Glyphosate Inhibition of 5-Enolpyruvylshikimate 3-Phosphate Synthase from Suspension-Cultured Cells of *Nicotiana silvestris*[1]

JUDITH L. RUBIN, C. GREG GAINES, AND ROY A. JENSEN*
Center for Somatic-cell Genetics & Biochemistry, Department of Biological Sciences, State University of
New York at Binghamton, Binghamton, New York 13901

ABSTRACT

Treatment of isogenic suspension-cultured cells of *Nicotiana silvestris* Speg. et Comes with glyphosate (*N*-[phosphonomethyl]glycine) led to elevated levels of intracellular shikimate (364-fold increase by 1.0 millimolar glyphosate). In the presence of glyphosate, it is likely that most molecules of shikimate originate from the action of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase-Mn since this isozyme, in contrast to the DAHP synthase-Co isozyme, is insensitive to inhibition by glyphosate. 5-Enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) from *N. silvestris* was sensitive to micromolar concentrations of glyphosate and possessed a single inhibitor binding site. Rigorous kinetic studies of EPSP synthase required resolution from the multiple phosphatase activities present in crude extracts, a result achieved by ion-exchange column chromatography. Although EPSP synthase exhibited a broad pH profile (50% of maximal activity between pH 6.2 and 8.5), sensitivity to glyphosate increased dramatically with increasing pH within this range. In accordance with these data and the pK_a values of glyphosate, it is likely that the ionic form of glyphosate inhibiting EPSP synthase is $COO^-CH_2NH_2^+CH_2PO_3^{2-}$, and that a completely ionized phosphono group is essential for inhibition. At pH 7.0, inhibition was competitive with respect to phosphoenolpyruvate (K_i = 1.25 micromolar) and uncompetitive with respect to shikimate-3-P (K_i' = 18.3 micromolar). All data were consistent with a mechanism of inhibition in which glyphosate competes with PEP for binding to an [enzyme:shikimate-3-P] complex and ultimately forms the dead-end complex of [enzyme:shikimate-3-P:glyphosate].

Glyphosate (*N*-[phosphonomethyl]glycine) is a broad-spectrum herbicide which is also a potent growth inhibitor of bacteria and algae (14). Although glyphosate may ultimately perturb a variety of biochemical processes including protein synthesis, nucleic acid synthesis, photosynthesis and respiration (16), the early conjecture (18) that the primary action of glyphosate can be localized to aromatic amino acid biosynthesis remains (with refinement) a credible explanation.

Rigorous evidence has awaited direct characterizations of plant enzymes. Duke and Hoagland (9) demonstrated elevated extractable levels of PAL[2] in dark-grown maize roots grown in the presence of glyphosate. Hence, it seemed that glyphosate might act by promoting a wasteful enhancement of carbon flow towards phenylpropanoid metabolites. However, since the highly effective PAL inhibitor, α-aminooxy-β-phenylpropionic acid, failed to relieve glyphosate toxicity (10), the induction of PAL is undoubtedly an effect rather than a cause of metabolic events initiated by glyphosate. A biosynthetic step between shikimate and chorismate was implicated as an enzyme target of glyphosate action because glyphosate-treated cell cultures of *Galium mollugo* L. accumulated massive amounts of shikimate and displayed diminished production of chorismate-derived anthraquinones (2). Indeed, EPSP synthase (EC 2.5.1.19) has been shown to be a sensitive enzyme target in *Neurospora crassa* (6), *Klebsiella pneumoniae* (1, 3, 34), *Corydalis sempervirens* (3), and *Pisum sativum* (27). These documentations include prokaryotes, lower eukaryotes, and higher plants, thereby implicating EPSP synthase as a general enzyme target of glyphosate action in nature.

Although we showed (29) that a pre-shikimate enzyme (a cobalt-dependent isozyme of DAHP synthase) in mung bean was glyphosate sensitive, treatment with glyphosate still caused accumulation of shikimate. We proposed that if glyphosate inhibits EPSP synthase of mung bean, then endogenous L-arogenate would be unavailable for its normal feedback inhibitor action against an isozyme of DAHP synthase (denoted DAHP synthase-Mn) which is glyphosate resistant. This picture of glyphosate action not only postulates depletion of aromatic amino acids, but also presents a basis for unrestrained early-pathway carbon flow which then imposes a severe energy burden as a consequence of PEP and ATP depletion. This altered biochemical state is not corrected by exogenous aromatic amino acids.

Nicotiana silvestris, an experimental system suited for somatic-cell genetics, was found to possess a pair of DAHP synthase isozymes reminiscent of the mung bean isozymes with respect to glyphosate sensitivity (30). In this paper, we characterize the glyphosate sensitivity of EPSP synthase activity partially purified from isogenic suspension-cultured cells of *N. silvestris*. These results contribute to a growing series of studies (3, 4, 20, 21, 27) of EPSP synthase from higher plants.

MATERIALS AND METHODS

Tissue Culture. Details of subculture routine for growth of suspension cultures, media composition, and other cell culture procedures were given by Gaines *et al.* (11). The cell line (denoted ANS-1) of *Nicotiana silvestris* Speg. et Comes (2N = 24) is an aneuploid derived from a haploid plant 3 years ago. An average chromosome number of 42 was determined (average of 10

[2] Abbreviations: PAL, phenylalanine ammonia-lyase; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; EPSP, 5-enolpyruvylshiki-mate 3-phosphate; PMSF, phenylmethylsulfonyl fluoride; PEP, phosphoenolpyruvate; PK/LDH, pyruvate kinase/lactate dehydrogenase; Epps, *N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid.

spreads) using previously described cytogenetic techniques (11).

Preparation of Crude Extracts. Cells were harvested 5 d after subculture on filters of Miracloth (Calbiochem) using vacuum filtration, washed with 3% (w/v) mannitol, and immediately frozen in liquid nitrogen. The cells were ground to a fine powder in liquid nitrogen in a Waring Blendor, and either used immediately to prepare crude extract, or stored at −20°C for no longer than 2 weeks. Crude extract was prepared and desalted as previously described (29), with the following modifications. Glycerol (15%, v/v) was included in all buffers, the ratio of plant material to extraction buffer was increased to 3:2 (w/v), and crude extract was centrifuged for 10 min at 40,000g at 4°C. All further procedures were conducted at 4°C.

Column Chromatography. Crude extract (170 mg protein) was applied to a DEAE-cellulose column (Whatman DE-52, 2.5 × 18 cm) previously equilibrated with 10 mM Pipes buffer, pH 7.2, containing 1 mM DTT, 0.1 mM PMSF, and 15% (v/v) glycerol. Fractions of 4.1 ml were collected. Equilibration buffer (200 ml) was applied, followed by 200 ml of 35 mM KCl in the same buffer. Protein eluted by the latter buffer contained EPSP synthase activity and was concentrated by ultrafiltration (Amicon PM-10 membrane, 45 p.s.i. N_2). The enzyme preparation was applied to a Sephadex G-25 column (2.5 × 53 cm) that was equilibrated with 10 mM Pipes buffer, pH 6.0, containing 1 mM DTT, 0.1 mM PMSF, and 15% (v/v) glycerol. The desalted protein was concentrated as described above to 12 ml and applied to a CM-cellulose column (Whatman CM-52, 2 × 6.5 cm), previously equilibrated with the pH 6.0 buffer. Equilibration buffer (46 ml) was applied, followed by a 400-ml linear gradient of 0 to 0.25 M NaCl in the same buffer, and fractions of 2.9 ml were collected. Phosphatase activity eluted mainly in wash fractions, although a minor peak of phosphatase eluted in gradient fractions overlapping the trailing portion of the EPSP synthase peak. While distinct peaks of EPSP synthase activity were detected in both wash and gradient fractions (28% and 53% of the total activity recovered from CM-cellulose column chromatography, respectively), 19% of the total activity was recovered in fractions between the two major peaks. These latter fractions were pooled and concentrated. No obvious differences were noted in catalytic properties or in glyphosate sensitivity when this enzyme preparation was compared to the phosphatase-free EPSP synthase described below.

When the two peaks of EPSP synthase were separately pooled, concentrated, desalted, and rechromatographed as described above on two identical CM-cellulose columns, EPSP synthase activity eluted in wash fractions from both columns, while all phosphatase activity present in the reapplied samples eluted as in the initial experiment. Both of these EPSP synthase preparations were highly sensitive to inhibition by glyphosate. While one enzyme preparation was contaminated with high levels of phosphatase activity and was therefore not utilized in additional experiments, the other enzyme preparation contained no detectable phosphatase activity and was the source of enzyme for most experiments. Details of the purification of the latter enzyme preparation are given in Table I.

Enzyme Assays. Routine assays were conducted at saturating substrate concentrations and under conditions of proportionality of enzyme activity with respect to both time and protein concentration. During purification procedures and for product (Pi) inhibition experiments, EPSP synthase was assayed by following the disappearance of PEP, using a modification of the method of Gollub *et al.* (12). Reaction mixtures (200 μl) contained 3 mM PEP, 3 mM shikimate-3-P, 2 mM $Na_2MoO_4 \cdot 2H_2O$, 50 mM Pipes buffer (pH 6.6), and enzyme. Control mixtures lacking enzyme or shikimate-3-P (to detect PEP disappearance unrelated to EPSP synthase activity) were prepared. Incubation at 37°C for 15 min was followed by incubation at 100°C for 3 min to stop the

reaction. After cooling to room temperature, the components of the PK/LDH reaction mixture were added, yielding a final volume of 2 ml. The final concentrations were as follows: 1 mM ADP, 25 mM KCl, 25 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris buffer (pH 7.4), 650 nmol NADH, 23.32 units pyruvate kinase (rabbit muscle), and 33.32 units lactate dehydrogenase (rabbit muscle). After 5 min at room temperature, the A at 340 nm was determined using a model 250 Gilford spectrophotometer. When crude extracts or other enzyme preparations with high protein concentrations were assayed, interfering coagulated protein was removed by centrifugation, and the absorbance of the supernatant was determined. Since PEP concentration was proportional to NADH oxidation, the mM extinction coefficient for NADH of 6.22 was used to calculate nmol PEP utilized by EPSP synthase.

Partially purified EPSP synthase preparations recovered from CM-cellulose column chromatography and containing no detectable phosphatase activity were assayed by following the appearance of product (Pi). Reaction mixtures (200 μl) contained 1 mM PEP, 1 mM shikimate-3-P, 50 mM Pipes buffer (pH 7.0), and enzyme, and were incubated at 37°C for 15 min. In some experiments, 2 mM $Na_2MoO_4 \cdot 2H_2O$ was included, but EPSP synthase activity was not affected by this addition. The reaction was stopped by the addition of 800 μl of the color reagent (malachite green-ammonium molybdate-Sterox) described by Lanzetta *et al.* (23) which was modified by increasing the concentration of Sterox 2-fold. One min after mixing, 100 μl of 34% (w/v) sodium citrate·2H₂O was added, and the A at 660 nm was determined after 30 min using a model 250 Gilford spectrophotometer equipped with a red filter. Using the modified color reagent, the chromophore was stable for at least 2 h, and proportional readings were obtained up to 20 nmol Pi. Phosphorous standard solution (Sigma) was utilized to prepare a standard curve, from which the molar extinction coefficient (77, 142) was calculated in order to determine nmol Pi produced by EPSP synthase.

Phosphatase activity was assayed by measuring the liberation of p-nitrophenol from p-nitrophenylphosphate. Reaction mixtures (200 μl) contained enzyme, 5 mM substrate and 50 mM Pipes buffer, pH 6.6. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 1 ml of 0.5 N NaOH. The A at 410 nm was determined, and nmol product formed was determined from a standard curve prepared using authentic p-nitrophenol.

Other Analytical Procedures. Shikimic acid content was measured in suspension-cultured cells of *N. silvestris* which had been subcultured 4 d prior to the addition of sterile glyphosate (final concentration, either 0.5 or 1.0 mM). After 3 d, the cell mass from each flask was harvested on a Miracloth filter for shikimic acid determination using the procedure of Amrhein *et al.* (2) which was modified as follows. AG 2-X8 (Bio-Rad) was used rather than Amberlite AG (type IRA-410) resin, the paper chromatography step was omitted and, thus, shikimate determinations were performed on the reconstituted material eluted from the AG 2-X8 resin, and nonradioactive shikimate was used in the controls to determine percent recovery.

Protein concentration was determined by the method of Bradford (7) using BSA as standard.

Biochemicals. The barium salt of shikimate-3-P was prepared by the method of Knowles and Sprinson (19). The shikimate-3-P content by weight was 79.3% of that expected for Ba₃ (shikimate-3-P)₂·5H₂O (35), with 6.4% shikimate present as a contaminant. The barium salt of shikimate-3-P was converted to the potassium salt by the addition of a 2-fold molar excess of K₂SO₄ to a solution of shikimate-3-P prepared in distilled H₂O, and centrifuged to remove the insoluble BaSO₄. Alternatively, the sodium salt of shikimate-3-P was prepared from an aqueous

solution of the barium salt, using AG 50W-X8 (H⁺, Bio-Rad) resin, and the pH was adjusted to 7.0 with NaOH. Prior to use as substrate, all shikimate-3-P solutions were assayed by determining shikimate (26) present in samples before and after hydrolysis with potato acid phosphatase (19).

When EPSP synthase was assayed by following the appearance of Pi, the sodium salt of PEP, which was prepared from the tri(cyclohexylammonium) salt of PEP (Sigma) via AG 50W-X8 (H⁺) resin, was used as substrate rather than the trisodium salt of PEP (Sigma), which contained interfering levels of Pi. After contact with the resin, the PEP solution was combined with an equal volume of 20 mM Pipes buffer (Na⁺), the pH was adjusted to 7.0 with NaOH, and the PEP concentration was determined using the PK/LDH reaction couple.

Analytical grade glyphosate (99.88% pure) was a gift from Monsanto Co. For *in vivo* experiments, glyphosate solutions were prepared in distilled H_2O, adjusted to pH 5.7, and filter-sterilized prior to addition to autoclaved media. Sterox was obtained from VWR Scientific, Inc. All other biochemicals were obtained from Sigma Chemical Co. and were of the highest grade available.

RESULTS

Interference by Phosphatases. EPSP synthase condenses two phosphorylated substrates to form a phosphorylated product, all of which are potential substrates for the variety of phosphatases present in crude extracts of suspension-cultured cells of *N. silvestris*. Although 2 mM molybdate efficiently prevents substrate and product dephosphorylation by low levels of contaminating phosphatases without affecting EPSP synthase activity, the addition of this inhibitor was inadequate to control the high levels of phosphatases present in crude extracts. Therefore, column chromatography was employed to resolve EPSP synthase from these interfering enzyme activities (Table I). Greater than 89% of the total phosphatase activity present in crude extract was not retained by DEAE-cellulose and was thus resolved from EPSP synthase in the first purification step. A subsequent step of CM-cellulose column chromatography yielded a peak of EPSP synthase in gradient fractions that was further resolved from those phosphatases that failed to bind to this resin. Rechromatography of this enzyme preparation on CM-cellulose as described in "Materials and Methods" yielded an EPSP synthase preparation lacking detectable phosphatase activity (Table I), and this was the source of enzyme for most experiments.

Since column chromatography successfully resolved all interfering phosphatase activity from this enzyme preparation, EPSP synthase was assayed in all further experiments by monitoring the appearance of nmol amounts of the product Pi, using a modification of the method of Lanzetta *et al.* (23). While only suitable for use with phosphatase-free enzyme preparations, this assay for EPSP synthase was far superior to the coupled assay (12) with respect to simplicity, sensitivity, and reproducibility. Furthermore, it was technically advantageous to assay EPSP synthase by following Pi appearance rather than PEP utilization in those kinetic experiments in which μM concentrations of PEP were utilized.

Physiological Effects of Glyphosate. Glyphosate was a potent growth inhibitor of suspension-cultured cells of *N. silvestris* (Fig. 1). Fifty% inhibition of growth was obtained with 0.12 mM glyphosphate, and growth inhibition exceeded 80% at glyphosate concentrations greater than 0.28 mM. Dramatic increases of intracellular shikimate accompanied growth inhibition. For example, after 3 d of growth inhibition imposed by the presence of 0.5 mM or 1.0 mM glyphosate, shikimate levels increased 214-fold or 364-fold, respectively (Fig. 1, inset).

Glyphosate Inhibition of EPSP Synthase. The massive levels of shikimate which accumulate in glyphosate-treated plant material (Fig. 1) suggest that an enzymic step beyond shikimate is the primary target of glyphosate action *in vivo*. EPSP synthase, partially purified from suspension-cultured cells of *N. silvestris*, proved to be inhibited by μM levels of glyphosate (Fig. 2). In the presence of 1 mM each of PEP and shikimate-3-P at pH 6.6, 18.3 μM glyphosate produced 50% inhibition of enzyme activity. Maximal inhibition of 96% was obtained with glyphosate concentrations in excess of 0.5 mM. Recasting the data from Figure 2 in the form of a Hill plot with log $[v_i/(v_o-v_i)]$ plotted *versus* log [glyphosate] (31) yielded a straight line with a slope of −0.944. This indicates that EPSP synthase from *N. silvestris* possesses one inhibitor binding site for glyphosate.

Glyphosate inhibition of EPSP synthase was highly dependent upon pH (Fig. 3), with the effectiveness of glyphosate as an inhibitor dramatically increasing with increasing pH. Since the enzyme displayed activity over a broad pH range (50% of maximal activity was obtained between pH 6.2 and pH 8.5 with maximal activity at pH 7.0 to 7.2, data not shown), it seemed likely that inhibition of EPSP synthase may require a particular ionic state of glyphosate. The data from Figure 3 were reexamined in view of the four pK_a values of glyphosate: $pK_{a1} < 2$, first dissociation of the phosphono group; $pK_{a2} = 2.6$, α-COOH; $pK_{a3} = 5.6$, second dissociation of the phosphono group; and $pK_{a4} = 10.6$, secondary amine (33). (It should be noted, however, that only the latter three pK_a values are usually given, and are cited as 2.3, 5.9, and 10.9 [16].) The data (Fig. 3) support our conclusion that the ionic form of glyphosate that inhibits EPSP synthase from *N. silvestris* is $COO^-CH_2NH_2^+CH_2PO_3^{2-}$. At pH 5.9 ($pK_{a3}$) protons have completely dissociated from the α-COOH and from one hydroxyl of the phosphono group, while the second ionization of this latter group is only 50% complete. In this configuration, glyphosate only weakly inhibits EPSP synthase (Fig. 3).

Table I. *Partial Purification of EPSP Synthase from Suspension-Cultured Cells of Nicotiana silvestris*

Enzyme Preparation	Volume	Total Protein	EPSP Synthase				Ratio[b] of Phosphatase: EPSP Synthase
			Total activity	Specific activity	Recovery	Purification factor	
	ml	*mg*	*units*[a]	*units/mg*	*%*	*-fold*	
Crude extract	81.9	170.0	17,124	100.7	"100"	"1.0"	17.53
DEAE-cellulose	12.0	30.33	13,752	453.4	80.3	4.5	2.34
CM-cellulose[c]							
I	12.0	0.876	3,782	4,317	22.1	42.9	0.59
II	6.1	0.061	1,033	16,935	6.0	168.0	0.00

[a] One unit of enzyme will utilize 1 nmol PEP/min at 37°C.

[b] Ratio of the specific activity of phosphatase to the specific activity of EPSP synthase, each expressed as nmol/min·mg at 37°C.

[c] The peak of EPSP synthase that eluted in gradient fractions from CM-cellulose (designated I) was reapplied to CM-cellulose (designated II) as described in "Materials and Methods."
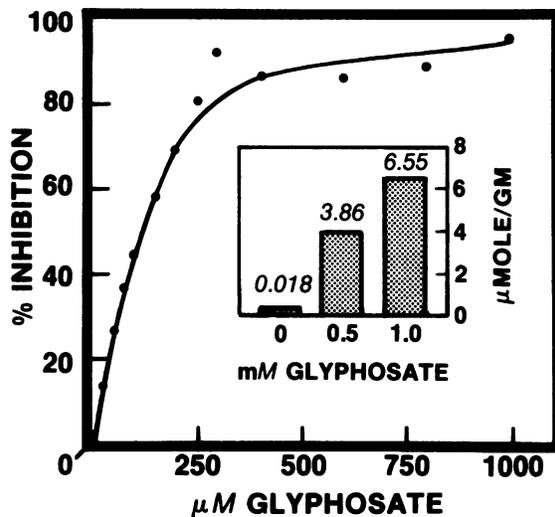
FIG. 1. Growth inhibition by glyphosate of suspension-cultured cells of *N. silvestris*. Cells were subcultured into media containing the indicated concentrations of glyphosate, grown for 8 d, and harvested for dry weight determinations. Per cent inhibition was calculated from the ratio (dry weight increase of cells grown in the presence of glyphosate) ÷ (dry weight increase (173.8 mg) of cells grown without glyphosate). The starting inoculum density of the control cultures was 1.0 mg/ml dry weight and the density at harvest was 9.0 mg/ml dry weight. The inset shows in histogram form the effect of glyphosate upon shikimate accumulation, with the experimental values expressed above each bar. In the latter experiments, subcultured cells were grown for 4 d prior to addition of glyphosate. Incubation was continued for 3 additional days prior to cell harvest and measurement of shikimate (which is expressed as μmol/g fresh weight).
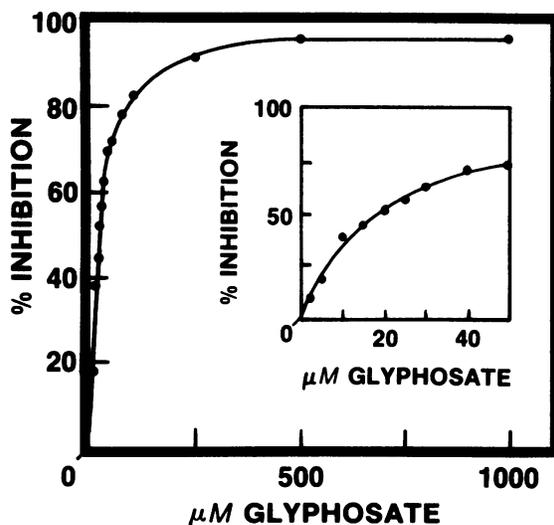

FIG. 2. Glyphosate inhibition of EPSP synthase activity. EPSP synthase was assayed at pH 6.6, with PEP and shikimate-3-P concentrations constant at 1 mM, in the presence of the indicated concentrations of glyphosate. The inset expands the scale in the range of 0 to 50 μM glyphosate.

However, at pH 7.9 the phosphono group is theoretically 99.09% ionized, and thus glyphosate exists as $COO^-CH_2NH_2^+CH_2PO_3^{2-}$. It is likely that a completely ionized phosphono group is a critical factor in the mechanism of inhibition of EPSP synthase by glyphosate.

Kinetic Effects of Glyphosate. The effect of glyphosate on the kinetic parameters of EPSP synthase was examined. Glyphosate displayed uncompetitive inhibition with respect to shikimate-3-
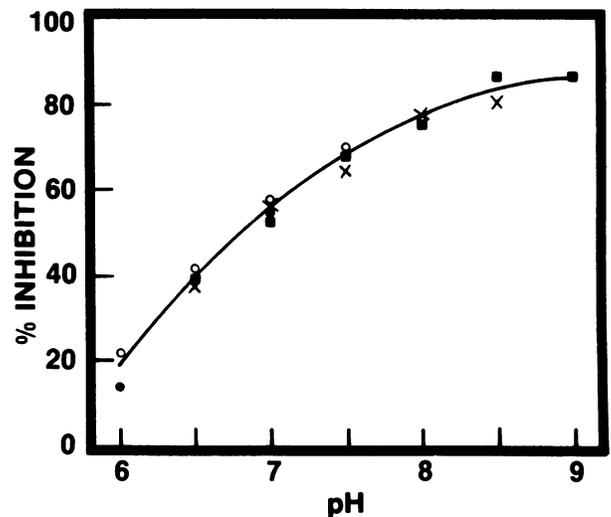

FIG. 3. Glyphosate inhibition of EPSP synthase as a function of pH. EPSP synthase was assayed in the presence of 100 mM Mes (●), Pipes (○), Hepes (×), or Epps (■) buffer at the indicated pH values in the presence or absence of 15 μM glyphosate. Data are expressed as the per cent inhibition obtained in the presence of glyphosate relative to control values.
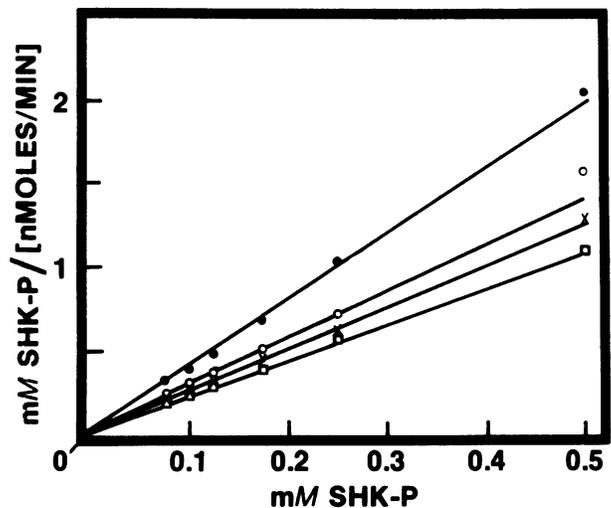

FIG. 4. Inhibition of EPSP synthase by glyphosate with respect to shikimate-3-P. Enzyme was assayed with PEP at a saturating concentration of 1 mM in the presence of glyphosate at the following concentrations: 20 μM (●), 10 μM (○), 5 μM (×), and none (□). That glyphosate is an uncompetitive inhibitor of EPSP synthase with respect to shikimate-3-P is indicated by the intersection of lines on the ordinate when these data were graphed as a Hanes-Woolf plot (32). SHK-P, shikimate-3-P.

P in the presence of saturating PEP (Fig. 4). Replotting these data (Fig. 5) yielded an estimate of the uncompetitive inhibition constant ($K_i' = 18.5$ μM). That inhibition was uncompetitive with respect to shikimate-3-P predicts that glyphosate can bind to the [enzyme:shikimate-3-P] complex, but not to free enzyme.

Inhibition of EPSP synthase by glyphosate was competitive with respect to PEP (Fig. 6). From a Dixon replot (32) of these data (Fig. 7), a K_i value of 1.25 μM was obtained. In accordance with the predictions of competitive inhibition, the degree of inhibition at a fixed concentration of glyphosate increased and approached 100% inhibition as PEP concentrations decreased (Fig. 8). Thus, it is evident that glyphosate is a highly effective inhibitor of EPSP synthase in the presence of low, physiological concentrations of PEP.
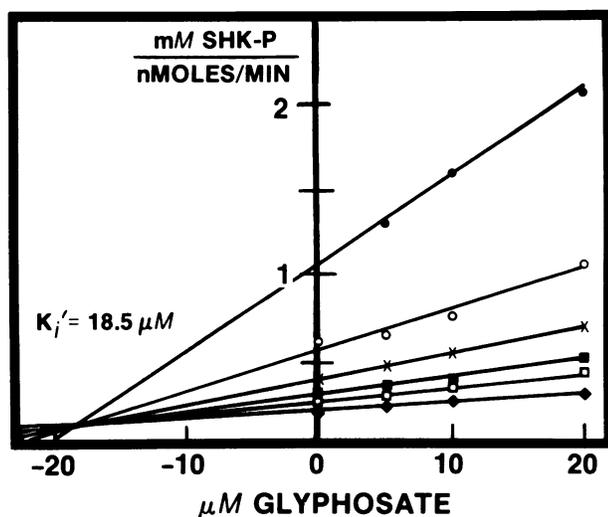
FIG. 5. Replotting the data from Figure 4 yields a value for K_i' of 18.5 μM. Shikimate-3-P concentrations were as follows: 0.50 mM (●), 0.25 mM (O), 0.175 mM (×), 0.125 mM (■), 0.10 mM (□), and 0.075 mM (♦). SHK-P, shikimate-3-P.
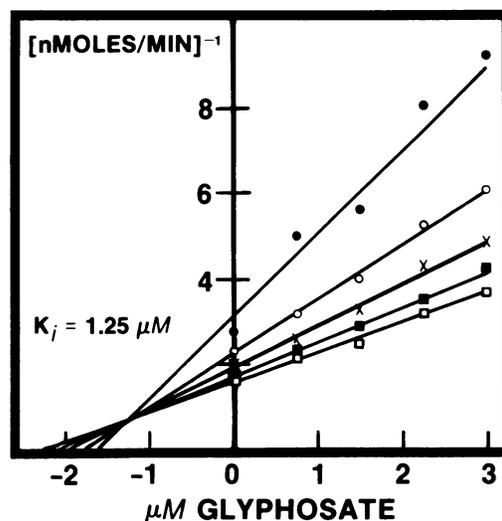

FIG. 7. Dixon replot of the data from Figure 6 yielding a K_i value of 1.25 μM. PEP concentrations were as follows: 40 μM (●), 60 μM (O), 80 μM (×), 100 μM (■), and 120 μM (□).
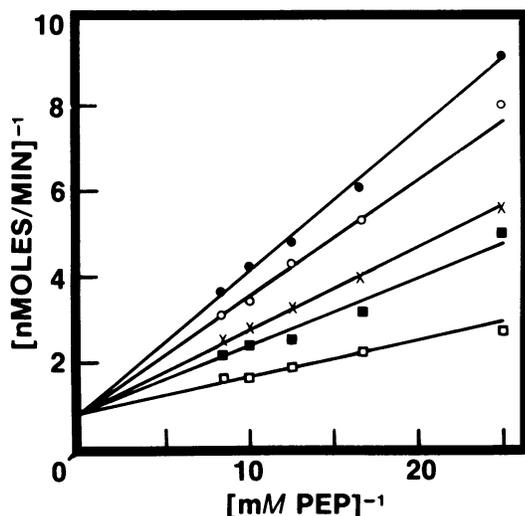

FIG. 6. Glyphosate as a competitive inhibitor of EPSP synthase with respect to PEP. EPSP synthase was assayed with shikimate-3-P fixed at a saturating concentration of 1 mM in the presence of the following concentrations of glyphosate: 3.0 μM (●), 2.25 μM (O), 1.5 μM (×), 0.75 μM (■), and none (□).
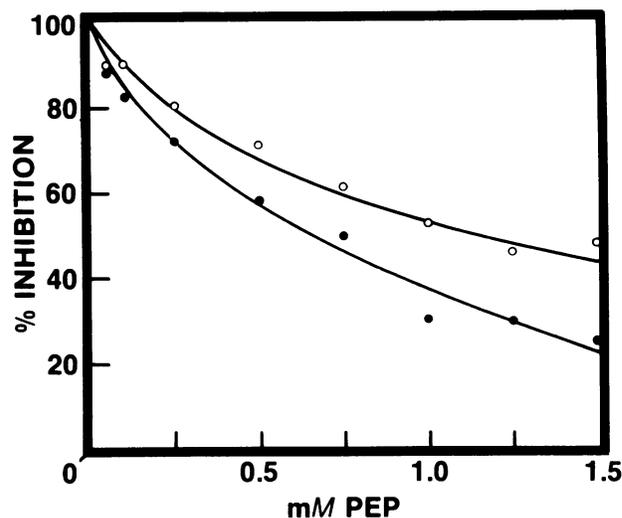

FIG. 8. Inverse dependence of glyphosate inhibition of EPSP synthase upon PEP concentration. EPSP synthase was assayed at the indicated concentrations of PEP with shikimate-3-P constant at 1 mM, in the absence and presence of 10 μM (●), or 20 μM (O) glyphosate. Data are expressed as the per cent inhibition obtained in the presence of glyphosate relative to control values.

DISCUSSION

Mechanism of Inhibition of EPSP Synthase by Glyphosate. We found glyphosate inhibition of EPSP synthase from *N. silvestris* to be uncompetitive with respect to shikimate-3-P, a result also obtained in *Neurospora crassa* (6). On the other hand, glyphosate inhibition was reported to be noncompetitive with respect to shikimate-3-P in the prokaryote, *Klebsiella pneumoniae*, (3), and in cultured cells of the higher plant, *Corydalis sempervirans* (3). In both cases, intracellular accumulation of shikimate-3-P is not expected to overcome glyphosate inhibition, as would be expected in the case of strictly competitive inhibition. Consistent with all systems so far examined (3, 6, 27), glyphosate inhibition of *N. silvestris* EPSP synthase was strictly competitive with respect to PEP. That glyphosate is an exceedingly potent enzyme inhibitor is reflected by the following K_i (PEP) values reported to date: *Pisum sativum*, 0.08 μM (27); *K. pneumoniae*, 1 μM (3); *N. crassa*, 1.1 μM (6); *N. silvestris*, 1.25 μM (this paper);

and *C. sempervirans*, 10 μM (3). Intracellular levels of PEP are undoubtedly a critical factor underlying sensitivity of EPSP synthase to glyphosate-mediated inhibition in all of the above-cited systems.

Mechanistic studies, utilizing partially purified bacterial enzyme (5, 15), suggest that EPSP synthase proceeds via a reversible addition-elimination mechanism in which the enolpyruvyl moiety of PEP is transferred apparently unchanged to shikimate-3-P. It has been proposed that EPSP synthase from *N. crassa* (6) and *Escherichia coli* (24) sequentially binds shikimate-3-P and PEP as the first and second substrates, respectively. In *N. silvestris*, uncompetitive inhibition by Pi with respect to shikimate-3-P and mixed inhibition by Pi with respect to PEP were noted utilizing a partially purified EPSP synthase preparation in experiments where the fixed substrate was present at saturating concentration. These product inhibition patterns imply that the *N. silvestris* enzyme possesses an order of substrate binding that would be consistent with the mechanism proposed by Boocock

and Coggins (6) for the *N. crassa* enzyme. This mechanism predicts that glyphosate competes with PEP for binding to an [enzyme:shikimate-3-P] complex and ultimately forms the dead-end complex of [enzyme:shikimate-3-P:glyphosate].

Multiple EPSP Synthase Targets? While there is considerable evidence to show that chloroplasts can catalyze all of the reactions necessary to form aromatic amino acids (25), it is likely that most or all shikimate pathway enzymes are simultaneously localized in both the cytosol and chloroplasts, as has been shown for enzymes of glycolysis and the oxidative pentose phosphate cycle (13). Multiple forms of chorismate mutase (8) and DAHP synthase (Ganson and d'Amato, unpublished data) occupy plastidic and cytosolic subcellular locations in *N. silvestris*. Shikimate dehydrogenase has also been detected in both of these compartments (28) in *Pisum sativum*. Thus, it seems quite possible that cytosolic and plastidic isozymes of EPSP synthase may exist as separate enzyme targets of glyphosate action. If so, it is interesting that the plastidic isozyme may be subject to periodic increases in sensitivity to inhibition in view of the light-induced pH increases (pH$_{dark}$ = 7.0; pH$_{light}$ = 8.0) that occur in the chloroplast stroma (36). Obvious implications of multiple enzyme targets exist with respect to the possibility that multiple gene manipulation events may be required to engineer a state of resistance to glyphosate in crop plants.

While only the presence of a single EPSP synthase has so far been detected in seedlings of pea (27) and mung bean (21), experiments are in progress to determine whether two spatially separate enzyme forms might be present in *N. silvestris*. Column chromatography has revealed two closely overlapping peaks of EPSP synthase activity that eluted from DEAE-cellulose. Different enzyme forms were found which initially differed in binding affinity for CM-cellulose. Each of these enzyme forms was similar in qualitative characteristics, including sensitivity to glyphosate. Since the cell line of *N. silvestris* originated from a haploid plant and is therefore isogenic, the possibility of isozyme forms originating as a trivial consequence of heterozygosity is unlikely. However, minor proteolytic clipping or other posttranslational modification events could account for chromatographically different enzyme forms.

Overall Impact of Glyphosate upon Aromatic Pathway Function. Suspension-cultured cells of *N. silvestris* resemble a variety of other cultured plant cells and organismal plants (1) in that the administration of glyphosate triggers a drastic mid-pathway imbalance of aromatic biosynthesis. Massive levels of shikimate accumulate in conjunction with slowed synthesis of aromatic compounds originating from chorismate. Although shikimate-3-P is probably the major compound accumulated, eventual transfer to phosphatase-containing vacuoles (17) may account for the usual detection of shikimate. Indeed, shikimate-3-P has been detected, in addition to shikimate, in glyphosate-treated tomato and buckwheat plants (3), and in *E. coli* and *Bacillus subtilis* cultures exposed to glyphosate (Fischer and Gaines, unpublished data). Although shikimate might accumulate as a result of potent product inhibition exerted upon shikimate kinase by shikimate-3-P, only a very weak sensitivity to product inhibition was observed with the mung bean enzyme (22).

N. silvestris, like mung bean (29), possesses a cobalt-dependent DAHP synthase isozyme which is sensitive to glyphosate inhibition in contrast to the glyphosate resistance of a second Mn-stimulated isozyme (30). In green leaves, DAHP synthase-Co and DAHP synthase-Mn have been shown to have spatially distinct locations in the cytosol and the chloroplast, respectively (Ganson and d'Amato, unpublished data). We suggest that, in the presence of glyphosate, most molecules of accumulated shikimate originate through the action of DAHP synthase-Mn in the chloroplast, since DAHP synthase-Co in the cytosol is glyphosate sensitive. Hence, as many as three enzyme targets of

glyphosate action may exist in *N. silvestris*, *i.e.* DAHP synthase-Co and EPSP synthase in the cytosol, in addition to EPSP synthase in the chloroplast.

A combination of molecular events can be envisioned to explain the highly effective action of glyphosate. First, starvation for aromatic amino acids undoubtedly limits protein synthesis. Second, shikimate-3-P may be inhibitory in view of the common finding that accumulation of phosphorylated compounds is often toxic. Third, as previously suggested (29), an energy-drain effect may be set into motion. Since DAHP synthase-Mn from *N. silvestris* appears to be subject to feedback inhibition by L-arogenate as in mung bean (Rubin, unpublished data), the glyphosate-promoted loss of early-pathway control (due to depletion of L-arogenate) would allow unrestrained loss of PEP and erythrose-4-phosphate into the aromatic pathway. Each molecule of shikimate-3-P accumulated represents an expenditure of one PEP molecule and one ATP molecule. The abnormal investment of PEP molecules in formation of shikimate-3-P creates an imbalance of the substrate ratio for EPSP synthase, PEP being depleted and shikimate-3-P being in huge excess. This imbalance would maximize the vulnerability of EPSP synthase to glyphosate action if the thesis is correct that glyphosate and PEP compete for binding to the [enzyme:shikimate-3-P] complex, while shikimate-3-P is incapable of overcoming the inhibition. This energy drain promoted by glyphosate ought not to be reversed very well by aromatic amino acids because DAHP synthase-Mn is not directly feedback inhibited by pathway endproducts. Indeed, we have found that aromatic amino acids (each at 1 mM) are only 25 to 30% effective in reversal of glyphosate inhibition of growth of suspension-cultured cell populations of *N. silvestris*.

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