Plant Physiol. (1990) 94, 1219–1224
0032-0889/90/94/1219/06/$01.00/0

Received for publication June 4, 1987
Accepted February 2, 1989

Turnover of Dhurrin in Green Sorghum Seedlings

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ABSTRACT

The turnover of dhurrin in green seedlings of *Sorghum bicolor* (Linn) Moench var Redland × Greenleaf, Sudan 70 has been investigated using glyphosate and pulse-labeling studies with ^{14}C-tyrosine and [^{14}C]shikimic acid. The rate of dhurrin breakdown was 4.8 nanomoles per hour in the shoot and 1.4 nanomoles per hour in the root. The rate of dhurrin accumulation in the shoot of 4- to 5-day-old seedlings was high but decreased with age until at the peak period of dhurrin accumulation, the rates of dhurrin synthesis and breakdown were equal. Using a first order equation (an approximation) the rate of dhurrin synthesis (which equals accumulation plus breakdown rates) was 17.4 nanomoles per hour in the shoot and 4.1 nanomoles per hour in the root. In both tissues, the breakdown rate was between 27 and 34% of their synthetic capacity within the experimental period. Dhurrin synthesis in green sorghum seedlings occurred in both the light and dark photoperiods but was faster in the dark period. The result is discussed in relation to the possible metabolic roles of the turnover.

The first observation that there might be a turnover of cyanogenic glycosides in plants was made by Blumenthal *et al.* (6) when they administered [^{14}C]HCN as a gas to young seedlings of sorghum, barley, pea, flax, and red and white clover and found that the radioactive HCN was incorporated in remarkable yield (20–50%) into the amide carbon atom of asparagine. Abrol and Conn (1) and Abrol *et al.* (2) extended these studies by feeding ^{14}C-labeled amino acids to *Lotus* spp. and *Nandina domestica* and observed that radioactivity was also incorporated into the amide carbon atom of asparagine. Further studies showed that the radioactivity found in asparagine could only be derived from the HCN moiety of the cyanogenic glycosides formed from the labeled amino acids. These experiments showed that a B-glucosidase has limited access to the cyanogens in these plants, thus releasing a small quantity of HCN, which is metabolized (via β-cyanoalanine formation) to asparagine. That asparagine is labeled when radioactive precursors are fed to cassava was demonstrated by Nartey (13), while Bediako *et al.* (5) showed qualitative evidence of the turnover of linamarin in cassava leaves.

Bough and Gander (7) performed by far the most sophisticated experiments on the turnover of a cyanogenic glycoside in a plant system. They pulse-labeled etiolated *Sorghum vulgare* seedlings with [^{14}C]tyrosine and chased it with cold L-tyrosine and/or water. They found a 40% decrease in the specific activity of dhurrin in a 10-h period while the total dhurrin content of the seedlings remained constant. They calculated the turnover rate of dhurrin to be 50 nmol/h/shoot in seedlings containing 1.0 μmol dhurrin/shoot. This corresponds to a metabolic half-life of 10 h and a complete breakdown and synthesis of 1.0 μmol dhurrin/shoot in a 20-h period. This pioneering work, however, had three major disadvantages. (a) The experiments were carried out using etiolated seedlings, which would not represent what normally occurs in green sorghum seedlings. (b) The turnover rate was calculated over a 10-h span, which seemed too short a period to estimate dhurrin turnover in green sorghum seedlings. (c) An exorbitant amount and price of radioactive chemicals was consumed, especially when ^{14}C-incorporation into dhurrin was only 3% or less (7).

It has been demonstrated that dhurrin and its catabolic enzymes are compartmentalized in green sorghum seedlings. The vacuole of the epidermal cells is the site of glycoside storage (14, 15), whereas the catabolic enzymes are found in the mesophyll cells (17). As a result of this distribution of substrate and enzymes, it becomes important to ask whether the turnover of dhurrin occurs in green sorghum seedlings and, if so, at what rate? Glyphosate, as shown in Figure 1, is a known inhibitor of the shikimic acid pathway (16), which leads to the formation of the aromatic amino acids including tyrosine, which is the precursor of dhurrin (8). Glyphosate, supplemented with the aromatic amino acids, has been successfully used to inhibit dhurrin synthesis in 4-day-old sorghum seedlings without adversely affecting other physiological processes for the next 5 days (Adewusi, unpublished data). This procedure has been used as an inexpensive method to estimate the turnover of dhurrin in the shoot and root of green sorghum seedlings over a 96-h period. Radioactive tyrosine and shikimic acid have also been fed as precursors of dhurrin for comparison with the above-described glyphosate method.

MATERIALS AND METHODS

Chemicals

High purity glyphosate (free acid) was a generous gift of Dr. E. G. Jaworski (Monsanto Agricultural Chemical Company, St. Louis, MO). [U-^{14}C]Tyrosine (specific activity, 410 mCi/mM) was purchased from ICN, and D-[2,3,4,5(*n*)-^{14}C]shikimic acid (specific activity, 84 mCi/mM) was obtained from Radiochemical Centre, Amersham. The aromatic amino acids and other reagents were obtained from Aldrich Chemical Company.

Plant Materials

Seeds of *Sorghum bicolor* (Linn) Moench Sudan 70 var Redland × Greenleaf were obtained from Northrup King and

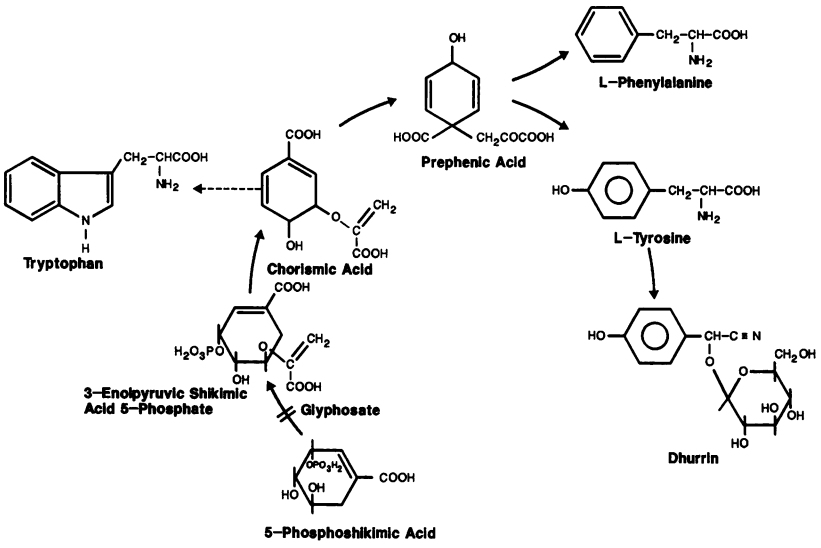

Figure 1. Site of glyphosate action on the shikimic acid pathway and biosynthesis of the aromatic amino acids (the primary products). The biosynthetic pathway for dhurrin shows the relationship between shikimic acid, tyrosine, and dhurrin. (Adapted from refs. 8, 9, and 16.)

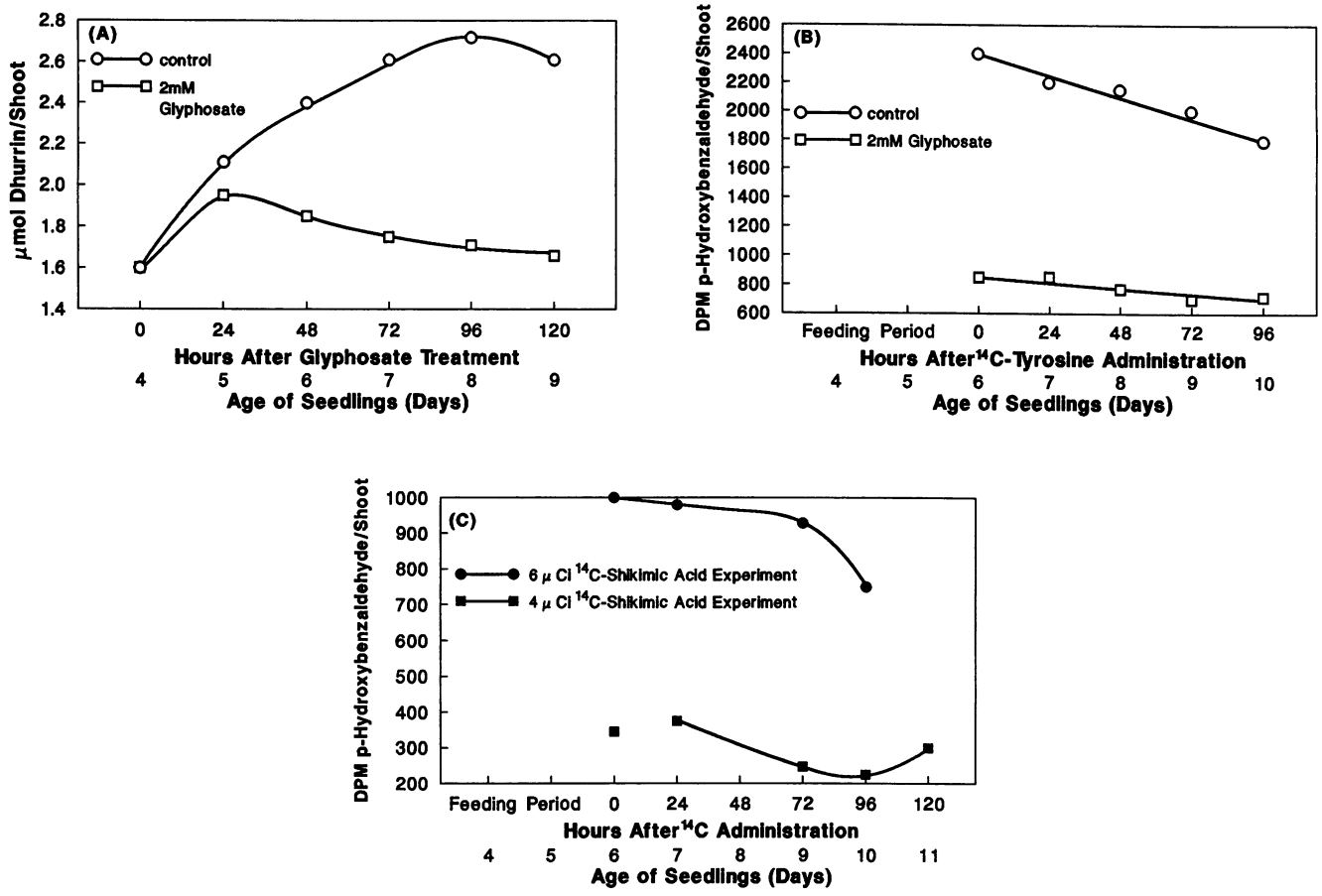

Figure 2. A, Effect of 2 mM glyphosate on dhurrin biosynthesis *in vivo* in shoots of green sorghum seedlings. Points, mean and SD of 20 seedlings analyzed in groups of two to four seedlings each and replicated five times. B, Loss of radioactivity from ^{14}C-labeled dhurrin in shoots of green sorghum seedlings fed 40 μCi of U-^{14}C-tyrosine in the presence or absence of 2 mM glyphosate. Points, average of 20 seedlings, replicated once. Growth conditions, administration of ^{14}C hydrolysis, and extraction of *p*-hydroxybenzaldehyde were as described in "Materials and Methods." Radioactivity was determined by liquid scintillation counting. C, Time course for breakdown of radioactive dhurrin in shoots of green sorghum seedlings fed 6 and 4 μCi of [^{14}C]shikimic acid. Points, average value of 20 shoots. All experimental procedures were as outlined for B.

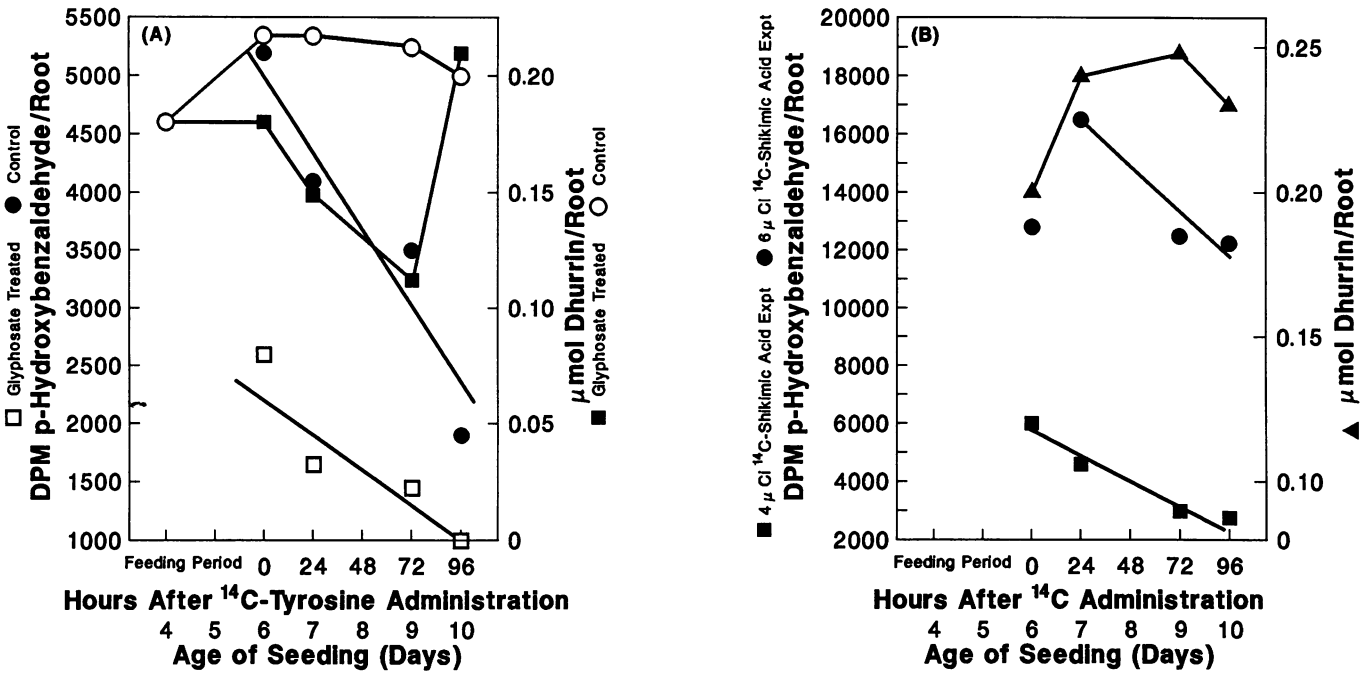

Figure 3. A, Time-course change in total and ^{14}C-labeled dhurrin in roots of green sorghum seedlings fed [U-^{14}C]tyrosine for 48 h in the presence or absence of 2 mM glyphosate. Roots of 20 seedlings were analyzed in quadruplicate and the average reported. All analytical procedures were as given for Figure 2B. B, Time-course change in total and radioactive dhurrin in roots of green sorghum seedlings fed 6 and 4 μCi of [^{14}C] shikimic acid for 48 h and monitored for 96 or 120 h. Points, average value of 20 seedlings. Procedures were those given in Figure 2B.

Company, Lubbock, TX. The seeds were soaked in aerated distilled water at room temperature for about 24 h. They were planted on water-saturated vermiculite in plastic trays and allowed to germinate in a growth chamber preset at 26°C light and 22°C dark temperature and 16:8 h light:dark photoperiod. All experiments including the feeding studies were carried out in the growth chamber.

Glyphosate Experiments

Glyphosate, neutralised with twice its concentration of NaOH, was supplemented with the aromatic amino acids and added to the vermiculite base of 4-d-old green sorghum seedlings such that the final concentration of glyphosate would be approximately 2 mM and the aromatic amino acids 1 mM each. Samples were taken and analyzed for total dhurrin content. The experiment was repeated five times, and the mean and SD are presented.

Total Dhurrin Content

This was estimated using the method of Gorz *et al.* (10).

U-^{14}C-Tyrosine Experiments

Four-day-old green sorghum seedlings were administered 40 μCi [U-^{14}C]tyrosine through the root in 5 ml containing 1 mM phenylalanine and 1 mM tryptophan with and without 2 mM glyphosate. After a 48-h feeding period, the roots were thoroughly washed with distilled water and the seedlings transferred to a solution containing 1 mM each of tyrosine, phenylalanine, and tryptophan alone or supplemented with 2 mM glyphosate. Samples were harvested immediately and at 24, 72, and 96 h. The roots were detached from the shoots and analyzed separately. The experiment was repeated once and average values are presented.

Hydrolysis, Extraction, and Analysis of Radioactive Dhurrin

Hydrolysis of radioactive dhurrin was by the method of Gorz *et al.* (10). The hydrolysate was reduced to 10 ml, and 2-mL aliquots were extracted 3 times with 2 mL of diethyl ether. Thin-layer chromatography of the ether extract was carried out on Bakerflex silica gel 1B flexible sheets in a benzene:ethyl acetate solution (5:1, v/v) for 3 to 4 h. The plates were sprayed with 0.2% solution of 2′,7′-dichlorofluorescein and viewed under UV light. The bands were also located using a Packard model 7201 radiochromatogram scanner. The radioactive bands were eluted with 1 mL of distilled water and counted in 10% aqueous scintillation fluid using a Beckman LS230.

D-[2,3,4,5(*n*)-^{14}C]Shikimic Acid Experiments

Four μCi ^{14}C-shikimic acid in 3 mL of cold 400 μM shikimic acid was fed to 100 4-d-old green sorghum seedlings through the root for 48 h. After the feeding period, the roots were washed thoroughly with distilled water and the seedlings were put into 400 μM cold shikimic acid. The roots were detached from the shoots and analyzed separately for radioactive dhurrin as indicated earlier at 0, 24, 72, 96, and 120 h after the

Table I. *Rate of Dhurrin (A) Breakdown, (B) Accumulation, and (C) Diurnal Variation in Shoots of Green Sorghum Seedlings*[a]

	Source	t_1	t_0	t	k	$t_{½}$ or D_t	Size of Dhurrin Pool	Turnover Accumulation Rate
				h			*μmol/shoot*	*nmol/h·shoot*
A								
	Fig. 2A							
	Glyphosate experiment	120	24	96	0.0016	433	2.7	3.1
	Fig. 2B							
	^{14}C-Tyrosine control experiment	96	0	96	0.0029	239	2.7	5.6
	^{14}C-Tyrosine + glyphosate experiment	96	0	96	0.0024	289	2.7	4.7
	Fig. 2C							
	6 μCi ^{14}C-shikimic acid experiment	96	0	96	0.0029	241	2.7	5.6
	Mean of all experiments				0.0026	279	2.7	4.8 ± 1.2
	Mean of ^{14}C-experiments				0.0027	256	2.7	5.3 ± 0.5
B								
	Fig. 2A							
	Control experiment	24	0	24	0.0110	63	1.6	25.4
		48	24	24	0.0056	124	2.1	16.9
		72	48	24	0.0036	208	2.4	11.5
		96	72	24	0.0016	433	2.6	6.0
C								
	Fig. 4							
	Photoperiod							
	Light	16	0	16	0.0085	81.5	1.51	18.5
	Dark	24	16	8	0.0206	33.6	1.73	51.5
	Light	40	24	16	0.0018	385.0	2.04	5.3
	Dark	48	40	8	0.0114	60.9	2.10	34.5
	Light	64	48	16	0.0013	515.6	2.30	4.5
	Dark	72	64	8	0.0102	67.9	2.35	34.6

[a] Equations of the first order kinetics:

$$ln \frac{n_0}{n} = kt \quad \text{(i)}$$

$$t_{½} \text{ or } D_t = \frac{0.693}{k} \quad \text{(ii)}$$

where n_0 = the size of dhurrin pool in the shoot at time zero (t_0); n = the size of dhurrin pool in the shoot at time t_1; t = the experimental period under consideration ($t_1 - t_0$); $t_{½}$ = half-life; D_t = doubling time, *i.e.* the period in which the dhurrin pool would be twice its size; k = rate constant.

Rate of dhurrin synthesis = Rate of accumulation + rate of breakdown

feeding period. In a repeat experiment, 6 μCi of ^{14}C-shikimic acid was fed to 80 4-d-old green sorghum seedlings for 48 h and analyzed at 0, 24, 72, and 96 h after the feeding period.

During both experiments, 400 μM cold shikimic acid was replenished such that the roots were fully immersed at all times.

Diurnal Variation of Dhurrin

This was estimated by analyzing for total dhurrin content of sorghum shoot at the beginning of both light and dark photoperiods. The experiment was repeated four times. In all experiments, total and/or radioactive dhurrin was expressed as its *p*-hydroxybenzaldehyde equivalent. Mean, SD, and regression coefficients were calculated using a Hewlett-Packard calculator.

RESULTS

Figure 2A shows that the total dhurrin content of control sorghum shoots increased by 1.1 μmol between d 4 and 9 (96 h), while that of glyphosate-treated samples decreased by 0.28 μmol between d 5 and 10 of growth (96 h).

Figure 2B shows that in the control shoots fed [^{14}C]tyrosine there was a 24% loss in dhurrin radioactivity and a 20% loss in glyphosate-treated samples over a 96-h period.

Figure 2C shows that when sorghum seedlings were fed 6 μCi of ^{14}C-shikimic acid, radioactivity in dhurrin remained virtually constant over the first 72 h before a decrease of 22% over the next 24 h. When fed 4 μCi of [^{14}C]shikimic acid, the radioactive content decreased by 40% over 72 h and finally increased to 320 dpm/shoot at 120 h. The late increase in radioactive dhurrin could have resulted from the proteolysis of reserve proteins coinciding with new growth (7).

Figure 3A shows that the radioactivity decreased by 64% in the root over 96 h while the total dhurrin content remained essentially constant at 0.22 μmol/root during that period. With glyphosate treated roots, radioactivity in dhurrin declined by 60% over 96 h while the total dhurrin content declined by 40% for 72 h and then increased dramatically to 0.21 μmol over the last 24 h. Since this coincided with the

Table II. *Rate of Dhurrin (A) Breakdown and (B) Accumulation in the Root of Green Sorghum Seedlings*
The reaction equation and other details are as given in Table I.

	Source	t_1	t_0	t	k	$t_{1/2}/D_t$	Size of Dhurrin Pool	Turnover/ Accumulation Rate
		h					*μmol/root*	*nmol/h·root*
A								
	Fig. 3A							
	^{14}C-Tyrosine experiment (control)	96	0	96	0.0105	65.8	0.22	1.7
	^{14}C-Tyrosine + glyphosate experiment	96	0	96	0.0096	72.1	0.22	1.5
	Dhurrin content in the presence of glyphosate	72	0	72	0.0068	101.2	0.22	1.1
	Fig. 3B							
	6 μCi ^{14}C-shikimic acid experiment	96	0	96	0.0096	72.0	0.24	1.7
	Mean				0.0085	75.3		1.4
B								
	Fig. 3B							
	Total dhurrin content	24	0	24	0.0076	91.2	0.20	2.2
	6 μCi ^{14}C-shikimic acid experiment	24	0	24	0.0108	64.2	0.20	3.1
	Average				0.0092	77.7	0.20	2.7

massive sprouting of secondary roots, the increase in the dhurrin content could represent dhurrin synthesis by these secondary roots.

Figure 3B shows that the total dhurrin content of the root increased during and after the feeding period while the radioactivity in dhurrin decreased by 24% over 72 h in the roots to which 6 μCi of ^{14}C-shikimic acid was fed. In the experiment involving 4 μCi of ^{14}C-shikimic acid, the radioactivity decreased by 60% over the 96 h experimental period.

When first order kinetics are applied to Figures 2 and 3, the half-life of dhurrin breakdown in the shoot, as estimated by dhurrin analysis in the presence of glyphosate, was found to be 433 h, while in the labeling studies, it was 256 ± 28 h. The breakdown rate estimated by glyphosate was 3.1 μmol/h/shoot, while estimation by ^{14}C-labeling was 5.3 ± 0.5 nmol/h·shoot (Table IA).

The data in Table IB show that the rate constant for dhurrin accumulation decreased by 50% every 24 h resulting in a variation in the rate of dhurrin accumulation from 25.4 to 6.0 nmol/h·shoot in the first and last 24 h periods of the experiment, respectively.

The half-life of dhurrin breakdown in the root of sorghum seedlings was 75 h (Table IIA), corresponding to a rate of breakdown of 1.4 nmol/h·root.

Table IIB shows that the doubling time of dhurrin accumulation in the root of green sorghum seedlings within the age of 6 to 7 days can be as high as 78 h, with the rate of accumulation being 2.7 nmol/h·root.

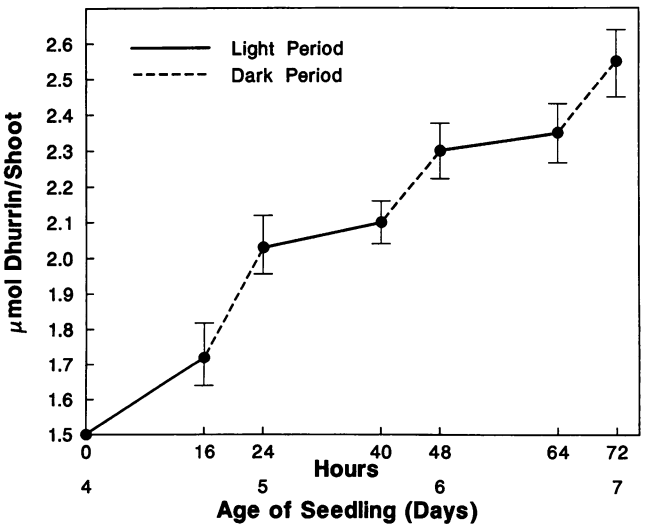

Figure 4. Diurnal variation of dhurrin synthesis/accumulation in shoots of green sorghum seedlings. Dhurrin was analyzed by the method of Gorz *et al.* (10). Points, mean value of four experiments; 25 shoots were analyzed for each point of the experiment. The experiment was repeated four times.

Diurnal Variation of Dhurrin Synthesis/Accumulation in Green Sorghum Seedlings

Figure 4 shows that the synthesis/accumulation of dhurrin occurred in both the light and dark periods of the day, but the rate in the dark was significantly higher ($P < 0.001$).

DISCUSSION

Glyphosate is a potent inhibitor of the shikimic acid pathway in buckwheat hypocotyls (3, 4, 16) and green sorghum seedlings (Adewusi, unpublished data) and should therefore be useful in dhurrin turnover studies. The rate of dhurrin breakdown estimated by this method was 3.1 nmol/h·shoot over the 96 h experimental period. This is low when compared with the breakdown rate of 5.3 ± 0.5 nmol/h·shoot obtained through radioactive labeling studies. This may be due to the fact that inhibition of the shikimate pathway by 2 mM glyphosate is not complete (only 90%), and second, 3 to 4% of the exogenous tyrosine could be converted to dhurrin (7 and SRA Adewusi, unpublished data). Supplementation of glyphosate solution by the aromatic amino acids including tyrosine was necessary to keep the seedlings in their physiological state for 5 days.

The rate of dhurrin breakdown in etiolated sorghum seedlings followed first order kinetics (7). The turnover of dhurrin obtained by radioactive labeling in this study also followed first order kinetics since the rate constant was the same (Table IA).

Our present results indicate that the turnover rate of dhurrin in green *S. bicolor* seedlings (4.8 nmol/h·shoot) is about 10% that of etiolated *Sorghum vulgare* seedlings (7). In addition, the half-life of dhurrin in green shoots is at least 20 times that observed in etiolated seedlings. The difference in the two results could be due to the fact that: (a) the earlier turnover rate (7) was calculated over a 10-h period when dhurrin breakdown was rapid; (b) metabolic pools of shikimate in green and etiolated seedlings might be different and thus account for rate differences; (c) compared with green seedlings, competition for tyrosine by several metabolic processes in etiolated seedlings would be minimal, thus providing abundant tyrosine to replace dhurrin already catabolized; and (d) there may also be varietal differences in the rate of dhurrin metabolism (SRA Adewusi, unpublished observations).

Dhurrin accumulation in the shoot and root did not follow first order kinetics. The rate constant obtained from Figure 2A (Table IB) suggested a second order reaction, but the plot of 1/C (concentration) against time did not give a straight line. The kinetics are complicated by being biphasic (Fig. 4). The first order equation used in the calculation can only be an approximation. From these approximations, the mean rate of dhurrin accumulation between days 4 and 9 was 12.6 nmol/h·shoot and 2.7 nmol/h in the root.

The rate of dhurrin synthesis (17.4 nmol/h·shoot) can be calculated by summation of the rates of accumulation (12.6) and breakdown (4.8). The rate of dhurrin breakdown therefore represented 27% of the synthetic capability of the shoot. In the root, the rate of dhurrin synthesis was 4.1 nmol/h·root, while the breakdown rate was 1.4 nmol/h/root, equivalent to 34% breakdown of the synthesized dhurrin in the root.

The results here reported tend to substantiate the hypothesis of Jones (11) that cyanogenic glycosides may serve both protective and primary metabolic functions; protective in the sense that sorghum seedlings accumulate more dhurrin than is catabolized with the result that the stored dhurrin could act as a deterrent to predators. That cyanogenic glycosides repel predators has been proved experimentally (11). The primary metabolic function of dhurrin in green sorghum seedlings could be through the provision of carbon atoms, for example in the formation of β-cyanoalanine and asparagine. Furthermore, Møller and Conn (12) have speculated that dhurrin may provide carbon atoms for ubiquinone biosynthesis on the basis of the *in vitro* oxidation of *p*-hydroxybenzaldehyde (a breakdown product of dhurrin) to *p*-hydroxybenzoic acid, a known precursor of ubiquinone in plants.

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