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Glyphosate Inhibits Photosynthesis and Allocation of Carbon to Starch in Sugar Beet Leaves[1]

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ABSTRACT

Application of glyphosate (*N*-[phosphonomethyl] glycine) to exporting leaves of sugar beet (*Beta vulgaris*, L.) during the day lowered stomatal conductance and carbon fixation. Allocation of newly fixed carbon to foliar starch accumulation was nearly completely inhibited, being decreased by the same amount as net carbon fixation. In contrast, decreasing net carbon fixation in untreated leaves by lowering CO_2 concentration caused starch accumulation to decrease, but only in the same proportion as net carbon fixation. Shikimate level increased 50-fold in treated leaves but the elevated rate of carbon accumulation in shikimate was only 4% of the decrease in the rate of starch accumulation. Application of steady state labeling with $^{14}CO_2$ to exporting leaves confirmed the above changes in carbon metabolism, but revealed no other major daytime differences in the ^{14}C-content of amino acids or other compounds between treated and control leaves. Less ^{14}C accumulated in treated leaves because of decreased fixation, not increased export. The proportion of newly fixed carbon allocated to sucrose increased, maintaining export at the level in control leaves. Returning net carbon exchange to the rate before treatment restored starch accumulation fully and prevented a decrease in export during the subsequent dark period.

Glyphosate ([*N*-phosphonomethyl] glycine), a nonselective, postemergence herbicide, appears to affect a number of major processes in plants (14). At low dose rate, glyphosate affects growth and the partitioning of carbon, without killing the plant. When applied to sugarcane at a sublethal concentration, glyphosate increases the amount of sugar stored in older stalks while lessening new growth. Sublethal treatment causes increased tillering in quackgrass (5) and wheat (3) and increased shoot proliferation in cranberry (15). We believe that similar effects occur shortly after application of lethal doses of glyphosate.

While many previous studies dealt with effects of glyphosate that occur one or several days after application there is value to studying early effects that may reveal mechanisms of glyphosate action. Gougler and Geiger (8) observed changes in diurnal translocation patterns in sugar beet plants beginning several hours after application of glyphosate. Accumulation of foliar starch decreased without observable effects on export but partitioning of carbon among sinks was altered.

The present study was conducted to determine the action of glyphosate on allocation of newly fixed carbon in source leaves and on NCE[2] during the first 24 h after application.

MATERIALS AND METHODS

Plant Material. Experiments were conducted on 5- to 6-week-old sugar beet plants (*Beta vulgaris* L. Klein E multigerm) grown in 1.5 L containers in a mixture of milled peat moss, vermiculite, and sand (1:1:2, v/v). Plants were raised in an environmental chamber (14-h photoperiod, 25°C d and 17°C night) and watered three times a day with a nutrient solution as described by Snyder and Carlson (17). Photon flux density was 450 μmol m^{-2} s^{-1} at leaf blade level for growth and during experiments.

Steady State Labeling and NCE Measurements. Steady state labeling was carried out in a closed system as described by Geiger and Fondy (7). Air containing $^{14}CO_2$ at a specific radioactivity of approximately 2.5 mCi g^{-1} C was circulated through a labeling system and over one or two source leaves (approximately 14 cm blade length). Rates of leaf NCE were determined from the timed depletion of CO_2. The CO_2 level was maintained at approximately 350 μl L^{-1} and varied between 300 and 400 μl L^{-1} during NCE measurements. Where indicated, stomatal conductance was measured with a steady state porometer (Licor model LI-1600C).

Export from a leaf was determined as the difference between the amount of carbon fixed and the amount accumulated in the source leaf during a given period. Accumulation of carbon in the leaf blade was measured either by gain of ^{14}C during steady state labeling (7) or by dry weight gain determined from the slope of a curve prepared by weighing leaf samples removed at hourly intervals.

Glyphosate Application. Solutions of analytical grade glyphosate, 99% purity (Monsanto Agricultural Products Co.) were prepared to give a final concentration of 17 mM, a concentration equivalent to 1% Round-up commonly used in herbicide applications. The pH of each solution was adjusted to 5.5 with isopropylamine and, unless stated otherwise in the text, were made to a final concentration of 0.01% (v/v) Tween-20 surfactant. The solution was distributed evenly over the leaf surface with an atomizer.

Leaf Sampling. To provide leaf material for replicate samples within leaves and among similar leaves, experiments were carried out on three or four adjacent source leaves, each successive leaf being approximately 2 d younger. Sampling was done by removing sets of four leaf discs (total area 0.64 cm^2), one disc from each quadrant of the source leaf blade, at intervals during and after labeling. During labeling, the leaf blade was sampled through a small opening in the lid of the labeling compartment to minimize changes in the specific radioactivity of CO_2. Sampled areas were chosen so as not to disrupt export by removal of a major vein.

Up to three duplicate sets of leaf samples were taken to provide adequate material for measurements of dry weight and labeled carbon present in entire leaf samples and in fractions thereof. Immediately upon collection, samples to be fractionated were extracted at 65°C in chloroform:methanol (1;4, v/v). Discs were then exposed to refluxing solvent mixtures at 65°C, first in the

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[2] Abbreviation: NCE, net carbon exchange.

chloroform:methanol mixture until white and then in 80% v/v ethanol for 30 min. Samples to be used for measuring total dry weight and labeled carbon were frozen on solid CO_2, dried under vacuum while frozen, weighed and prepared for scintillation counting by the technique of Mahin and Lofberg (9).

Analysis of Soluble Extract. Labeled carbon present in the chloroform:methanol-soluble fraction was determined by scintillation counting of ^{14}C in an aliquot from each sample. The remainder of the extract was separated into chloroform-soluble and water-soluble phases and the latter was passed through Sephadex ion-exchange columns to separate neutral, organic, and amino acid fractions according to the method of Redgwell (12). Aliquots from each fraction were counted to determine the content of labeled carbon.

Analysis of Shikimate. Shikimate was separated and measured with a Waters HPLC (Milford, MA) equipped with a model 450 variable wavelength UV detector set at 230 or 215 nm for methods I and II, respectively. In method I, a 5-μm C-18 Waters Radial Pak column, 250 × 4.6 mm, preceded by a guard column of similar composition, was used. The mobile phase was 50 mM KH_2PO_4 buffer, adjusted to pH 2.4 with H_3PO_4. Retention time for shikimate was 5.2 min. In method II a 5-μm silica-bonded NH_2 column, Merck Lichrosorb, 250 × 4.6 mm, preceded by a guard column of similar composition, was used. The solvent system consisted of acetonitrile:water:H_3PO_4, 95:4:1 v/v. Retention time for shikimate was 9.7 min. The flow rate for both systems was 1 ml min^{-1}.

Analysis of Insoluble Residue. One set of extracted discs was analyzed for starch by a method modified from Outlaw and Manchester (10) after Fondy and Geiger (6). These discs were dried at 65°C and then incubated at room temperature in 300 μl of 0.2 N KOH in 100 mM ethanol for 10 to 12 h. Discs were homogenized for 2 min and the starch solubilized by heating the homogenate for 2 h. Volume was adjusted to 500 μl with deionized H_2O and the mixture was clarified by centrifugation (1000*g*, 10 min) in a clinical centrifuge to remove all insoluble material such as cell walls. A 25-μl aliquot of the supernate was removed to determine labeled starch. The KOH extract probably also contained some labeled protein but after the relatively short labeling period most of the label was likely to be in starch. Hydrolysis of some samples of the extract, separation of the products by TLC, and location of label by autoradiography confirmed that most of the label was in glucose under these circumstances (data not shown).

Starch was then hydrolyzed with amyloglucosidase. The pH of the homogenate was adjusted to 4.8 by adding 10 μl of glacial acetic acid. To each sample, 500 μl of a solution containing 1 mg ml^{-1} amyloglucosidase (Sigma) in 0.2 M potassium acetate buffer (pH 4.8) was added and the volume adjusted to 1 ml. The samples were allowed to incubate for 18 to 24 h at room temperature and glucose derived from starch was measured.

Labeled carbon present in the residue remaining after alcohol extraction was measured in a second set of samples after digestion for scintillation counting (9). The label present in samples after extraction by alcohol and KOH was calculated by subtracting the label in the KOH extract following centrifugation, from the label present in these alcohol-extracted samples. This value likely included the radioactivity present in some proteins, as well as in cell-wall and other structural materials.

RESULTS

Starch Accumulation. Accumulation of foliar starch stopped 4 to 6 h after application of glyphosate (Fig. 1). Treatment of source leaves was delayed until 3 to 4 h into the light period to allow the diurnal pattern of starch accumulation to be established in the leaf (6). By the end of the light period, the amount of starch stored in treated leaves was 30% less than in control leaves, a difference amounting to approximately 45 μg C cm^{-2}.

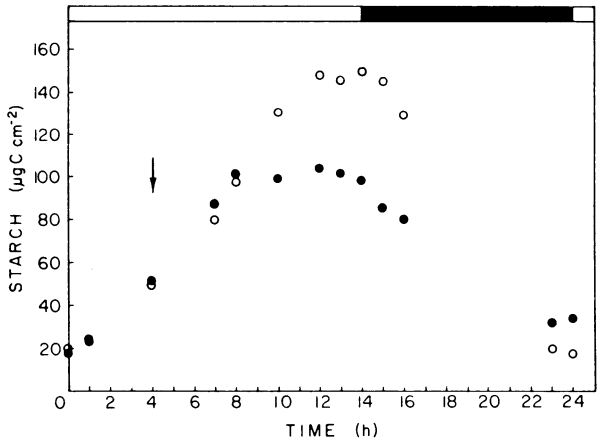

FIG. 1. Accumulation of starch in glyphosate-treated (●) and control (○) source leaves of sugar beet. Glyphosate (17 mM) was applied to the leaf at the time indicated by the arrow. The dark bar represents the dark period. Each point is the average of five experiments.

Allocation of Newly Fixed Carbon. Changes in allocation of newly fixed carbon following glyphosate application were traced by steady state labeling of leaves in $^{14}CO_2$. Less labeled carbon accumulated in treated leaves indicating inhibition of NCE (Fig. 2C). Allocation of newly fixed carbon into foliar starch was also less in glyphosate-treated leaves (Fig. 2A). By the end of the light period there was 150 nCi cm^{-2} or 60 μg C cm^{-2} less ^{14}C-starch. This value is close to the difference in starch shown in Figure 1 for another set of plants and is similar to the difference in total accumulated ^{14}C (Fig. 2C). In contrast, there were similar amounts of labeled carbon in both the chloroform:methanol-soluble fraction (Fig. 2B) and in the residue remaining after starch extraction (Table I) in both sets of leaves during this period.

Net Carbon Exchange. From 4 to 5 h after glyphosate application, NCE rate began to decline and continued to do so for the rest of the light period (Fig. 3B). These data explain the smaller total amount of ^{14}C per area of leaf in glyphosate-treated leaves (Table I; Fig. 2C). Glyphosate minus surfactant caused a similar lowering of the NCE rate while water alone had no effect (data not shown).

Stomtal Conductance. Periodic measurements showed that stomatal conductance began to decrease in leaves treated with 17 mM glyphosate, either with or without surfactant, approximately 4 h after treatment (Table II). This marked decrease in conductance was not observed in leaves treated with surfactant solution or with water.

Starch Accumulation and Net Carbon Accumulation. Comparison of glyphosate-treated and control leaves shows that the decrease in amount of carbon accumulated as starch is similar to the decrease in the total amount of carbon fixed (Table III). To provide a comparison, CO_2 concentration was lowered around leaves not treated with glyphosate, reducing NCE to an extent similar to that caused by glyphosate treatment (Fig. 4C). Starch accumulation decreased (Fig. 4B) but only in proportion to the decrease in NCE. The ratio of starch accumulation rate to NCE rate remained nearly unchanged from that of leaves in the air level of CO_2 (Fig. 4A). In contrast, this same ratio dropped to zero as NCE decreased several hours after glyphosate treatment (Fig. 3A).

When the concentration of CO_2 supplied to treated source leaves was raised, the glyphosate-induced drop in NCE was reversed and starch accumulation was comparable to that of the control leaves (Fig. 5).

Starch Degradation and Mobilization. During the ensuing dark

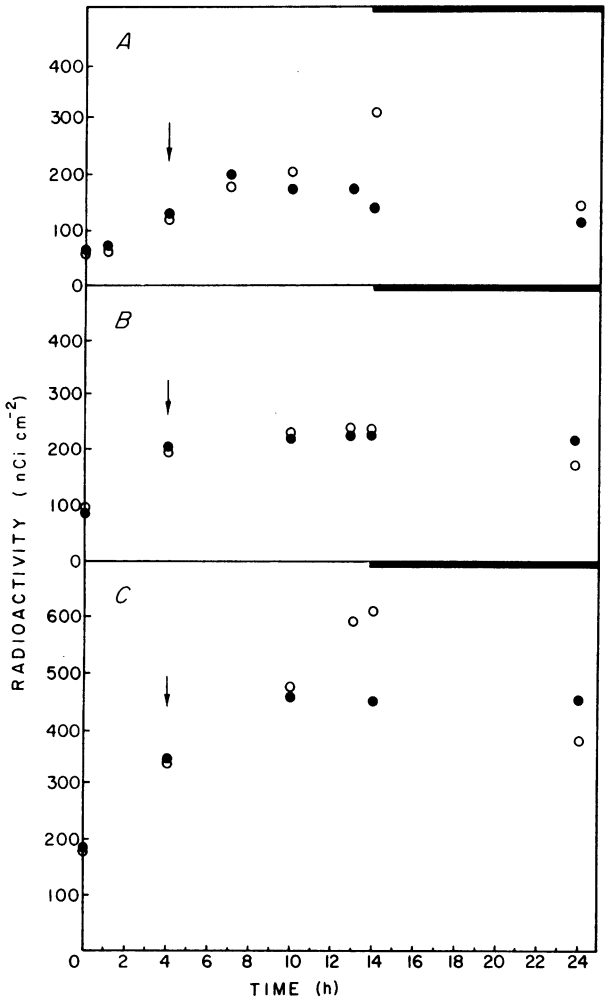

FIG. 2. Diurnal pattern of ^{14}C content of starch (A), soluble leaf extract (B), and total leaf (C) in glyphosate-treated (●) and control source leaves (○). Source leaves were labeled throughout the 14-h light period. Symbols as in Figure 1. Each data point is the average of two experiments.

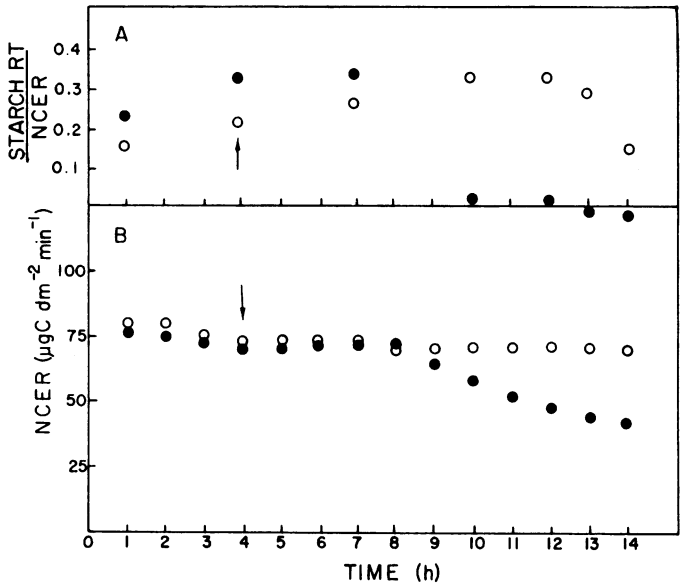

FIG. 3. Ratio of starch accumulation rate:NCE rate (A) and NCE rate (B) throughout a light period of glyphosate-treated (●) and control source leaves (○). Each point is the average of five experiments.

Table II. *Effect of Glyphosate on Stomatal Conductance in Sugar Beet Source Leaves*

Stomatal conductance was measured periodically before (1–3.5h) and after (3.5–7.5 and 7.5–9.5h) glyphosate treatment. Change in conductance was calculated from values taken at the start and end of an interval. Increased conductance is indicated by (+), decreased by (−). Stomatal conductance for the four groups observed during the initial 2.5-h period ranged from 3.0 to 4.3 mm s^{-1}. Times given are in hours after start of illumination.

Treatment	Change in Stomatal Conductance		
	1–3.5 h	3.5–7.5 h	7.5–9.5 h
	Δ *mm s*$^{-1}$		
Water (control)	+0.10	−0.20	+0.10
Surfactant	−0.20	−0.15	−0.60
Glyphosate	−0.20	−0.10	−3.1
Glyphosate + surfactant	−0.25	+0.10	−1.6

Table I. *Distribution of Radioactivity in Glyphosate-Treated and Untreated Sugar Beet Source Leaves following Steady State Labeling for 14 h*

At 4 h after the beginning of the light period, 17 mM glyphosate was applied to leaves. Values listed are the mean of data from two experiments followed by the relative size of the difference between the two values ([100 × absolute difference]/mean).

Fraction	^{14}C Content	
	Control	Glyphosate
	nCi cm^{-2}	
Insoluble (total)	370 (4.2)	213 (5.9)
Starch	307 (3.2)	154 (8.7)
Nonstarch	61 (9.1)	68 (8.2)
Soluble (total)	238 (5.0)	230 (1.8)
Water-soluble	175 (4.0)	172 (3.2)
Neutral	127 (4.4)	130 (7.5)
Organic acid	28 (13.0)	31 (17.6)
Amino acid	23 (15.0)	24 (11.3)
Chloroform-soluble	62 (6.7)	63 (6.4)
Total leaf	613 (4.7)	454 (6.2)

period, degradation of accumulated starch occurred in both glyphosate-treated and control leaves (Fig. 1). In treated leaves, degradation sometimes began before the end of the light period. Glyphosate lowered the amount of starch present at the end of the light period and, although starch was degraded, little or none was exported from the source leaf (Table IV). On the other hand, when NCE was maintained in glyphosate-treated leaves, carbon derived from starch was exported at night. The level of sucrose at night was slightly lower in the glyphosate-treated leaves.

Comparison of ^{14}C-content of different fractions during the night period revealed a decline of radioactivity in the soluble fraction of control leaves that was not observed in treated leaves (Fig. 2B). Sucrose level was similar in both treated and control leaves (data not shown). Shikimate accumulation in glyphosate-treated plants began between 0 and 5.5 h after treatment and continued day and night following glyphosate application (Fig. 6). In the 24 h following application, shikimate accumulated to 325 μg g^{-1} fresh weight, an 80-fold increase over the level found in control leaves.

DISCUSSION

Effects of Glyphosate on Allocation of Newly Fixed Carbon. Glyphosate inhibited allocation of newly fixed carbon to starch

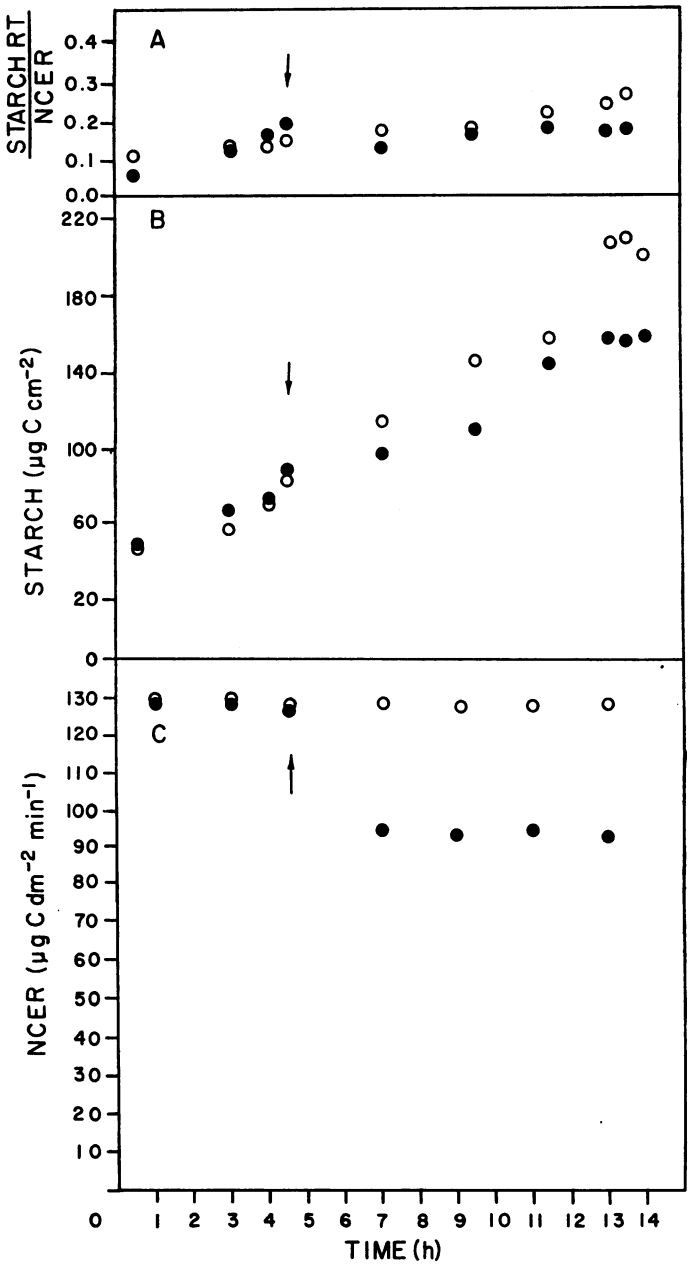

FIG. 4. Ratio of starch accumulation rate:NCE rate (A), starch accumulation (B), and NCE rate (C) throughout a light period for leaves under a lowered CO_2 level (●) and for control leaves (○). Each point is the average of three experiments.

Table III. *Decreases in Carbon Observed in Glyphosate-Treated Source Leaves Compared with Control Leaves at the End of the Light Period*

Sugar beet source leaves were treated with glyphosate 4 h after the start of the light period. Differences in cumulative carbon fixed and starch accumulation are from curves obtained by periodic measurements of NCE and starch. Values listed are the mean of data from two experiments followed by the relative difference between the two values (100 × absolute difference/mean).

Source	Control	Glyphosate	Difference
		$\mu g\ C\ cm^{-2}$	
Starch	147.2 (18.8)	99.5 (14.6)	47.6 (12.1)
Total carbon accumulated	622.1 (25.8)	566.3 (22.5)	57.1 (14.4)

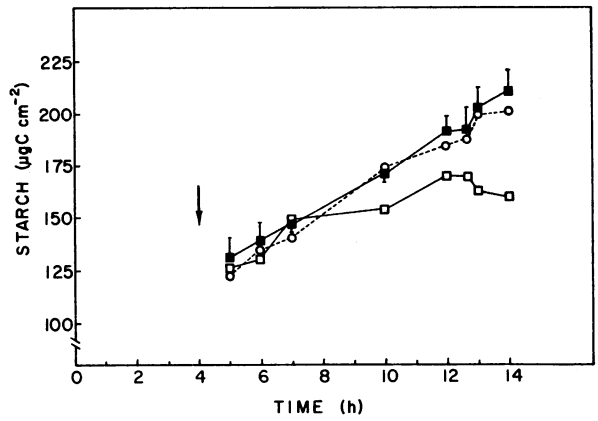

FIG. 5. Starch accumulation in sugar beet source leaves. One leaf on a plant served as a control (■). A second leaf was treated with glyphosate and the NCE rate was allowed to decline (□). Average values (○) were obtained from the third and fourth leaves which were treated with glyphosate and for which the NCE rate was maintained at the control level by elevating CO_2.

Table IV. *Carbon Exported From Source Leaves of Sugar Beet during the Day and Night*

Glyphosate was applied to source leaves 4 h after the light period began. For one set of treated leaves NCE was maintained at control level by raising the CO_2 level. During the 14-h light period, export was calculated from cumulative NCE minus leaf dry weight accumulation. Export at night was calculated from the change in leaf dry weight considering respiratory loss to be small by comparison. Values for each group is the mean of data for two plants followed by relative difference between the two values (100 × absolute difference/mean).

Treatment	Export	
	Day	Night
	$\mu g\ C\ cm^{-2}$	
Control	302 (54)	104 (20)
Glyphosate	328 (40)	13 (18)
Glyphosate + added CO_2	341 (29)	92 (14)

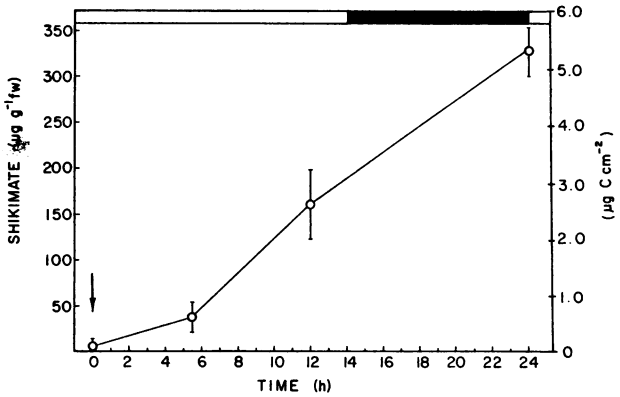

FIG. 6. Accumulation of shikimate in glyphosate-treated sugar beet source leaves. Glyphosate was applied at the arrow. The bars represent the range for valued from two leaves.

within several hours after application to sugar beet leaves (Fig. 1), a result previously reported by Gougler and Geiger (8). Steady state labeling with $^{14}CO_2$ throughout a light period revealed that there was less labeled carbon in treated than in control leaves (Table I). This result is consistent with the observed decrease in stomatal conductance and NCE. The difference in ^{14}C-content

of leaves was the same as the difference in amount of ^{14}C-starch in the two groups of leaves (Table I; Fig. 2A). The ^{14}C-content of all other fractions in treated and control leaves was similar, as was daytime export.

Shikimate accumulation increased its concentration dramatically over the level in control leaves (Fig. 6). Previous studies have shown that shikimate accumulates in tissues treated with glyphosate (1, 2). The carbon diverted to shikimate during 10 h after glyphosate application was approximately 2 μg C cm^{-2} (Fig. 6), only 4% the size of the approximately 50 μg C cm^{-2} difference in foliar starch accumulation (Table III). These data show that inhibition of starch accumulation was not the direct result of competition with shikimate for newly fixed carbon.

Relation of Decreased Starch Accumulation to Decreased NCE in Glyphosate-Treated Leaves. A connection between decreased NCE and the decline in starch storage is indicated by the similarity in size of the two (Fig. 2; Tables I and III). Full restoration of starch accumulation when NCE was maintained at the level found in control leaves (Fig. 5) supports the likelihood that decreased NCE caused decreased starch accumulation in treated leaves.

In the present study, the drop in the NCE rate in glyphosate-treated leaves (Fig. 3) coincided with a decrease in stomatal conductance (Table II). This pattern is in accord with previous studies that found that the major effects on photosynthesis result from mechanisms other than a direct effect on carbon pathways (4, 11, 13, 16, 18). Shaner and Lyon (16) noted that both NCE and stomatal conductance decreased in response to application of glyphosate. Brecke and Duke (4) observed a marked decrease in stomatal conductance within 1 h of applying 1 mM glyphosate. Inhibition of starch accumulation is caused, at least in part, by the glyphosate-induced reduction in stomatal conductance.

Glyphosate-Induced Change in Allocation of Carbon. Inhibition of NCE, by itself, is not able to account for the marked inhibition of starch accumulation following glyphosate application. A change in allocation of newly fixed carbon to starch and to sucrose also occurred. This additional effect of glyphosate can be seen by comparing the ratio of rates of NCE and starch storage in glyphosate-treated (Fig. 3A) and in control leaves under lowered CO_2 (Fig. 4A). In leaves treated with glyphosate, not only was NCE inhibited but the proportion of newly fixed carbon allocated to starch fell to zero while the proportion that went to sucrose increased. When NCE was decreased in control leaves by lowering CO_2 concentration, accumulation of starch continued to follow the usual pattern (6) and fell only to the extent that NCE decreased. As a consequence of glyphosate treatment, virtually no carbon accumulated in starch, while daytime export continued nearly unchanged. The small amount of carbon diverted to shikimate may have come at the expense of starch.

Although the amount of starch present in the glyphosate-treated leaves following the light period was considerably smaller, it still was degraded at night. However, export of carbon derived from the mobilization of starch was not detected during the night (Table IV). When the rate of starch accumulation was maintained at the control level by increasing NCE rate in glyphosate-treated leaves, export occurred at night as usual. This pattern implies that glyphosate did not inhibit the machinery involved in export directly but likely altered allocation of carbon to sucrose synthesis because the available store of starch was low. Gougler and Geiger (8) earlier demonstrated that glyphosate did not interfere with processes for phloem loading or export. During the night, shikimate continued to accumulate in treated leaves, possibly competing with sucrose synthesis.

Future Studies. Mechanisms responsible for glyphosate-induced inhibition of NCE and starch accumulation and for the change in allocation of carbon have yet to be identified. Decreased stomatal conductance, as well as effects on starch accumulation and allocation of carbon to starch and sucrose may result from the direct action of glyphosate or be mediated by shikimate. Altered carbon flow caused by competition for carbon or by changes in enzymes or regulatory metabolites also may be involved. The present study does not address these questions, which are the subject of our current research.

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