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Effect of Glyphosate on Carrot and Tobacco Cells[1]

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ABSTRACT

The growth of suspension-cultured carrot (*Daucus carota* L.) and tobacco (*Nicotiana tabacum* L. cv. Xanthi) cells was inhibited by glyphosate (N-[phosphonomethyl]glycine). This inhibition was reversed by adding combinations of phenylalanine, tyrosine, and tryptophan or casein hydrolysate. Casein hydrolysate and phenylalanine + tyrosine + tryptophan were the most effective treatments. Reversal of glyphosate-induced inhibition occurred only if the aromatic amino acids were added during the first 8 days of glyphosate incubation. Glyphosate uptake was not reduced when the aromatic amino acids or casein hydrolysate were added.

Even though phenylalanine biosynthesis is a suggested site for glyphosate action, inhibitory levels of glyphosate did not lower free phenylalanine concentrations in carrot cells within 10 days. ^{14}C-Phenylalanine studies indicated that the metabolic pool size was, likewise, not decreased.

In carrot cells total free amino acids increased within 6 hours after glyphosate addition. Cell protein levels declined within 48 hours following glyphosate treatment.

Studies on ^{14}C-thymidine and ^{14}C-uridine incorporation were complicated by rapid metabolism of these compounds to $^{14}CO_2$.

Glyphosate (N-[phosphonomethyl]glycine) is a nonselective herbicide with particular potential for perennial weed control due to excellent translocation (4, 7, 13).

Initial studies on the mechanism of glyphosate action indicated that phenylalanine biosynthesis was inhibited (9). Later, glyphosate was shown to reduce photosynthesis in whole plants by 72 hr (13), affect chloroplast ultrastructure (2), and induce chromosome aberrations (1). Jaworski (9) found that the addition of phenylalanine with glyphosate partially reversed glyphosate inhibition of *Lemna gibba* L. Tyrosine and tryptophan did not prevent glyphosate inhibition, but when the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, were combined, growth inhibition was reversed. Some phenylalanine precursors and derivatives also helped reduce growth inhibition. Other amino acids, some cofactors, and cinnamic acid did not reverse the inhibition. Similar results were noted for *Rhizobium japonicum* (9). Phenylalanine content decreased whereas the tyrosine level increased in glyphosate-treated *Lemna* (9). Because of these observations, an inhibition of phenylalanine biosynthesis at or near chorismate mutase or prephenate dehydratase was proposed as the primary mechanism of action. Roisch and Lingens (11) also found that *Escherichia coli* growth inhibition from glyphosate was prevented by all three aromatic amino acids. They found that glyphosate had no effect on the activities of chorismate mutase, prephenate dehydrogenase, or prephenate dehydratase. However, glyphosate did inhibit 3-deoxy-2-oxo-D-arabinoheptonic acid 7-phosphate synthetase and 5-dehydroquinic acid synthetase both of which are enzymes in the shikimic acid pathway.

The objective of the studies reported here was to determine the effects of glyphosate on carrot and tobacco cells in suspension culture and to elucidate further the biochemical mechanisms of glyphosate inhibition on cell growth.

MATERIALS AND METHODS

General Methods. Tobacco pith (*Nicotiana tabacum* L. cv. Xanthi) and garden carrot root (*Daucus carota* L.) cells were cultured as described by Widholm (15) with 1 g fresh weight inocula except that the liquid medium was half-strength (unless otherwise stated).

Since preliminary studies indicated that glyphosate (technical, 98% pure, Monsanto) was biologically and chemically stable to autoclaving, glyphosate and other compounds were autoclaved, either as a stock solution or in the nutrient media at pH 5.8. Glyphosate concentrations which inhibited cell growth 70 to 90% were used in duplicate flasks.

When radioisotopes were used, radioactivity of aqueous samples was determined in Kinard's scintillator (10) with a Packard Tri-Carb scintillation spectrometer. Radioactivity in solid material was determined after oxidation with a Packard Tri-Carb sample oxidizer by liquid scintillation spectrometry.

Glyphosate Uptake. Methylene-^{14}C-glyphosate (0.083 μCi/ml, 1.83 mCi/mmol) diluted with technical glyphosate (0.75 mM) was added to carrot cells (10 mg fresh weight/ml medium). Cells were harvested as described previously (6) except that filter papers were tared and prewashed with 3 ml of wash solution (water containing 10^{-3} M glyphosate) to fill glyphosate-binding sites. The cells were washed four times with 3 ml each of the glyphosate wash solution. Cells on filter paper were air dried, weighed, oxidized, and counted for ^{14}C.

Glyphosate Effects on Free Amino Acid and Protein Levels. Carrot cells were homogenized in a glass Kontes homogenizer (with power-driven pestle) containing 10 ml cold, 80% ETOH, then placed in a freezer for 30 min. Homogenates were centrifuged 10 min at 12,000g and the supernatant decanted into 100-ml round bottom flasks. The precipitated material was washed three times by homogenizing it in 10 ml cold, 80% ETOH and centrifugation as described above. The supernatants from each homogenate were combined and evaporated to dryness on a rotary flash evaporator. The extracts were taken up in 3% trichloroacetic acid and were analyzed for total free amino nitrogen by the ninhydrin method (3) and for individual amino acids with a TSM Technicon automatic amino acid analyzer.

Total protein was measured in the residual precipitates by dissolving each precipitate in 10 ml of 0.5 N NaOH and assaying by a modified Schacterle and Pollack method (12). Copper and NaOH were added separately in this determination. The protein standard was BSA.

Even though the amino acid and protein data reported here

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were from different experiments, cell growth for both experiments was similar.

Glyphosate Effect on Phenylalanine Metabolic Pool Size. At several times after 0.75 mM glyphosate addition to 3-day-old carrot cells (in full strength medium) L-phenylalanine-UL-^{14}C (0.1 μCi/4 ml, 455 mCi/mmol), and L-leucine-UL-^{14}C (0.1 μCi/4 ml, 310 mCi/mmol) were added to flasks of glyphosate-treated and untreated cells and incubated for 2 hr. Cells were harvested on tared filters and washed four times with 3 ml water or methanol-chloroform-water (MCW) solutions. Each wash solution contained phenylalanine and leucine at 1 mM each. The MCW wash technique permits measurement of ^{14}C-amino-acid incorporation into protein (6). Cells were air-dried, weighed, oxidized, and the ^{14}C counted. When dried cell weights differed significantly between treated and untreated cells adjustments in ^{14}C activity were made on a cell weight basis.

RESULTS

Inhibition of Cell Culture Growth by Glyphosate. Glyphosate inhibited the growth of suspension-cultured carrot and tobacco cells with tobacco cells being most sensitive (Fig. 1). At glyphosate concentrations of 0.05 mM, tobacco cell growth was inhibited about 70% and carrot cells by only 10%. Due to some variability in cell response to glyphosate, growth curves as in Figure 1 could be shifted slightly to the right or left. For example, in Table I, 1 mM glyphosate was required for 79% growth inhibition whereas in Figure 4, 0.3 mM was sufficient for similar inhibition. This variation seemed to be dependent upon the culture age of the cells used to initiate the experiments.

Reversal of Growth Inhibition. Various combinations and concentrations of the amino acids phenylalanine, tyrosine, and tryptophan as well as casein hydrolysate were used to prevent the cell growth inhibition caused by glyphosate.

The addition of the aromatic amino acids in combination at 0.25 mM each and casein hydrolysate at 1 mg/ml significantly reduced glyphosate inhibition of tobacco cell growth. Casein hydrolysate was more effective at reducing inhibition than were the amino acids.

When the three aromatic amino acids were combined all concentrations above 0.075 mM each significantly decreased glyphosate-induced carrot cell inhibition. When the aromatic amino acids were added individually and in combinations at 0.5 mM each to carrot cells, only the amino acid combinations gave appreciable prevention of growth inhibition (Table I). The best two-amino acid combination was phenylalanine plus tyrosine where a 43% inhibition resulted compared to a 79% inhibition with glyphosate alone. All three amino acids in combination decreased glyphosate inhibition to 29%. Casein hydrolysate at 10 mg/ml plus tryptophan (0.3 mg/ml) overcame glyphosate inhibition completely in carrot cells. Casein hydrolysate without tryptophan was also effective (data not shown).

Glyphosate inhibition of carrot cell growth was reversed only when phenylalanine + tyrosine + tryptophan (0.5 mM each) were added within 8 days after cell inoculation into the inhibitory medium (Fig. 2).

Glyphosate Uptake. The uptake of methylene-^{14}C-glyphosate by carrot cells was rapid the first hr followed by a slower but linear increase through 96 hr. The estimated concentration within the cells after 24 hr was 0.19 mM which is similar to the cellular concentrations of some free amino acids. After 12 and 96 hr, 0.2 and 0.8% of the glyphosate in the medium was taken up by the cells, respectively.

Since the reversing agents might act by decreasing glyphosate uptake, studies to test this were carried out. The presence of

Table I. Effect of adding the aromatic amino acids (individually and in all combinations), glycine, and casein hydrolysate (plus tryptophan) on the reversal of glyphosate inhibition of carrot cell growth.

Cell weight was determined in duplicate flasks 10 days following inoculation with 0.5 g fresh weight of 10-day-old cells into 100 ml of half strength nutrient medium. All amino acids were 0.5 mM; Casein hydrolysate (acid, vitamin-free) was 10 mg/ml and Trp was 0.3 mg/ml; all chemicals were added prior to inoculation. Glyphosate concentration was 1.0 mM. Percent inhibition is a comparison of +glyphosate cell weights with the control (no additions).

Additions	Cell weight (g) -glyphosate	Cell weight (g) +glyphosate	% Inhibition with glyphosate
Control	9.2	1.9	79
Phe	6.3	2.5	73
Tyr	8.9	2.5	73
Trp	10.3	2.1	77
Phe + Tyr	7.6	5.2	43
Phe + Trp	7.3	4.1	56
Tyr + Trp	9.9	3.9	58
Phe + Tyr + Trp	8.8	6.5	29
Gly	7.8	1.3	86
Casein hyd. + Trp	9.7	10.9	-19
$LSD_{0.05}$	1.5	1.5	

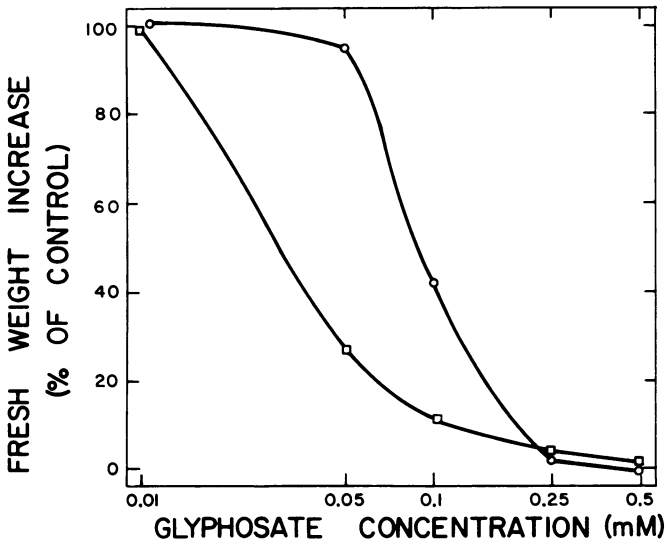

FIG. 1. Inhibition of carrot (○) and tobacco (□) cell growth by glyphosate. Cells were harvested 10 days after inoculation with 0.5 g fresh weight, 12-day-old cells into 100 ml half-strength fresh nutrient medium containing glyphosate. Cell weight increase for control (no glyphosate) carrot and tobacco cells were 6 and 13.8 g, respectively.

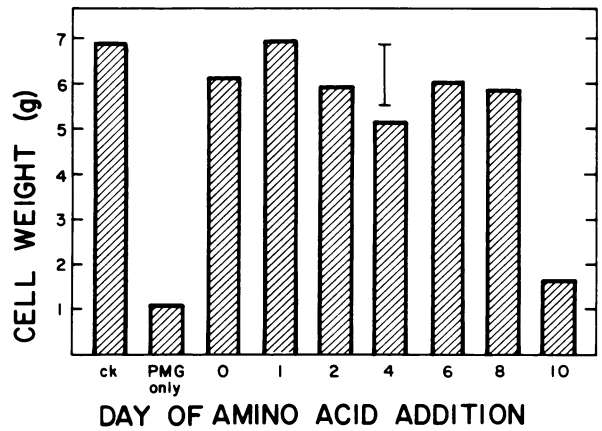

FIG. 2. Response of glyphosate-treated carrot cells to additions of phe + tyr + trp (0.5 mM each) at various times after inoculation with 0.5 g fresh weight, 10-day-old cells into 100 ml half-strength medium containing 1 mM glyphosate (PMG). The cells were allowed to grow for 10 days after the last chemical addition before weighing. $LSD_{0.05}$ is inserted.

phenylalanine or all three aromatic amino acids did not inhibit ^{14}C-glyphosate uptake by carrot cells in 3-, 6-, or 12-hr incubations. Casein hydrolysate decreased glyphosate uptake by about 30% but only at 12 hr.

Experiments in which glyphosate was washed away from the cells after incubation times of 24 and 48 hr showed that the cells could recover and grow. The loss of glyphosate from carrot cells was measured by incubating cells with ^{14}C-glyphosate for 3 or 6 hr and then incubating without ^{14}C-glyphosate for another 3 or 6 hr. Up to 54% of the cell-bound glyphosate was lost within 6 hr.

Glyphosate Effects on Free Amino Acid and Protein Levels. Carrot cell weight and total free amino acid levels with and without glyphosate are shown for a 10-day growth period in Figure 3. Total free amino nitrogen increased within 6 hr after inoculation. The largest increases were in glyphosate-treated cells. Free amino acid concentrations were lower in untreated cells than in glyphosate-treated cells. A decline in free amino nitrogen between 6 and 48 hr correlated with the increases in cellular protein noted in both treated and untreated cells (Fig. 4).

Higher concentrations of free amino acids in the treated cells were due, mainly, to larger quantities of ammonia, histidine, arginine, and glutamic acid (including glutamine) (Table II). This nitrogen fraction from treated cells accounted for a 13- and 15-μmol/g fresh weight increase over the free amino acid content in control cells on days 3 and 4, respectively.

The concentrations of individual free amino acids in treated cells usually paralleled those of the control for the first 24 hr (Table II). Phenylalanine concentrations were of particular interest in treated cells. Phenylalanine was slightly lower in treated cells at day 2, but at day 3 phenylalanine concentrations were above those of the controls, and remained higher throughout the growth period.

Glyphosate was also added to carrot cell cultures 6 days after inoculation (early log phase) as compared to day 0 for the experiment discussed above. Cell weights, total free amino acids, and individual amino acids were measured during the next 48 hr. Inhibition of growth was observed after 24 hr and slight increases in total free amino acids were noted 6 through 48 hr after treatment. Changes in individual free amino acid and ammonia concentrations were similar to those when glyphosate was added at day 0 (Table II). The accumulation of arginine was inhibited markedly by glyphosate. Arginine concentrations were

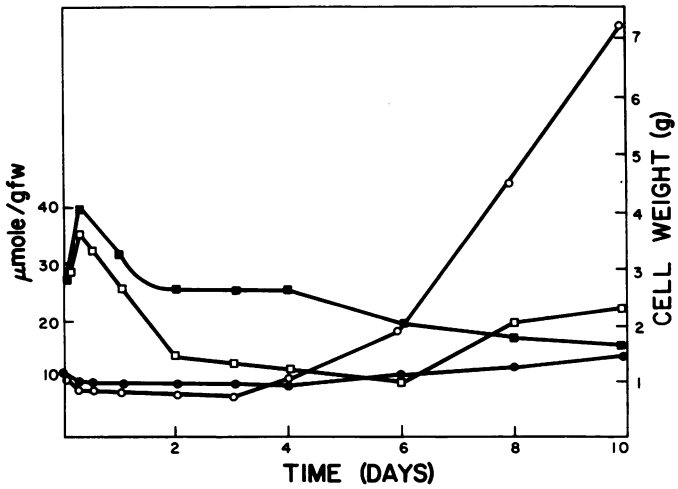

FIG. 3. Effect of glyphosate (0.3 mM) on cultured carrot cell weights and free amino acid levels. One g fresh weight of 11-day-old cells was inoculated into 100 ml half-strength medium and measurements made as under "Materials and Methods." Cell fresh weight with (●) and without (○) glyphosate; total free amino acid with (■) and without (□) glyphosate.

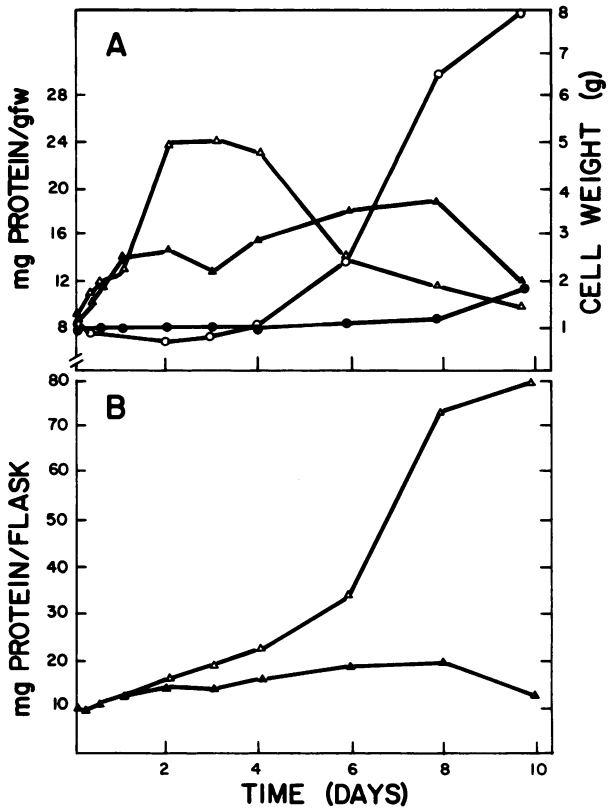

FIG. 4. Carrot cell weight and protein as affected by 0.5 mM glyphosate. Carrot cells (1 g fresh weight, 11-day-old) were inoculated into 100 ml half-strength medium. A: total protein/g fresh weight with (▲) and without (△) glyphosate; cell fresh weight with (●) and without (○) glyphosate. B: total protein/flask with (▲) and without (△) glyphosate.

0.8 μmol/g fresh weight prior to glyphosate addition, but after 48 hr was 7.1 μmol/g fresh weight in control cells and 1.6 μmol/g fresh weight in glyphosate-treated cells. Phenylalanine decreased slightly in treated cells relative to untreated up to 24 hr, but increased at 48 hr.

Glyphosate Effect on Phenylalanine Metabolic Pool Size. The uptake and incorporation of ^{14}C-leucine and ^{14}C-phenylalanine were measured between 3 and 48 hr after glyphosate addition (Table III). Glyphosate had little affect on ^{14}C-leucine and ^{14}C-phenylalanine incorporation (into protein) for 6 hr. At 12 hr uptake of phenylalanine was reduced by glyphosate and at 24 hr incorporation of both phenylalanine and leucine was increased in glyphosate-treated cells. Incorporation of both amino acids into protein was comparable in most assays suggesting that glyphosate affects incorporation of aromatic and aliphatic amino acids into protein in a similar way.

Uptake and Incorporation of ^{14}C-Thymidine and ^{14}C-Uridine. Attempts were made to determine the effects of glyphosate treatment on RNA and DNA synthesis in carrot cells by measuring the incorporation of ^{14}C-labeled precursors, but preliminary experiments showed that thymidine-2-^{14}C and uridine-2-^{14}C were rapidly metabolized to $^{14}CO_2$ which was evolved from the medium. The amount of $^{14}CO_2$ evolved within 3 hr amounted to 80% of the total thymidine added and about 70% of the uridine added. Others have reported metabolism of thymidine-2-^{14}C and uridine-2-^{14}C to $^{14}CO_2$ in cultured plant cells (5, 8, 14).

DISCUSSION

The growth inhibition caused by glyphosate in cultured carrot and tobacco cells was reversed by the addition of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, and by

Table II. Free amino acid concentrations from carrot cells with glyphosate (PMG, 0.3 mM) or without (ck). One g fresh weight of 11-day-old cells was inoculated into 100 ml of half strength nutrient medium. After amino acids were extracted at times shown, analyses were made with a TSM Technicon amino acid analyzer followed by computer calculation of LSD from duplicate samples.

Amino Acid	μmole/g fresh wt																			
	0 hr	6 hr		12 hr		24 hr		48 hr		72 hr		96 hr		6 days		8 days		10 days		$LSD_{0.05}$
	ck	ck	PMG	ck	PMG	ck	PMG	ck	PMG	ck	PMG	ck	PMG	ck	PMG	ck	PMG	ck	PMG	
NH_3	4.85	7.68	12.29	9.20	12.67	4.73	9.48	3.96	7.38	4.48	8.85	4.36	11.02	4.06	6.12	5.13	6.23	6.64	10.52	2.51
Orn	0.51	0.57	0.61	0.54	0.64	0.45	0.52	0.24	0.34	0.20	0.34	0.15	0.28	0.07	0.15	0.17	0.08	0.29	0.15	0.09
His	1.77	2.21	2.36	2.36	2.48	1.87	2.14	1.27	1.91	0.98	2.27	0.57	2.21	0.29	1.46	1.28	0.91	1.64	0.88	0.87
Lys	0.60	0.65	0.67	0.58	0.59	0.36	0.36	0.10	0.24	0.08	0.23	0.08	0.18	0.07	0.04	0.18	0.07	0.33	0.19	0.16
Arg	8.14	9.41	9.91	9.54	9.60	8.66	7.92	4.97	6.75	1.63	6.16	0.62	4.99	0.15	2.12	3.39	0.88	4.47	0.60	1.53
Asp	0.76	1.00	1.13	1.02	1.03	0.53	0.77	0.35	0.42	0.33	0.55	0.30	0.47	0.32	0.48	0.51	0.50	0.63	0.69	0.19
Thr	2.05	3.90	2.40	2.13	2.52	0.77	1.06	0.30	1.47	0.48	1.19	0.41	1.28	0.54	2.61	2.82	1.11	1.83	1.24	0.85
Ser	0.65	0.90	a	1.05	0.92	0.43	a	0.29	0.49	0.49	a	0.45	0.34	0.38	0.46	0.85	0.44	0.98	0.74	0.44
Glu	2.04	3.11	4.23	3.31	4.02	1.64	3.18	1.24	3.08	1.45	3.82	1.37	3.37	1.58	3.60	2.57	3.24	2.36	4.04	1.04
Gly	0.16	0.15	0.17	0.11	0.11	0.07	0.07	0.07	0.05	0.07	0.13	0.12	0.14	0.17	0.09	0.21	0.10	0.23	0.15	0.09
Ala	0.91	1.12	1.21	0.86	0.89	0.50	0.69	0.42	0.43	0.26	0.97	0.51	1.02	0.69	0.84	1.69	0.73	1.97	1.01	0.59
Val	0.89	0.94	1.04	0.82	0.88	0.43	0.55	0.32	0.36	0.38	0.53	0.34	0.54	0.32	0.51	1.03	0.40	1.12	0.65	0.23
Met	0.33	0.41	0.43	0.31	0.33	0.10	0.12	0.05	0.05	0.05	0.07	0.05	0.06	0.04	0.05	0.35	0.03	0.39	0.05	0.09
Ile	0.59	0.56	0.61	0.50	0.61	0.25	0.40	0.13	0.25	0.15	0.43	0.15	0.47	0.15	0.47	0.49	0.42	0.60	0.48	0.10
Leu	0.43	0.51	0.55	0.41	0.45	0.17	0.18	0.15	0.09	0.10	0.14	0.10	0.18	0.10	0.14	0.31	0.13	0.41	0.18	0.09
Trp	0.29	0.29	0.35	b	0.30	0.08	0.17	0.05	0.12	0.12	0.08	0.10	0.14	0.07	b	0.22	0.11	0.09	b	0.18
Tyr	0.60	0.63	0.66	0.51	0.54	0.20	0.25	0.07	0.07	0.07	0.12	0.07	0.11	0.07	0.08	0.31	0.07	0.31	0.11	0.10
Phe	0.22	0.25	0.27	0.16	0.16	0.10	0.08	0.16	0.08	0.13	0.24	0.16	0.31	0.12	0.26	0.15	0.31	0.08	0.51	0.15
Total	25.75	32.86	38.83	33.42	38.55	20.18	27.93	13.20	22.87	10.99	26.04	9.87	27.07	9.16	19.49	21.65	15.75	24.30	22.17	5.77

[a]Serine's value is included with threonine.
[b]No value obtained.

Table III. Uptake and incorporation of L-phenylalanine-UL-^{14}C (0.1 μCi/4 ml, 455 mCi/mmole) and L-leucine-UL-^{14}C (0.1 μCi/4 ml, 310 mCi/mmole) in rapidly metabolizing (3-day-old) carrot cells as affected by glyphosate. The ^{14}C-amino acids were added to the cells at the times shown after glyphosate addition and incubated for a 2-hr period before harvesting and assay as described in Materials and Methods. The results listed are means of duplicates. Without glyphosate (ck), with 0.75 mM glyphosate (PMG).

Time (hr)		^{14}C-phenylalanine dpm X10^{-2}			^{14}C-leucine dpm X10^{-2}		
		Uptake	Incorporation	% Incorporation	Uptake	Incorporation	% Incorporation
3	ck	483	428	89	390	318	82
	PMG	475	392	83	309	295	95
6	ck	511	436	86	417	343	82
	PMG	515	437	85	418	323	78
12	ck	524	462	88	438	346	80
	PMG	484	464	97	452	346	77
24	ck	534	324	61	403	255	64
	PMG	532	419	79	390	295	76
48	ck	403	264	66	332	232	71
	PMG	414	284	69	231	156	68

casein hydrolysate (Table I, Fig. 2). Inhibitory levels of glyphosate did not, however, appreciably reduce the cellular concentrations of the free aromatic amino acids (Table II). Incorporation studies of ^{14}C-leucine and ^{14}C-phenylalanine into protein also indicated that the metabolic pool of free phenylalanine was similar to that of leucine (Table III). Although our data indicate that phenylalanine is not a limiting amino acid for protein synthesis in glyphosate-treated cells, there was a decrease in the phenylalanine content at 48 hr (Table II), but, at no other time in treated cells. This agrees with Jaworski's (9) data with *Lemna* where he measured phenylalanine concentrations at 48 hr after glyphosate treatment. We conclude that phenylalanine levels do not limit growth.

The ability of the aromatic amino acids to reverse cell growth inhibition when added up through 8 days (Fig. 2) demonstrated that the cells remained alive during glyphosate inhibition. Support for cells being alive during glyphosate inhibition is also given by studies where glyphosate was removed from the cell culture which permitted cell growth to occur. The inability of the aromatic amino acids to reverse glyphosate action in carrot cells after 8 days incubation with glyphosate suggested that glyphosate could affect growth irreversibly if it was in contact with the cells long enough. Decreases in protein and free amino acid concentrations were noted in cells that were beyond the growth inhibition reversal stage (Figs. 3 and 4).

Nitrogen utilization was markedly affected by glyphosate in carrot cells. Nitrogen-storing amino acid levels decreased rapidly in control cells, when mature cells were placed in fresh medium and protein synthesis was intensified. The decrease in basic amino acid levels was slower in treated cells and may have been due to slower growth rates and lower demands for amino acids. Total soluble amino-nitrogen remained higher in treated cells until 8 days, at which time untreated cells began storing free amino nitrogen and glyphosate-treated cells did not (Fig. 3). Accumulations of ammonia, lysine, aspartic acid, serine, glutamic acid, glycine, valine, and phenylalanine were not affected by glyphosate between 8 and 10 days.

Since the data indicate that synthesis of the aromatic amino acids is not specifically inhibited by glyphosate and since reversal of glyphosate-induced inhibition is not due to decreased glyphosate uptake, no conclusion can be made concerning the mechanism of glyphosate action with cultured cells or the reason for reversal of this inhibition by certain amino acids.

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