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The Site of the Inhibition of the Shikimate Pathway by Glyphosate

I. INHIBITION BY GLYPHOSATE OF PHENYLPROPANOID SYNTHESIS IN BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH)[1,2]

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ABSTRACT

The nonselective herbicide glyphosate (N-[phosphonomethyl]glycine) inhibited the light-induced accumulation of phenylpropanoid substances (chlorogenic acid, procyanidin, rutin, anthocyanin) in etiolated buckwheat hypocotyls 90% at 1 millimolar. Structurally related compounds, such as N,N-*bis*[phosphonomethyl]glycine, aminomethylphosphonate, methylglycine, and iminodiacetate, had little or no inhibiting effects. Of all amino acids tested, only L-phenylalanine reversed the inhibition, and partial reversal of anthocyanin synthesis was achieved with chorismate, phenylpyruvate, *trans*-cinnamate, *p*-coumarate, and naringenin. Phenylalanine concentrations were reduced in glyphosate-treated hypocotyls, and glyphosate effectively reduced the high level of phenylalanine that was caused by the phenylalanine ammonia-lyase inhibitor L-α-aminooxy-β-phenylpropionate. Glyphosate had no significant effect on the time course of phenylalanine ammonia-lyase activity in hypocotyls incubated either in the dark or in the light. Under appropriate feeding conditions, glyphosate inhibited the incorporation of [^{14}C]shikimate into all three aromatic amino acids, and radioactive shikimate accumulated in the tissue. The results lead to the conclusion that glyphosate interferes with the shikimate pathway at or prior to the formation of chorismate.

Glyphosphate (N-[phosphonomethyl]glycine) is being widely used as a nonselective, broad-spectrum, postemergence herbicide that is readily translocated in plants and is biodegradable by soil microorganisms (14). Its molecular mode of action has been proposed by Jaworski (20) to be through inhibition of aromatic amino acid biosynthesis, presumably by interference with chorismate mutase and/or prephenate dehydratase. The proposal was based on the observation that growth inhibition of *Lemna gibba* in the presence of the herbicide was alleviated by phenylalanine, whereas growth inhibition of *Rhizobium japonicum* was overcome only by the addition of both phenylalanine and tyrosine. Addition of tryptophan was not absolutely essential for reversal of the inhibition and, therefore, it was thought unlikely that glyphosate blocks a reaction prior to chorismate synthesis. Roisch and Lingens (25) reported that *Escherichia coli* recovered from growth inhibition by glyphosate, when all three aromatic amino acids were added to the growth medium, but no effect of glyphosate was found on chorismate mutase, prephenate dehydrogenase, and prephenate dehydratase. High concentrations of the herbicide inhibited the two initial enzymes of the shikimate pathway, but this inhibition was apparently too slight to account for growth inhibition caused by low glyphosate concentrations. Inhibition by glyphosate of the growth of carrot and tobacco cells was alleviated by the addition of the three aromatic amino acids, but Haderlie *et al.* (15) hesitated to conclude that the synthesis of the aromatic amino acids was inhibited by glyphosate because glyphosate did not appreciably reduce the cellular concentrations of these amino acids. Just recently, Greshoff (14) extended the earlier studies of Jaworski (20) and Haderlie *et al.* (15) with an investigation on glyphosate's growth inhibition of *E. coli*, *Chlamydomonas reinhardtii*, carrot and soybean cell cultures, and *Arabidopsis thaliana* seedlings. In all cases, phenylalanine and tyrosine acted synergistically in the alleviation of the inhibitory effect of glyphosate. It was suggested that some essential process or compound derived from phenylalanine and/or tyrosine was inhibited by glyphosate or one of its metabolites (14). Nilsson (23) reported an increase in the amount of free amino acids and in NH_3 in wheat plants sprayed with glyphosate, but the percentage of phenylalanine and tyrosine of the total amino acids was strongly reduced. In a series of recent papers, Duke and Hoagland (9-11, 16, 17) hypothesized that glyphosate might exert its effect at least in part through induction of PAL[4] activity, resulting in one or more of the following growth-limiting conditions: (*a*) depletion of free phenylalanine and, possibly, tyrosine levels and coincident inhibition of protein synthesis; (*b*) toxic levels of ammonia provided that the rate of deamination exceeds the rate of amination; and (*c*) accumulation of growth-limiting phenolics derived from *trans*-cinnamic acid, the product of the PAL reaction.

In spite of the attractiveness of this proposal, there are alternative explanations for the induction of PAL by glyphosate (see under "Discussion"), and this hypothesis would not explain the growth-inhibitory action of glyphosate in organisms, such as bacteria, which do not have PAL. Furthermore, if conditions *b* or *c* were pertinent, one would expect that phenylalanine would increase the inhibitory effect of glyphosate rather than alleviate it. PAL, however, may contribute only partially to aromatic amino acid depletion, as was recently pointed out by Duke *et al.* (11). It was also suggested (11) that glyphosate's effect on aromatic compounds may not be its principal mode of action. In the present

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[2] A preliminary report of part of this work was presented at the ASPP meeting in July 1979 (18).

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[4] Abbreviations: PAL, phenylalanine ammonia-lyase (EC 4.3.1.5), AOPP, L-α-aminooxy-β-phenylpropionic acid; glyphosate, *N*-[phosphonomethyl]glycine.

study, we used an approach basically similar to that of Duke and Hoagland (9, 16, 17). We reasoned that, due to the possible functioning of a homeostatic control system and/or the possible involvement of more than one pool of aromatic amino acids (24), it might be difficult to detect a decreased level of a primary metabolite, such as phenylalanine, which, in addition, is likely to have a high turnover rate. We decided instead to measure the effect of glyphosate on the accumulation of phenylalanine-derived metabolic end products with little or no turnover and to avoid the complexity of growth phenomena. We used etiolated buckwheat seedlings, which upon illumination rapidly accumulate phenylpropanoid substances, that are exclusively derived from phenylalanine (2, 26). We can manipulate the endogenous phenylalanine pool in this system by the application of the PAL-inhibitor AOPP (5), which blocks the flow of phenylalanine into phenylpropanoid compounds (19). Under these conditions, it should be easier to detect an effect of glyphosate on the phenylalanine concentration in the tissue. Furthermore, we traced the metabolic fate of labeled shikimate in glyphosate-treated tissue in order to define the site of action of the herbicide more closely.

MATERIALS AND METHODS

Glyphosate (N-[phosphonomethyl]glycine) and glyphosine (N,N-bis[phosphonomethyl]glycine) were provided by Dr. E. G. Jaworski, Monsanto Co. AOPP was synthesized according to reference 7, and chorismate was prepared according to reference 13. Aminomethylphosphonate, 3-aminopropylphosphonate, methylglycine, and iminodiacetate were obtained from Sigma; shikimate, aromatic acids, and naringenin were from Carl Roth KG, Karlsruhe. D-[2,3,4,5(*n*)-^{14}C]Shikimate (84 mCi/mmol) was provided by the Radiochemical Centre, Amersham. All other chemicals in reagent grade were obtained from Merck, Darmstadt.

Seeds of buckwheat (*Fagopyrum esculentum* Moench) were germinated in the dark as described previously (26). Six-day-old seedlings were used in all experiments. Details of incubation conditions of isolated hypocotyls and derooted seedlings have already been reported (19). Isolated cotyledons were incubated under the same conditions as excised hypocotyls.

Extraction and quantitative determination of anthocyanin, rutin, chlorogenic acid, and leucoanthocyanin (procyanidin) have been described (26). Chl content of cotyledons was determined according to reference 28.

Procedures for extraction and measurement of amino acids and the measurement of the distribution of radioactivity in hypocotyl extracts have been described (19). PAL was extracted and assayed spectrophotometrically (6).

RESULTS

Effect of Glyphosate and Related Compounds on the Synthesis of Phenylpropanoid Compounds in Hypocotyls. Anthocyanin synthesis in illuminated, excised hypocotyls was severely depressed in the presence of increasing concentrations of glyphosate (Fig. 1). Fifty per cent inhibition of anthocyanin accumulation was achieved with 0.1 to 0.2 mM glyphosate and 1 mM caused a 85 to 90% inhibition. Glyphosate was most inhibitory when taken up through the roots of intact seedlings, but it was also effective as a spray (Fig. 1). Examination of other phenylpropanoid compounds, the accumulation of which is promoted by light (26), showed that glyphosate reduced their formation to the same extent as that of anthocyanin (Table I). The fact that glyphosate inhibited the production of the non-flavonoid compound chlorogenic acid indicates that the herbicide is a general inhibitor of phenylpropanoid synthesis. Structural variations on the glyphosate molecule, such as introduction of a second phosphonomethyl group (glyphosine), substitution of the phosphonyl group by a carboxyl group (iminodiacetate), or loss of the carboxymethyl group (aminomethylphosphonate), drastically reduced inhibitory potency (Table II).

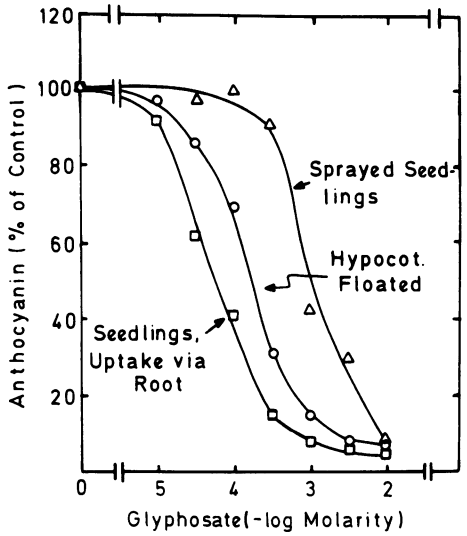

FIG. 1. Inhibition of anthocyanin synthesis in buckwheat hypocotyls as a function of glyphosate concentration. (□——□): intact seedlings, roots immersed in glyphosate solution; (○——○): excised hypocotyls floating in glyphosate solution; (△——△): above-ground parts of seedlings sprayed with glyphosate solution. Seedlings were grown for 6 days in the dark and then treated with glyphosate and illuminated under the indicated conditions. Anthocyanin content was determined after a 24-h illumination period. Controls produced 0.5 μmol (intact seedlings) or 0.37 μmol (excised hypocotyls) of anthocyanin/20 hypocotyls.

Table I. *Inhibition of Light-induced Phenylpropanoid Synthesis in Excised Buckwheat Hypocotyls by Glyphosate*

The hypocotyls floated in 10 ml 1 mM glyphosate solution in Petri dishes.

Phenylpropanoid	Treatment			Inhibition of Light-induced Increase
	None (0 h)	24 h Light	24 h Light + Glyphosate	
	μmol/20 hypocotyls			%
Cyanidin	0	0.37	0.05	86
Rutin	0.18	0.74	0.22	93
Procyanidin	1.22	1.83	1.30	87
Chlorogenic acid	0.47	1.20	0.52	93

Table II. *Effect of Glyphosate and Structurally Related Compounds on Anthocyanin Synthesis in Excised Buckwheat Hypocotyls*

Hypocotyls in 1 mM solutions of the compounds were illuminated for 24 h. Controls produced 0.38 μmol cyanidin/20 hypocotyls.

Compound	Inhibition of Anthocyanin Synthesis
	%
Glyphosate	87
Glycine	0
Methylglycine	13
Iminodiacetate	0
Aminomethylphosphonate	5
3-Aminopropylphosphonate	0
Glyphosine	16

Complementation experiments with precursors of cyanidin biosynthesis, carried out analogously to previous experiments with α-aminooxyacetic acid (2), revealed that L-phenylalanine was the

only aromatic amino acid that effectively reversed inhibition of anthocyanin synthesis (Table III). This result agrees with the previous finding that buckwheat does not utilize tyrosine for the synthesis of hydroxycinnamic acids and their derivatives (2). Furthermore, it indicates that glyphosate either blocks phenylalanine production or diverts endogenously produced phenylalanine into other metabolic pathways. As was expected, cinnamate, *p*-coumarate, and naringenin partially alleviated glyphosate inhibition of anthocyanin synthesis (Table III). The toxicity of the two acids and naringenin, combined with apparently poor transport and distribution within the tissue (as evident from pigmentation in patches), did not permit full reversal, however. Chorismate and phenylpyruvate produced a slight, but reproducible, reversal of the inhibition, whereas shikimate and *p*-OH-benzoate [one decomposition product of chorismate (13)] were inactive. Since phosphonates are ion-complexing agents (21), we checked the effect of the chlorides of Ca^{2+}, Al^{3+}, Co^{2+}, and Fe^{3+} on glyphosate-mediated inhibition of anthocyanin, but none of these cations reversed the inhibition.

Effect of Glyphosate on Amino Acid Levels in Hypocotyls. Amino acids extractable in 80% ethanol generally decline during incubation of excised hypocotyls, especially in the light (19). In the presence of light and glyphosate, the total amount of amino acids remained constant, even though the amounts of individual amino acids varied (Table IV): glyphosate either prevented, retarded, or had no effect on the decline of some amino acids, and it increased the amounts of others. Phenylalanine declined the most in the presence of glyphosate and, in some experiments, was hardly detectable. The marked decline in tyrosine content in the light was not affected by glyphosate. Accurate determination of phenylalanine was difficult due to its low level and to interfering ninhydrin-positive material. Therefore, we prevented the flow of phenylalanine into phenylpropanoid compounds by use of a specific PAL-inhibitor, AOPP (5). We have previously shown (19) that, in the presence of AOPP, buckwheat hypocotyls accumulate large amounts of phenylalanine and that this accumulation is due to *de novo* synthesis from shikimate. Glyphosate greatly reduced the accumulation of phenylalanine in the presence of AOPP, and, even at high concentrations, glyphosate could not completely prevent the increase in phenylalanine caused by AOPP (Table V). We inferred from these results that glyphosate inhibits the biosynthesis of phenylalanine in buckwheat. To learn more about the site of inhibition in the shikimate pathway, we monitored the metabolism of labeled shikimate in the presence and absence of glyphosate.

Table III. *Effectiveness of "Reversal Agents" in Counteracting Glyphosate Inhibition of Anthocyanin Formation in Illuminated Excised Buckwheat Hypocotyls*

All chemicals were added simultaneously to the incubation medium at the beginning of the 24-h illumination period. The following compounds (5 mM) did not counteract the effect of 1 mM glyphosate: shikimate, quinate, *p*-OH-phenylpyruvate, L-tyrosine, L-tryptophan, *o*-coumarate, *p*-OH-benzoate, $AlCl_3$, $FeCl_3$, $CoCl_2$, and $CaCl_2$; 10 mM D-phenylalanine, glucose, or 0.1% casein hydrolysate was ineffective.

Incubation Medium	Anthocyanin Content
	% control
Buffer only (control)	100[a]
1 mM glyphosate	12
1 mM glyphosate + 5 mM chorismate	26
1 mM glyphosate + 5 mM phenylpyruvate	28
1 mM glyphosate + 10 mM L-phenylalanine	114
1 mM glyphosate + 5 mM cinnamate	62
1 mM glyphosate + 5 mM *p*-coumarate	38
1 mM glyphosate + 0.5 mM naringenin	40

[a] 100% = 0.37 μmol/20 hypocotyls.

Table IV. *Profiles of 80% Ethanol-Soluble Amino Acids in Buckwheat Hypocotyls*

The amino acid profiles were derived from hypocotyls incubated for 24 h in light ± 1 mM glyphosate fed to derooted seedlings. Methionine and cysteine were not determined.

Amino Acid	Treatment		
	None (0 h)	24 h Light	24 h Light + Glyphosate
	nmol/g fresh wt		
Asp	488 a[a]	253 b	463 a
Thr	433 a	332 b	431 a
Ser[b]	1,109 a	764 b	1,674 c
Glu	847 a	648 b	1,006 c
Gly	172 a	69 b	102 b
Ala	264 a	172 b	363 c
Val	553 a	153 b	314 c
Ile	272 a	54 b	100 c
Leu	395 a	192 b	193 b
Tyr	228 a	59 b	76 b
Phe	116 a	44 b	20 c
Trp	159 a	101 a	171 a
His	454 a	291 b	409 a
Lys	90 a	51 b	93 a
Arg	112 a	25 b	100 a
Total	5,692 a	3,208 b	5,514 a

[a] Individual amino acids followed by the same letter are not significantly different at the 95% level.

[b] Ser, Asn, and Gln were not separated in the system used.

Table V. *Effect of AOPP and Glyphosate on Phenylalanine Content in Excised Buckwheat Hypocotyls*

Treatment	Phenylalanine Content
	nmol/20 hypocotyls
None (0 h)	153
24 h light, buffer only (control)	50
24 h light, 0.1 mM AOPP	1687
24 h light, 1 mM glyphosate	24
24 h light, 0.1 mM AOPP + 1 mM glyphosate	493
24 h light, 0.1 mM AOPP + 3 mM glyphosate	344

Effect of Glyphosate on the Metabolism of [^{14}C]Shikimate. Ring [^{14}C]shikimate was supplied to derooted seedlings in the light and the kinetics of incorporation into hypocotyl fractions were recorded (Fig. 2) as described (19). The uptake of shikimate was somewhat reduced in the presence of 1 mM glyphosate (Fig. 2a). The average reduction in six experiments was 14% after 24 h. The accumulation of radioactivity in the 80% ethanol-soluble fraction of the hypocotyls was not affected by glyphosate for 6 to 8 h, declined in the controls during further incubation, and continued to increase in the glyphosate-treated hypocotyls (Fig. 2b).

Most of the radioactive material in the 80% ethanol extract was not retained on the cation-exchange resin, and the time course of the accumulation of radioactivity in the neutral and acidic fractions (Fig. 2c) is, therefore, similar to that in the total 80% ethanol-soluble fraction (Fig. 2b). Two-dimensional paper chromatography of the 80% ethanol extract and autoradiography of chromatograms showed that shikimic acid accumulated and was metabolized to only a minor degree in glyphosate-treated tissue, but phenylalanine, tyrosine, rutin, anthocyanin, and possibly phenylpyruvate were labeled in control tissues (data not shown). Incorporation of shikimate into the amino acid fraction of the extract was reduced by glyphosate (Fig. 2d), and the label was found only

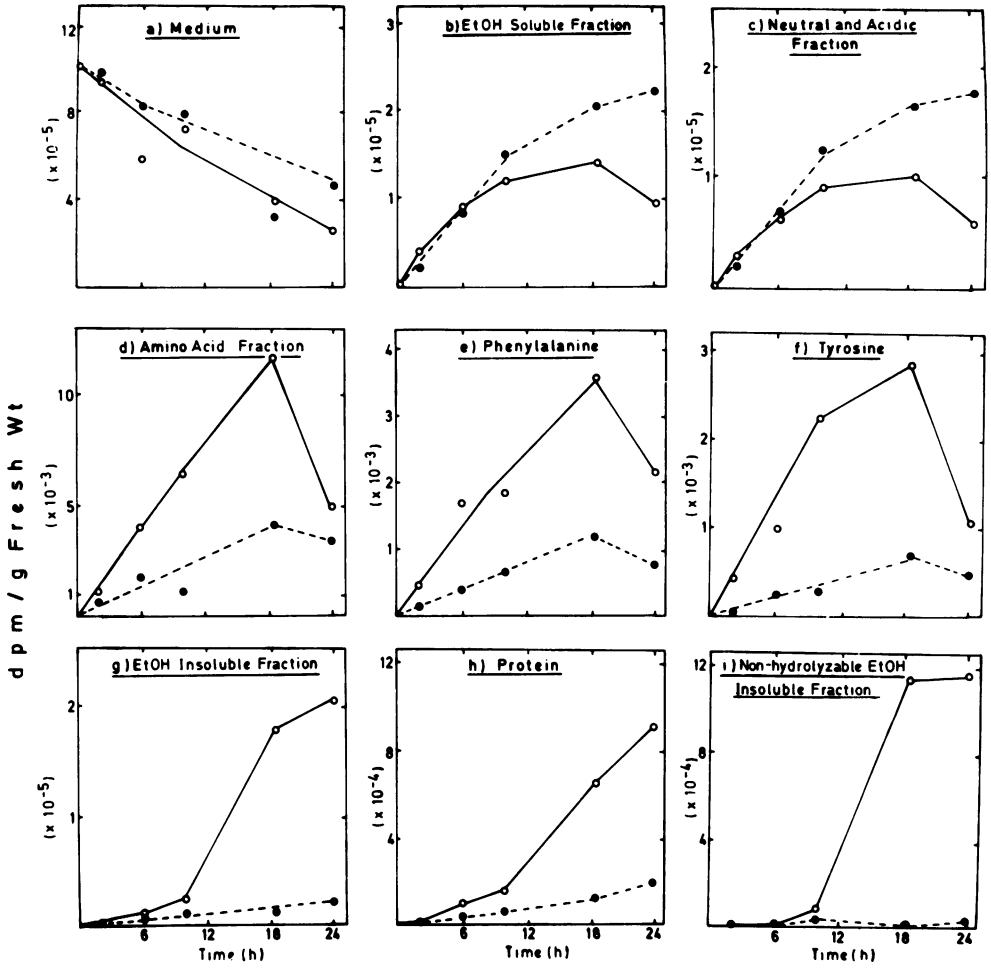

FIG. 2. Uptake and metabolic fate of [^{14}C]shikimate in buckwheat hypocotyls during a 24-h incubation period in the light. Open symbols: controls; closed symbols: 1 mM glyphosate. Incorporation of radioactivity into the nonhydrolyzable 80% ethanol-insoluble fraction (i) was determined as the difference between incorporation of radioactivity into the 80% ethanol-insoluble fraction (g) and into the protein fraction (h). Shikimate concentration was 3 μM (0.5 μCi/2 ml).

in phenylalanine (Fig. 2e) and tyrosine (Fig. 2f), not in tryptophan. The time courses of the incorporation of shikimate into these two amino acids were similar. The preferential enhancement of the accumulation of labeled phenylalanine in the presence of AOPP (19) was effectively curtailed by glyphosate (Table VI). Incorporation of shikimate into the 80% ethanol-insoluble fraction was drastically reduced in the presence of glyphosate (Fig. 2g). Acid hydrolysis of this material released only labeled tyrosine and phenylalanine, which indicated that the radioactivity was in protein (Fig. 2h). More than 60% of the radioactivity in the 80% ethanol-insoluble material from controls was not released by acid hydrolysis and, presumably, was in ligneous material. Incorporation of label into both these fractions was greatly reduced by glyphosate (Fig. 2h, 2i). Although the results presented in Figure 2 indicate that glyphosate inhibits the incorporation of shikimate into tyrosine and phenylalanine, and consequently, into protein and nonhydrolyzable material, it is important to consider whether tryptophan synthesis is also inhibited. Is the site of inhibition chorismate mutase as suggested by Jaworski (20) or possibly an earlier step in the biosynthetic sequence from shikimate to chorismate? A step prior to shikimate formation seemed unlikely because shikimate did not alleviate glyphosate-inhibition of anthocyanin synthesis (Table III) and the shikimate feeding studies indicated that glyphosate interfered in steps subsequent to shikimate formation.

Table VI. *Effect of AOPP and Glyphosate on Incorporation of [^{14}C]-Shikimate into Free Phenylalanine in the Hypocotyls of Derooted Buckwheat Seedlings*

[^{14}C]Shikimate, 0.5 μCi in 2 ml medium, was fed to 20 seedlings for 24 h in the light.

Treatment	Radioactivity Incorporated into Phenylalanine
	dpm
Buffer only (control)	2415
0.1 mM AOPP	40432
1 mM glyphosate	<500
0.1 mM AOPP + 1 mM glyphosate	5429

Even though it is generally accepted that shikimate is a precursor of tryptophan in higher plants, it has repeatedly been reported that incorporation of labeled shikimate into tryptophan was either undetectable or much lower than into tyrosine or phenylalanine (22, 27). We have reported (19), and now confirm, that labeled shikimate is effectively incorporated into tryptophan when either phenylalanine or tyrosine are supplied with shikimate in the incubation medium.

For the present study, it is relevant that glyphosate inhibited the incorporation of shikimate into all three aromatic amino acids, when 0.05 or 0.1 M phenylalanine was present in the incubation medium to ensure incorporation of shikimate into tryptophan in

the controls (Fig. 3). This result indicates that glyphosate blocks the formation of chorismate rather than its utilization by chorismate mutase.

Effect of Glyphosate on PAL-Activity in Buckwheat Hypocotyls. Glyphosate elevates PAL activity in maize and soybean plants (9, 10, 17), and its inhibition of growth has been thought to be the consequence of this induction of PAL activity. However, in excised buckwheat hypocotyls, PAL activity was not affected by glyphosate up to 24 h in the dark or in the light (Fig. 4), so that it seems unlikely that the induction of PAL by glyphosate is a general phenomenon. When anthocyanin synthesis in excised illuminated hypocotyls was allowed to proceed for 10 h and then continued in the presence of 3 mM glyphosate, its rate was reduced within less than 1 h (Fig. 5). This rapid inhibition is more likely to be due to

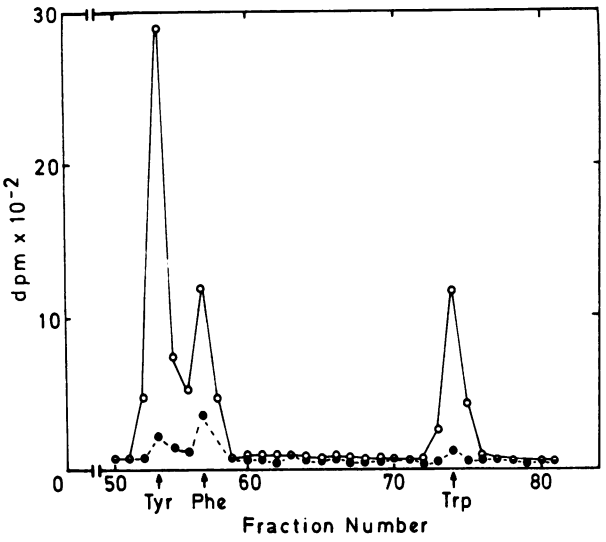

FIG. 3. Incorporation of radioactivity from [^{14}C]shikimate into the aromatic amino acids of buckwheat hypocotyls. Derooted buckwheat seedlings were incubated for 24 h in the light with 0.5 μCi [^{14}C]shikimate and 0.1 M L-phenylalanine in the absence (O——O) or presence (●- -●) of 1 mM glyphosate. The soluble amino acid fraction of the hypocotyls was subjected to amino acid analysis, and fractions were assayed for radioactivity after staining with ninhydrin (amino acid analyzer tracing not shown).

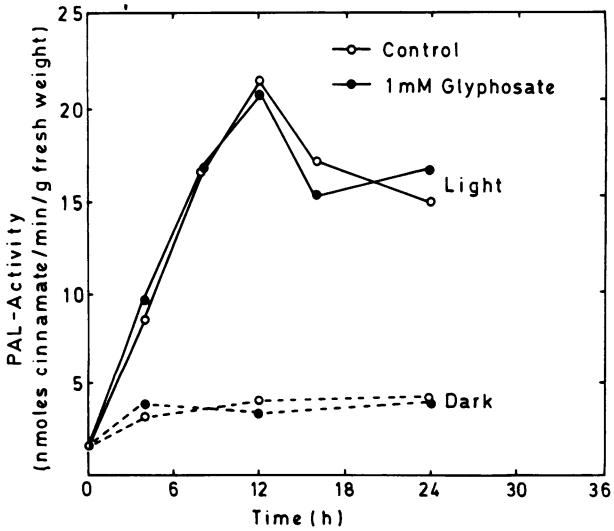

FIG. 4. Time course of PAL activity in excised buckwheat hypocotyls incubated in darkness (- - -) or light (——) in the absence (O) or presence (●) of 1 mM glyphosate.

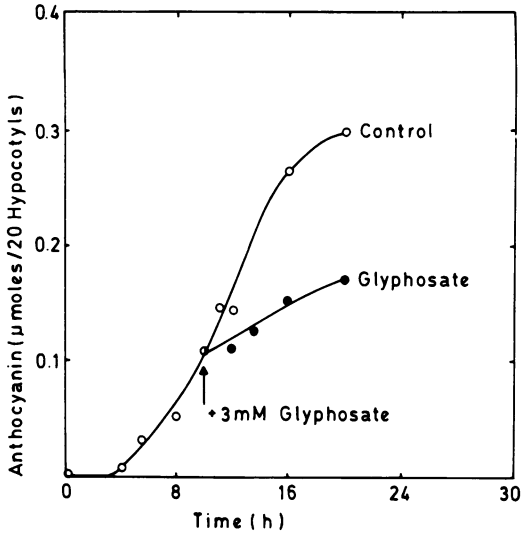

FIG. 5. Midcourse inhibition by glyphosate of anthocyanin production in excised buckwheat hypocotyls. Excised hypocotyls were illuminated in Petri dishes and anthocyanin content was determined at intervals (O). After 10 h, hypocotyls were transferred to 3 mM glyphosate (●) or fresh buffer (O), and the incubation was continued for a total of 20 h.

the direct inhibition by glyphosate of a metabolic step in the biosynthetic pathway leading to anthocyanin rather than to the induced appearance (or disappearance) of an enzyme.

Effect of Glyphosate on the Formation of Anthocyanin and Chl in Buckwheat Cotyledons. When derooted etiolated seedlings were supplied with 1 mM glyphosate in the light, it was noted that not only anthocyanin, but also Chl, formation was inhibited in the cotyledons. To investigate this effect of glyphosate in more detail, excised cotyledons were incubated in the light in the presence of increasing concentrations of glyphosate. Inhibition of anthocyanin formation was more sensitive than that of Chl by 1 order of magnitude (Fig. 6). The increase in the cotyledon fresh weight was not affected by glyphosate during the 24-h incubation. In agreement with the complementation experiments carried out with hypocotyls (Table III), phenylalanine alone was able to alleviate glyphosate inhibition of anthocyanin formation (Table VII). The presence of both phenylalanine and tyrosine was required, however, to achieve partial alleviation of the inhibition on Chl formation. Additional tryptophan did not further increase the Chl content of the cotyledons (Table VII).

DISCUSSION

The fact that growth inhibition by glyphosate of diverse organisms (*Rhizobium*, *E. coli*, *Chlamydomonas*, *Arabidopsis*, *Lemna*, and cultured carrot, soybean, and tobacco cells) is synergistically reversed by phenylalanine and tyrosine (and, at least in some cases, to a lesser extent also by tryptophan) strongly suggests a site of action of the herbicide which is common to all these organisms and which is not plant-specific (14). Induction of PAL activity, which has been reported to be brought about by glyphosate in maize and soybean seedlings (9, 10), therefore, cannot be the common determinant because of the restricted occurrence of PAL (8). We have recently shown that PAL activity in gherkin seedlings is enhanced by α-aminooxyacetic acid and AOPP, whereas hydroxycinnamic acid synthesis is reduced in the presence of these agents (1, 4). Therefore, an elevated level of PAL in a tissue does not necessarily indicate an enhanced rate of synthesis of phenolic compounds. We suggest that the enhancement of PAL activity by glyphosate in some plant tissues is secondary to the inhibition of the production of some phenolic compound(s), which regulate the level of PAL (4, 12). It was, in fact, shown recently (11) that, in

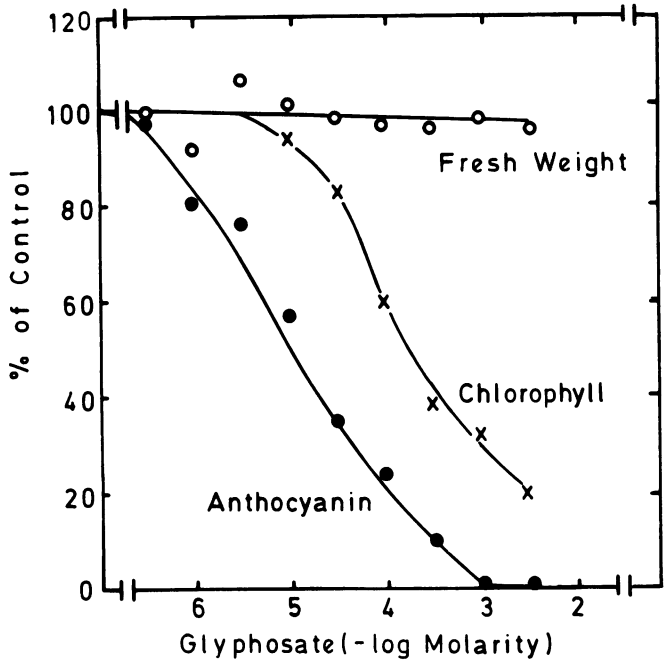

FIG. 6. Effect of glyphosate on fresh-weight increase (○——○), Chl content (×——×), and anthocyanin content (●——●) in excised buckwheat cotyledons. Cotyledons of 6-day-old etiolated seedlings were incubated at the indicated glyphosate concentrations for 24 h in the light. The initial weight of 20 pairs of cotyledons was 303 mg; the fresh weight increase was 60%. Controls produced 0.31 μmol anthocyanin and 295 μg Chl/20 pair of cotyledons.

Table VII. *Reversal Treatments of the Inhibition by Glyphosate of Anthocyanin and Chl Formation in Cotyledons Excised from 6-Day-Old Etiolated Buckwheat Seedlings*

All chemicals were added simultaneously to the incubation medium at the beginning of the 24-h incubation period. See the legend to Fig. 6 for further information.

Incubation Medium	Anthocyanin	Chlorophyll
	% control	
Buffer only (control)	100[a]	100[b]
1 mM glyphosate	0	36
1 mM glyphosate + 10 mM phenylalanine	87	38
1 mM glyphosate + 3 mM tyrosine	0	39
1 mM glyphosate + 3 mM tryptophan	0	38
1 mM glyphosate + 3 mM phenylalanine + 3 mM tyrosine	45	68
1 mM glyphosate + 3 mM phenylalanine + 3 mM tyrosine + 3 mM tryptophan	43	66

[a] 100% = 0.29 μmol anthocyanin/20 pairs of cotyledons.
[b] 100% = 315 μg Chl/20 pairs of cotyledons.

soybean seedlings, treatment with either glyphosate or AOPP increases extractable PAL-activity. Alternatively, glyphosate might induce PAL by interfering with the transport of phenolic compounds or their precursor, phenylalanine, from one cell compartment to another (12). In buckwheat seedlings, PAL is not induced by inhibitors of phenylpropanoid synthesis (1) and, thus, it is not surprising that the activity of PAL in buckwheat hypocotyls is not affected by glyphosate (Fig. 4). Glyphosate inhibits the production of phenylpropanoid compounds in this tissue (Table I), for which phenylalanine serves as the precursor. The complementation studies (Table III), as well as feeding experiments with labeled shikimate (Figs. 2 and 3; Table VI), suggest that the site of inhibition is at or prior to the formation of chorismate. Positive growth reversal by chorismate in *Lemna* and *Rhizobium* was also observed by Jaworski (20), but Gresshof (14) did not find an effect in systems which he studied. The instability of chorismate, as well as its poor uptake, might account for this failure. Tyrosine, which does not serve as an intermediate in hydroxycinnamic acid synthesis in buckwheat, did not alleviate glyphosate inhibition of anthocyanin synthesis (Table III) but acted synergistically with phenylalanine in alleviating inhibition of Chl production in excised cotyledons (Table VII). Tryptophan was required in glyphosate-inhibited carrot cells for optimal reversal of the growth inhibition (15) but was not required in *E. coli*, *C. reinhardtii*, or the carrot and soybean cultures employed by Gresshoff (14). Variation in response to glyphosate by various systems may well be associated with differential uptake, translocation, and metabolism of the herbicide, as well as with differences in the regulatory controls of the system(s), with which glyphosate interferes.

We have shown herein, that glyphosate inhibited the incorporation of labeled shikimate, not only into phenylalanine and tyrosine but also into tryptophan, when conditions favorable for shikimate incorporation into tryptophan were chosen for the control incubations (Fig. 3). Since chorismate is the common precursor of the three aromatic amino acids, we conclude that glyphosate either inhibits chorismate formation or a step prior to its formation.

The lack of metabolism of labeled shikimate in the presence of glyphosate argues against interference of the herbicide in a step prior to shikimate formation. An accompanying report (3) provides the following evidence as unequivocal proof that glyphosate interferes with the biosynthesis of aromatic amino acids prior to chorismate formation: (*a*) shikimate accumulates in tissues treated with glyphosate, (*b*) glyphosate inhibits the synthesis of chorismate-derived anthraquinones in cultured cells of *Galium mollugo*, and (*c*) glyphosate inhibits the formation of chorismate from shikimate in a cell-free system from *Aerobacter aerogenes* 62-1.

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