doses of glyphosate inhibitor.

DISCUSSION

A complete set of enzymologic determinations is needed in a single glyphosate-sensitive plant system in order to document a rigorous definition of herbicide action. Even then, comparative data from a number of plant systems will be necessary prior to generalization, particularly in view of current data suggesting the possibility of multiple pathway targets. At present, the data base supporting interpretations about the action of glyphosate is derived from a melange of experimental systems. For example, the simple reversal of glyphosate inhibition by L-phenylalanine alone in *Lemna* (18) contrasts with the requirement for multiple aromatic amino acids to reverse inhibition in several other plant systems (15, 16) and in *E. coli* (25). It follows that the lack of sensitivity of chorismate mutase or prephenate dehydratase to inhibition by glyphosate (25, 26) and the inhibition of EPSP synthase by glyphosate (31) in enteric bacteria are not compelling data to negate (31) predictions made (18) that glyphosate must target to the phenylalanine branchlet in *Lemna gibba*. Prephenate dehydratase has never been detected in any plant system, and various

attempts to define possible enzyme targets have apparently been thwarted in plants. The prospects for aromatic pathway diversity justify conservativeness in generalizing about the mechanism of glyphosate action in plants, even after rigorously established in a single plant species.

Mung bean seedlings offer an advantageous experimental plant system for characterization of aromatic biosynthesis at the enzymologic level (27). This system is glyphosate-sensitive and accordingly is an excellent model system for the study of glyphosate targeting to aromatic biosynthesis. Partial purification of enzymes was necessary, not only to resolve multiple enzyme species, but also to avoid utilization of substrates or products of a given reaction by other enzymes. A satisfactory removal of phenolics, and the separation of proteases and phosphatases was also achieved.

Our *in vitro* data show the DAHP synthase-Co isozyme to be a candidate enzyme target of glyphosate inhibition in mung bean. Although glyphosate is a metal chelator, it is unlikely that inhibition is the result of cobalt chelation. Inasmuch as cobalt is a V_{max} stimulator, a mechanism of inhibition based upon cobalt chelation should result in kinetics of glyphosate inhibition that are noncompetitive with respect to either substrate. However, glyphosate inhibition is competitive against E-4-P. These data may explain the apparent ability of cobalt to overcome glyphosate inhibition found by Roisch and Lingens (25) as follows. At saturating substrate *in vitro*, cobalt activation is relatively dramatic. Under these conditions, glyphosate inhibition is not overcome by cobalt, but is offset or masked by activation which operates by a separate mechanism. At low substrate levels *in vivo*, effects upon velocity are much less significant than effects upon affinity. Since glyphosate inhibition is competitive with respect to E-4-P and the intracellular concentration of E-4-P in plants is thought to be exceedingly low (distinctly lower than that of PEP [J. Bassham, personal communication]), this suggests that DAHP synthase-Co of mung bean may be quite vulnerable to glyphosate *in vivo*. However, the additional presence of a glyphosate-resistant isozyme, DAHP synthase-Mn, may significantly compensate for inhibition of DAHP synthase-Co by glyphosate in mung bean. Unpublished, preliminary data have been cited (31) in support of the sensitivity of EPSP synthase of mung bean to inhibition by glyphosate. Since glyphosate-treated seedlings of mung bean accumulated shikimate, the DAHP synthase-Co enzyme blockade is apparently bypassed through the activity of DAHP synthase-Mn (which is glyphosate-insensitive).

However, the possibility that inhibition of EPSP synthase is the ultimate herbicidal event in mung bean is not consistent with the distinctly incomplete ability of aromatic pathway endproducts to reverse glyphosate inhibition (although essentially trivial explanations could apply). Perhaps the developmental stage of seedling germination presents unique complications in terms of molecular targets of glyphosate action or in transport of aromatic amino acids. In *Phaseolus vulgaris*, L-tyrosine and L-phenylalanine in combination were completely effective in reversing glyphosate inhibition of transpiration (28). One possible mechanism of glyphosate action is consistent with EPSP synthase being blocked effectively without complete reversal by pathway endproducts. DAHP synthase-Mn is feedback inhibited by L-arogenate but not by L-tyrosine or L-phenylalanine (in preparation). Glyphosate inhibition of EPSP synthase depletes intracellular levels of L-arogenate, which normally inhibits the activity of DAHP synthase-Mn. This allows the futile loss of E-4-P and PEP into the aromatic pathway, resulting in an unacceptable energy drain. Since L-tyrosine and L-phenylalanine do not directly feedback inhibit either DAHP synthase isozyme, they do not reverse glyphosate inhibition very well. It is possible that other plant systems may possess only a single glyphosate-sensitive DAHP synthase, in which case this enzyme may be the prime target of herbicidal action. The several multi-targeting possibilities for glyphosate action may account for its extraordinary effectiveness against both plants and microorganisms.

At least in mung bean, we can dismiss possible enzyme targets of glyphosate that were previously eliminated only indirectly through use of nonplant materials. Pathway enzymes such as prephenate aminotransferase and arogenate dehydrogenase have been described only very recently (6, 27), and are here reported to be glyphosate-insensitive for the first time in any system.

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