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EXOGENOUS L-TYROSINE METABOLISM AND DHURRIN TURNOVER IN SORGHUM SEEDLINGS*

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Abstract—It was shown that dhurrin (*p*-hydroxymandelonitrile-β-D-glucopyranoside) was actively metabolized by etiolated *Sorghum vulgare* seedlings. A turnover rate of about 0·05 μmole/hr was found in seedlings containing 1·0 μmole dhurrin/shoot. These studies showed a logarithmic decrease in the specific activity of ^{14}C labelled dhurrin when unlabelled L-tyrosine was administered to the seedlings. The increase in specific activity of dhurrin was shown to be a logarithmic function of time when seedlings were administered [2-^{14}C]-L-tyrosine. In addition, it was concluded that a major fraction of the dhurrin in the seedlings was derived from endogenous sources rather than from exogenously supplied L-tyrosine. Sorghum seedlings were shown to metabolize [1-^{14}C]-, [2-^{14}C]-, [3-^{14}C]-, and [U-^{14}C]-L-tyrosine to $^{14}CO_2$. Based on the time course of $^{14}CO_2$ released from L-tyrosine labelled in carbon atom 1, 2 or 3 as compared to uniformly labelled L-tyrosine, the data suggest that sorghum seedlings catabolized a portion of the aromatic ring carbon atoms to $^{14}CO_2$. The dhurrin present 50 hr after administering [2-^{14}C]-L-tyrosine represented only 3% of the ^{14}C found in the various extracts, residues and CO_2. A procedure for isolating dhurrin from perchloric acid extracts is described.

INTRODUCTION

CYANOGENIC glucosides are a class of compounds whose role in plant metabolism is not well understood. The cyanogenic glucoside in *Sorghum vulgare*, dhurrin (*p*-hydroxymandelonitrile-β-D-glucopyranoside), is derived from L-tyrosine[1–4] and 1·0–1·2 μmole/shoot is found in 7-day-old seedlings (see Results and Ref. 5). This quantity remains constant or decreases slightly during the first month of growth.[5]

Sorghum vulgare seedlings contain enzymes which hydrolyze the glucose moiety of dhurrin (β-glycosidase)[6] and dissimilate *p*-hydroxymandelonitrile to hydrogen cyanide which with cysteine forms β-cyano-L-alanine (β-cyanoalanine synthetase). The β-cyano-L-alanine is subsequently hydrolyzed yielding asparagine.[7,8] The quantitative importance of these enzymes in dhurrin metabolism is not known. Furthermore the rate of synthesis of dhurrin from L-tyrosine is not known. In addition the relative proportion of exogenously supplied L-tyrosine converted to dhurrin has not been determined.

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This investigation was undertaken:

(1) To determine if dhurrin was synthesized actively and degraded by etiolated seedlings or was a "dead end" metabolic product.
(2) To determine the quantity of exogenously supplied L-tyrosine converted to dhurrin/hr *in vivo* in each seedling, and
(3) To determine the relative quantities of ^{14}C from (1-^{14}C)-, (2-^{14}C)-, (3-^{14}C)-, and (U-^{14}C)-L-tyrosine metabolized to CO_2.

RESULTS

Catabolism of L-*Tyrosine to* CO_2 *and Distribution of* ^{14}C *in Sorghum Seedlings*

Figure 1 shows the time course of $^{14}CO_2$ evolution obtained after administering specifically labelled L-tyrosine to 30 etiolated seedlings growing aseptically in vermiculite in 500 ml wide mouth Erlenmeyer flasks as described in the Experimental. The values for total $^{14}CO_2$ evolved during 50 hr as percentages of the ^{14}C-tyrosine taken up during that period are: (1-^{14}C), 59%; (2-^{14}C), 24%; (3-^{14}C), 20%; and (U-^{14}C), 35%. In control experiments in which no seeds were planted negligible $^{14}CO_2$ was released.

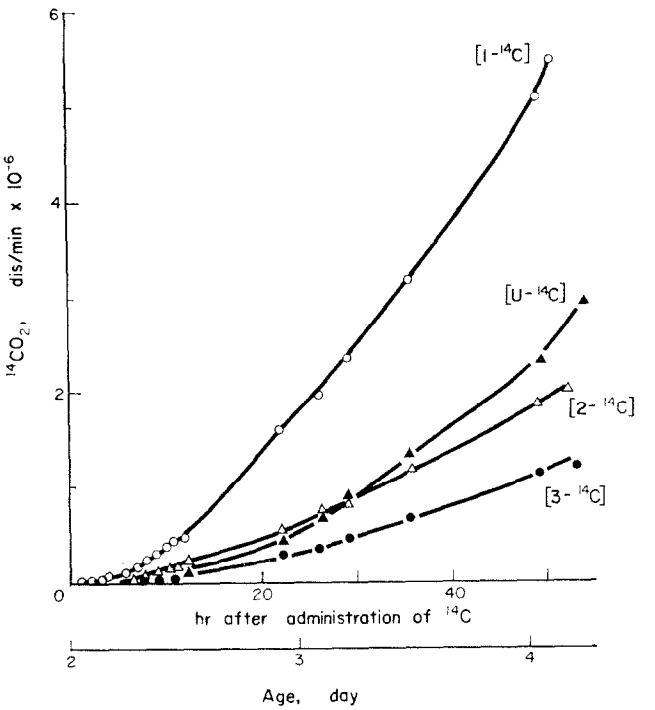

FIG. 1. TIME-COURSE OF $^{14}CO_2$ EVOLUTION FROM ETIOLATED SEEDLINGS ADMINISTERED A PARTICULAR ^{14}C-LABELLED L-TYROSINE.

Seedlings, 30 in a flask, were administered 5 μc of either [1-^{14}C] (–○–○–); [U-^{14}C] (–▲–▲–); [2-^{14}C] (–△–△–); or [3-^{14}C]-L-tyrosine (–●–●–) in 15 ml of a pH 6 solution containing 25 μmole unlabelled L-tyrosine. The $^{14}CO_2$ was collected in traps containing 400 ml 5 N NaOH. Duplicate 0·1 ml aliquots were removed at times corresponding to points of the time-course curves and each placed into 15 ml of scintillation cocktail.

Table 1 shows the percentage of radioactivity taken up as (U-^{14}C)-L-tyrosine found in various extracts of sorghum roots, seeds and shoots. This table shows that after 50 hr about 10% of the ^{14}C taken up was found in the shoots, 44% in the roots, 24% in the seeds and the remainder (22%) was released as CO_2. Approximately 50% of the ^{14}C in the acid extract of the shoots but only 1·4% of the ^{14}C in the acid extract of the roots was located in dhurrin (Table 2). In contrast, tyrosine contained approximately 38% of the ^{14}C in the roots; however, there was negligible tyrosine in the shoots. These tables show that dhurrin contained about 3 per cent of the total ^{14}C taken up, and tyrosine 5·6%.

TABLE 1. DISTRIBUTION OF ^{14}C DERIVED FROM [U-^{14}C]-L-TYROSINE IN SORGHUM SEEDLINGS

Extract*	Shoots (9·4 g) (%)	Roots (2·0 g) (%)	Seeds (10·15 g) (%)
0·3 M $HClO_4$, 3 vol., 4°	6·3	15·1	4·6
1 N KOH, 25 ml, × 3, 25°	3·2	19·2	8·2
2 N KOH, 25 ml, autoclaved 45 min	0·9	9·6	10·5
Residue after above extractions	0·04	0·2	0·3
% Of ^{14}C in a particular organ	10·44	44·1	23·6

* The extraction procedure was as follows: The excised tissue was extracted with 3 vol. of 0·3 M $HClO_4$ at 4°. This mixture was centrifuged at 18,000 *g* for 20 min. The supernatant fluid was designated as the 0·3 M $HClO_4$ extract. The residue was then extracted 3 times with 1 N KOH, followed by an additional extraction in 2 N KOH at 20 p.s.i. for 45 min at 127°. The material solubilized by each of these treatments was designated as 1 N and 2 N KOH extract, respectively. The radioactivity in each fraction was estimated as detailed in the Experimental.

TABLE 2. DISTRIBUTION OF ^{14}C IN SUBSTANCES ISOLATED FROM PERCHLORIC ACID EXTRACTS OF SHOOTS AND ROOTS

Substance isolated	% Of total ^{14}C in the 0·3 M $HClO_4$ extract	
	Shoots	Roots
Dhurrin*	48·7	1·4
Tyrosine†	—	37·7
Unidentified	2·4	3·6
p-Hydroxybenzaldehyde	0·8	—

* The ^{14}C in dhurrin amounted to only 3% of the total ^{14}C found in the various extracts, residues and CO_2.

† The ^{14}C in tyrosine amounted to only 5·6% of the total ^{14}C found in the various extracts, residues and CO_2.

Dhurrin Concentration and Specific Activity

The time course of the change in the quantity and specific activity of dhurrin obtained after administering (2-^{14}C)-L-tyrosine is shown in Fig. 2. The specific activity increased logarithmically after 10 hr. The highest specific activity shown represents a dilution factor (ratio of the specific activity of the tyrosine administered to the dhurrin isolated) of 42. Figure 2 also shows the quantity of dhurrin expressed both in terms of μmole/g fr. wt. of shoots and in μmole/shoot. The value shown here of 1·0 μmole/shoot after 7 days of growth in moist vermiculite compares closely with the values reported by Stafford of "about 1·2 μmole/shoot at 5–8 days of growth in solution culture".[5]

One week seemed to be the maximum period of time etiolated seedlings could be grown in the dark before the cell wall structure ceased to support the shoots in a vertical position. At 7 days the shoots were 10–12 cm long and the first leaf soon emerged.

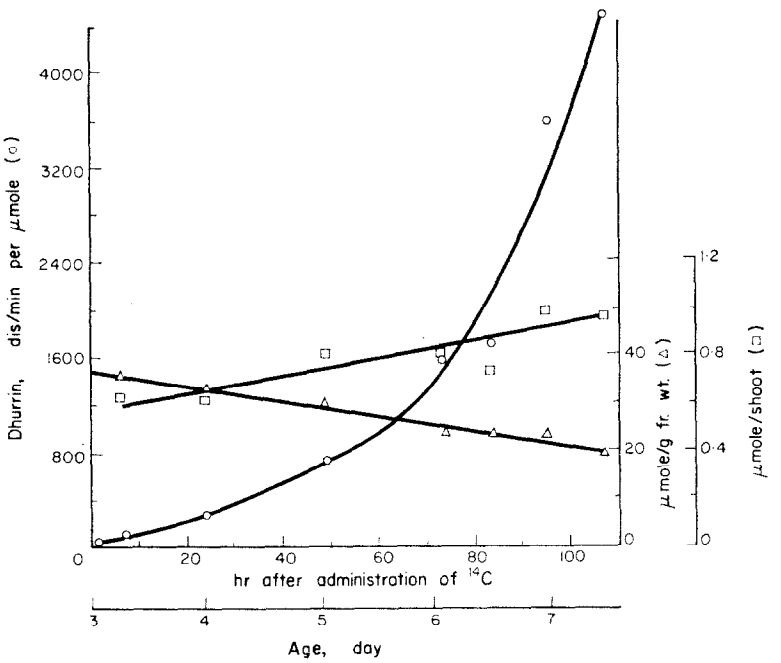

FIG. 2. TIME-COURSE OF THE CHANGE IN DHURRIN SPECIFIC ACTIVITY AND LEVEL IN ETIOLATED SHOOTS.

Etiolated seedlings, grown at 22 ± 1° in vermiculite, were administered 10 μc [2-^{14}C]-L-tyrosine in 50 ml 2·5 mM L-tyrosine. Samples were taken by excising 20-shoot samples. Samples were extracted in 7 ml cold 0·3 M $HClO_4$. The $HClO_4$ extract was adjusted to pH 3·5–4·0 with 10 N and 1 N KOH, cooled, filtered at 4°, and the residue washed with 8 ml cold water. The filtrate was evaporated *in vacuo* at 30° to dryness and stored at −15° until chromatographed. The residue after evaporation was dissolved in 2 ml water and applied to a 1·5 × 40 cm column of washed PVP. The column was eluted with water at 4° at a rate of approximately 10 ml/hr, collecting 5 ml fractions. Dhurrin elutes from such a PVP column giving a symmetrical profile between 135–165 ml. The concentration of dhurrin was determined based on E_{230} of 1·07 × 10^4. ^{14}C was determined by liquid scintillation counting.

Metabolic Turnover of Dhurrin Pool

Figure 3 shows the results of our first tyrosine "chase" experiment. Two-day-old etiolated seedlings rooted in vermiculite were administered 125 μmole (2-^{14}C)-L-tyrosine (spec. act. 1·68 × 10^5 dis/min μmole^{-1}) in 50 ml of H_2O. 48 hr later the ^{14}C-tyrosine was washed out of the vermiculite growth medium by repeated washing with 2·5 mM unlabeled L-tyrosine. This technique for "chasing" the ^{14}C-tyrosine was effective as shown by the immediate cessation of $^{14}CO_2$ evolution at 48 hr. This figure shows an unexplained 5-hr delay between adding unlabelled tyrosine and the anticipated onset in the decline in dhurrin specific activity. The logarithmic increase in specific activity for 5 hr following "chasing" with unlabelled tyrosine was similar to that shown in Fig. 2. These results show that the major dhurrin precursor pool was unaffected by tyrosine immediately after tyrosine was infiltrated into the plant.

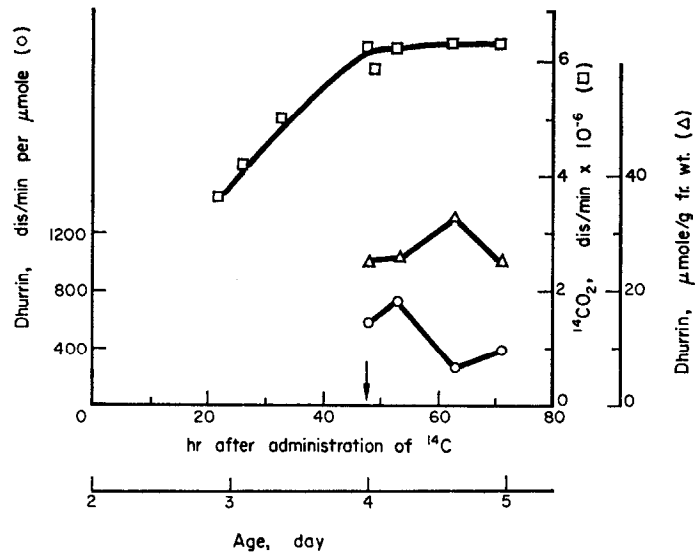

FIG. 3. TIME-COURSE OF $^{14}CO_2$ EVOLUTION AND DHURRIN SPECIFIC ACTIVITY AND LEVEL AS INFLUENCED BY 'CHASING' ^{14}C- WITH UNLABELLED-L-TYROSINE.
Conditions of growth and administration of ^{14}C were the same as for Fig. 2. The ^{14}C was "chased" at the time indicated by the arrow on the abscissa by rinsing the vermiculite-root mat with 2l. 2·5 mM L-tyrosine. The seedlings rooted in vermiculite were not dislodged during the rinsing because the dish in which they were growing was covered with a double layer of cheese cloth. After rinsing, the seedlings were administered 50 ml 2·5 mM unlabelled L-tyrosine. Other details as for Fig. 2.

Dhurrin specific activity decreased 64% during the 10 hr period of decline in specific activity. Furthermore it appeared that the sharp decline in specific activity was followed by a period in which there was little change in specific activity. Since these data were based on only two points on the specific activity curve, the experiment was repeated and the results are shown in Fig. 4.

The "chase" with unlabelled L-tyrosine was initiated 72 hr after administering the ^{14}C-L-tyrosine so that both the concentration and specific activity of dhurrin would be high. The specific activity of dhurrin decreased 40% in the 10 hr period (75–85 hr) following an initial increase as observed in the previous experiment. This decrease in specific activity

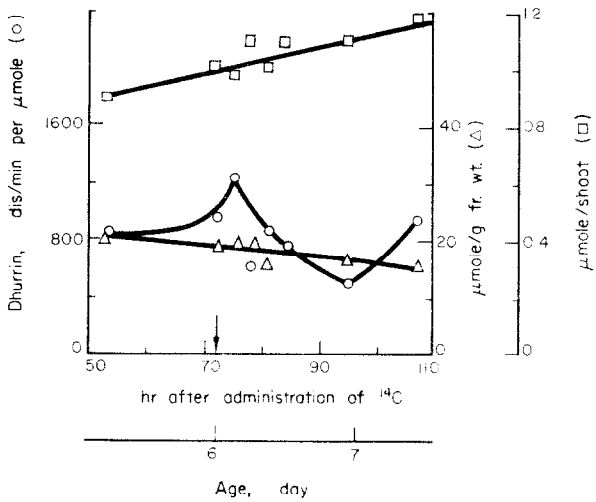

FIG. 4. TIME-COURSE OF DHURRIN SPECIFIC ACTIVITY AND LEVEL AS INFLUENCED BY "CHASING" ^{14}C- WITH UNLABELLED-L-TYROSINE.
Conditions and procedures were the same as for Fig. 3.

followed a negative logarithmic curve. The quantity of dhurrin remained nearly constant at 1·02–1·06 μmole/shoot. The increase in specific activity at 117 hr coincided with the emergence of the first leaf.

The 40% decrease in dhurrin specific activity in a 10-hr period described above was verified in a subsequent experiment in which the seedlings were germinated and grown on moist filter paper discs. The "chase" was initiated by washing the roots with sterile tap water and the roots of the seedlings placed in a 2·5 mM solution of unlabelled L-tyrosine. The results are shown in Fig. 5. The specific activity of dhurrin was greater in this experiment because the specific activity of ^{14}C-L-tyrosine was 5-fold greater than in the previous 'chase' experiments. The seedlings grew much more slowly on filter paper than when rooted in vermiculite. The 7-day-old seedlings grown on filter paper were only 2·0–2·5 cm tall as compared to 10–12 cm when grown in vermiculite. However, the decrease in dhurrin specific activity, starting at 6 hr after initiation of the "chase", appeared to be similar to that when the seedlings were grown in vermiculite.

Since "chasing" with unlabelled L-tyrosine resulted in a 40% decrease in dhurrin specific activity in a 10-hr period, it was of interest to determine if "chasing" with water would have a similar or different effect. The results of such an experiment (Fig. 6) show that "chasing" with water had the same effect on dhurrin specific activity (45% decrease in a 10-hr period) as did "chasing" with unlabelled L-tyrosine. Furthermore, the quantity of dhurrin was also unaffected by L-tyrosine (compare the data in Fig. 6 with that in Table 1). They show that both the quantity of dhurrin present/g fr. wt. and the amount/shoot are unaffected by supplying exogenous L-tyrosine.

DISCUSSION

The results of this investigation indicate that etiolated *Sorghum vulgare* seedlings catabolized both the aromatic ring and side chain carbon atoms of L-tyrosine to CO_2. Since data on degradation of aromatic amino acids in plants are meager, only a few comparisons

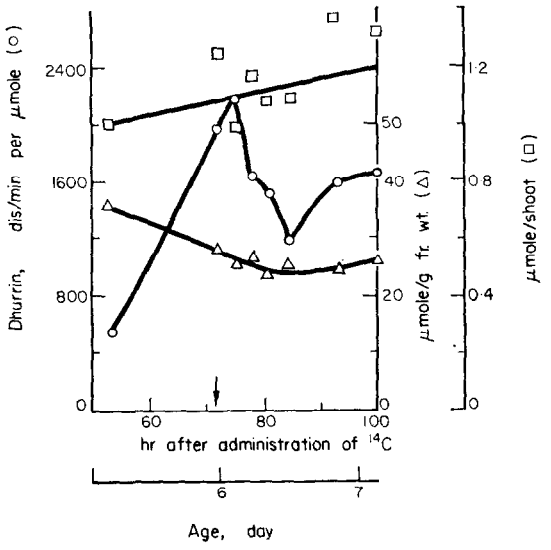

FIG. 5. TIME-COURSE OF DHURRIN SPECIFIC ACTIVITY AND LEVEL AS INFLUENCED BY "CHASING" ^{14}C- WITH UNLABELLED-L-TYROSINE.

Etiolated seedlings, grown at 22 ± 1° on filter paper, were administered 10 μc [2-^{14}C]-L-tyrosine in 10 ml 2·5 mM L-tyrosine. The ^{14}C was "chased" at the time indicated by the arrow on the abscissa by removing the seedlings, washing them thoroughly with sterile water and placing them in 10 ml 2·5 mM L-tyrosine on filter paper. Samples were taken by removing five seedlings and excising the shoots. For other details see Fig. 2.

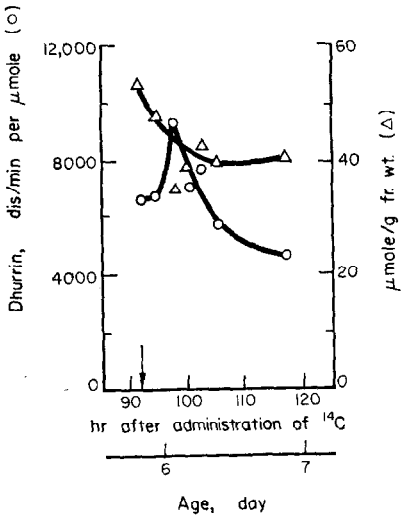

FIG. 6. TIME-COURSE OF DHURRIN SPECIFIC ACTIVITY AND LEVEL AS INFLUENCED BY "CHASING" ^{14}C-L-TYROSINE WITH WATER ONLY.

Conditions and procedures were the same as for Fig. 3 except that water was used to "chase" the ^{14}C instead of 2·5 mM L-tyrosine.

can be made.[9] Ibrahim *et al.*[10] observed that over 33% of the total radioactivity added as (U-^{14}C)-L-tyrosine could be accounted for in identifiable aliphatic compounds. They concluded that three or more carbon atoms of the tyrosine molecule were utilized and suggested that ring cleavage was a possibility. Evidence is also meager concerning the metabolic activity (turnover) of secondary plant metabolites in general and of cyanogenic glucosides in particular. Abrol and Conn[10] found that ^{14}C-valine and ^{14}C-isoleucine were incorporated by *Lotus* seedlings into cyanogenic glucosides which were degraded at a sufficient rate to incorporate radioactivity from the nitrile carbon atom of the cyanogenic glucosides into asparagine. Thus they concluded that cyanogenic glucosides were metabolically active rather than inert end products. The same conclusion was reached by Abrol *et al.*[11] concerning *p*-glucosylmandelonitrile, the cyanogenic glucoside found in *Nadina domestica* (Thumb). The nitrile moiety of the cyanogenic glucoside was metabolized and incorporated into the amide carbon atom of asparagine.

The extent of degradation of (U-^{14}C)-L-tyrosine as compared to (2-^{14}C)- and (3-^{14}C)-L-tyrosine suggests that the aromatic ring was cleaved and at least partially degraded to CO_2. However, over 50 per cent of the tyrosine initially decarboxylated was not further degraded as shown by the extent of degradation of (1-^{14}C)- and (2-^{14}C)-L-tyrosine.

Although enough (U-^{14}C)-L-tyrosine was taken up by the seedlings to account for 24 per cent of the dhurrin synthesized actually only 1·45 per cent of the ^{12}C tyrosine taken up was found in dhurrin at any one time. Table 1 showed that a large fraction of the ^{14}C remained in the roots and that 5·6 per cent of the tyrosine taken up was located in tyrosine in the roots. In contrast, the shoots contained very little tyrosine and the ^{14}C was located primarily in dhurrin.

Data presented here and our unpublished experiments strongly suggest that exogenous L-tyrosine constitutes only a small fraction (invariably less than 3%) of the total precursor in dhurrin biosynthesis. Therefore, it is not possible to ascertain whether L-tyrosine is an obligatory intermediate in dhurrin biosynthesis or if other biosynthetic pathways exist. Table 2 again gives evidence to this point. Tyrosine in the roots has a specific activity that is 52 times greater than dhurrin isolated from the shoots. Therefore, the majority of the dhurrin must be derived from endogenous precursors. It seems unlikely that CO_2 fixation would be of major importance in etiolated plants metabolizing in the dark.

The main objective of this study was to determine whether or not dhurrin is a "dead end" product of metabolism. If it is a "dead end" product the seedlings would synthesize and accumulate a genetically determined amount of dhurrin, in this case about 1 μmole/shoot, and additional dhurrin synthesis would stop. If this were true one should observe little or no decrease in the specific activity of dhurrin by "chasing" with unlabelled L-tyrosine or water. The results of this investigation showed that "chasing" ^{14}C-L-tyrosine with either unlabelled L-tyrosine or water resulted in about a 40% decrease in the specific activity of dhurrin in a 10-hr period while the quantity of dhurrin remained nearly constant at 1 μmole/shoot. The data from several experiments, summarized in Table 3, were obtained by calculating the rate of change of specific activity of dhurrin at the indicated times from Figs. 2–6. The data when plotted on semilogarithmic graph paper gave straight lines both for the increase in dhurrin specific activity as well as its decrease in specific activity following

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TABLE 3. METABOLIC TURNOVER OF DHURRIN

t_0 (hr)	k (hr^{-1})	Rate of change of spec. act. at t		Half-life at t (hr)	Turnover time of pool (hr)	Size of pool (μmole/shoot)	Turnover rate (μmole/hr)
		t (hr)	DPM/mole/hr				
0	0·035	72	520	13·6	—	—	—
0	0·079	96	855·0	6·4	—	—	—
72	−0·046	72	−64·4	10·9	21·75	1·0	0·046
72	−0·059	72	−145·8	8·5	16·96	1·1	0·065
96	−0·040	96	−384·0	12·5	—	—	—

the chase. This table shows that the increase in specific activity is of the same order of magnitude as the rate of decrease in specific activity following the addition of unlabelled L-tyrosine. The decrease in specific activity cannot result from an exchange reaction of the nitrile group; otherwise, the data of Koukol *et al.*[13] would have shown that the bond between the α- and β-carbon atoms was severed when L-tyrosine was converted to dhurrin. Therefore, assuming that the dhurrin synthesized from its endogenous precursors goes into the same pool as that synthesized from the exogenous L-tyrosine, the rate of dhurrin synthesis and degradation appears to be approximately constant at about 0·5 μmole/10 hr under the conditions of these experiments. From this we conclude that dhurrin is not an end-product of L-tyrosine metabolism.

These experiments provide no evidence on the fate of the atoms of dhurrin. Perhaps dhurrin serves as a limited precursor which can be utilized in asparagine biosynthesis,[14] lignin biosynthesis,[15] or alternatively it might be degraded for the purpose of yielding energy and the synthesis of other cellular constituents as Waller and Lee[16] have shown when castor leaves metabolized the α-pyridone ring of ricinine.

It is of interest that the increase in the specific activity of dhurrin at 93 hr shown in Fig. 5 coincides with the emergence of the first leaf as was observed in a previous experiment (Fig. 4). This late increase could result from the proteolysis of reserve proteins coinciding with the emergence of the first leaf. The reserve proteins could have acquired radioactivity from ^{14}C-L-tyrosine administered to the seedling in the early phase of the experiment.

EXPERIMENTAL

Reagents

The [1-^{14}C]-, [2-^{14}C]-, and [U-^{14}C]-L-tyrosine were purchased from Calbiochem. [3-^{14}C]-DL-Tyrosine was purchased from New England Nuclear Corp. D-Amino acid oxidase and catalase were purchased from Worthington Biochemical Corp. All other chemicals used were reagent grade.

Enzymatic Conversion of DL- *to* L-*Tyrosine*

[3-^{14}C]-DL-tyrosine was converted to [3-^{14}C]-L-tyrosine enzymatically using D-amino acid oxidase. The incubation mixture contained 5·3 mg (200 μc) [3-^{14}C]-DL-tyrosine, 0·5 ml D-amino acid oxidase (1·12 units), 0·1 ml 3 mM FAD, and 9·4 ml 0·1 M pH 7·2 phosphate buffer. The mixture was incubated at 37° for

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3 hr. Catalase (4750 units) in 2 ml 0·1 M pH 7·2 phosphate buffer was added and allowed to incubate 30 min to decompose H_2O_2. The reaction mixture was acidified with 1 ml conc. HCl, then continuously extracted with ether overnight. The aqueous phase after ether extraction was heated at 100° for 5 min then filtered to remove the protein precipitate. The filtrate was concentrated *in vacuo* at 35° to 5 ml applied to a 1·2 × 85 cm column of Sephadex G-10 (100 ml vol.). The column was eluted with water and 5 ml fractions were collected. Tyrosine eluted between 115 and 135 ml. About 100 μc of ^{14}C-tyrosine was collected from the column. There were 200 μc in the original DL mixture and 99·9 μc were isolated after treatment with D-amino acid oxidase. The resolution was assumed to be complete and that 99·9 μc of product was [3-^{14}C]-L-tyrosine.

Sterilization, Planting and Growth

Seeds of *Sorghum vulgare* var. Rox Orange were a gift of Northrup King and Company. Seeds were sterilized by soaking in a 1% solution of sodium hypochlorite for 30 min followed by rinsing with sterile tap water. The seeds were planted in vermiculite which had been wetted with tap water and sterilized by autoclaving for 20 min.

The planting operation was done aseptically in a cardboard glove box, a gift of Will Scientific, Inc. In one experiment, noted later, the seeds were germinated and grown on moist filter paper discs. The seedlings were germinated and grown in darkness where the temperature was controlled at 22 ± 1°, unless otherwise noted. All operations which involved manipulations on the seedlings or support medium were performed in a dim green light.

Catabolism of L-*Tyrosine to* CO_2

Sterilized seeds were planted on 1 cm sterile moist vermiculite in four 500-ml-wide-mouth Erlenmeyer flasks modified to have one side arm at 2 cm height and another at 6 cm height. The seeds were covered with 0·5 cm sterile moist vermiculite and allowed to germinate in the dark at 27°. Two days after planting the number of seedlings/flask was aseptically decreased to 20. To each flask was then added 5 μc of either [1-^{14}C]-, [2-^{14}C]-, [3-^{14}C]-, or [U-^{14}C]-L-tyrosine in 15 ml of a pH 6 solution containing 25 μmole unlabelled L-tyrosine. The seedlings were grown in the dark at 23°. Air was bubbled through 2 water reservoirs then across the seedlings. The CO_2 was collected in traps containing 400 ml 5 N NaOH. Secondary CO_2 traps were also used but contained no ^{14}C at the end of the experiment. The $^{14}CO_2$ was periodically sampled by removing duplicate 0·1 ml aliquots from the primary CO_2 trap. These were placed into 15 ml of a scintillation cocktail containing toluene–methylcellosolve–absolute ethanol (110:88:13, v/v/v), and 2,5-diphenyloxazole (5 g/l. cocktail). The vials were cooled, then counted for 4 min at 0° in a Nuclear Chicago 720 series scintillation spectrometer. At the conclusion of the experiment the uptake of ^{14}C was determined for each flask by extracting the vermilculite medium with 500 ml 0·2 N HCl. Uptake was calculated as the difference between total ^{14}C administered and ^{14}C left at the end of the experiment. The uptake values expressed as percentages were: [1-^{14}C], 84%; [2-^{14}C], 75%; [3-^{14}C], 56%; and [U-^{14}C], 77%.

Dhurrin Specific Activity, Level and "Chase" Experiments

Sterilized seeds (10 g) were planted on 3 cm sterilized wet vermiculite in a 4 × 8 cm cylindrical crystallization dish and covered with 1 cm sterilized wet vermiculite. The dish was covered with a double layer of cheese cloth which was secured to the dish by rubber bands. At either 2 or 3 days after planting, depending on the germination rate, 10 μc of [2-^{14}C]-L-tyrosine in 50 ml 2·5 mM L-tyrosine in sterile tap water was added to the vermiculite. The dish was placed in a Desaga Controlled Atmosphere Developing tank which was wrapped in black cloth. Air was drawn through a drying tube packed with glass wool, into the tank (growth chamber), across the seedlings, then into a CO_2 trap containing 2l. 2 N NaOH followed by a secondary trap containing 500 ml 1 N NaOH. The secondary trap was connected to a water aspirator.

At the appropriate time in the case of the "chase" experiments the ^{14}C was washed out of the vermiculite with either 2 l. of 2·5 mM L-tyrosine (unlabelled) or sterilized tap water. The seedlings grew up through the cheese cloth cover which also held the vermiculite-root mat in place. Thus the dish could be filled and emptied repeatedly in order to wash out the ^{14}C. After washing out the ^{14}C 50 ml of the appropriate solution was added to the vermiculite to complete the 2·5 mM L-tyrosine of water "chase" and the dish returned to the growth chamber.

In one "chase" experiment the seeds were planted on moistened, sterilized discs of Whatman No. 1 filter paper in a 4 × 8 cm crystallization dish. The seedlings were administered 10 μc [2-^{14}C]-L-tyrosine in 10 ml 2·5 mM L-tyrosine. The ^{14}C was "chased" by removing the seedlings, washing them thoroughly, and placing them in a 2·5 mM L-tyrosine solution on filter paper in another dish.

Samples were harvested (usually 20 shoots) at the appropriate times by excision. The dish and remaining seedlings were promptly returned to the growth chamber. All operations which involved removing the seedlings from the growth chamber were done in a dim green light. The excised shoots were weighed and extracted with 7 ml 0·3 M $HClO_4$ at 4° by grinding with a mortar and pestle using sand as an abrasive. The extract was adjusted to pH 3·5–4 using KOH, cooled, filtered at 4°, and the residue washed with 8 ml

cold water. The filtrate and washings were concentrated *in vacuo* at 30° to dryness then stored at −15° until chromatographed on a column of PVP.[17–19]

PVP was boiled 10 min in 10% HCl three times, washed to neutrality with distilled water, followed by five washings with deionized distilled water. PVP washed by this procedure was chloride free and did not decolorize a ferricyanide solution.[18] For chromatography, a sample was removed from the freezer, dissolved in 2 ml water and applied to a 1·5 × 40 cm column of PVP at 4°. The column was eluted with water and 5 ml fractions were collected at a rate of approximately 10 ml/hr. Dhurrin eluted from such a PVP column between 135–165 ml. The u.v. spectrum of dhurrin so isolated was identical to that reported for crystalline dhurrin.[6] The dhurrin concentration was determined using the molar extinction coefficient of $1{\cdot}07 \times 10^4$ at 230 nm in acid or neutral solution.[6]

The radioactivity in dhurrin was determined by pooling the appropriate fractions, adjusting the vol. to 50 ml, and counting triplicate 1·0 ml aliquots in 20 ml of the scintillation cocktail already described. The efficiency of counting was 60–65%.

In one experiment [U-^{14}C]-L-tyrosine was fed to 2-day-old etiolated seedlings. After 50 hr the shoots, roots, and seeds were harvested separately and washed. The 0·3 M $HClO_4$ extract of the shoots was chromatographed on PVP and dhurrin isolated as already described. The 0·3 M $HClO_4$ extract of the roots was adjusted to pH 5 with KOH, cooled, filtered at 4°, and applied to a 1·5 × 40 cm column of Dowex-1-Acetate. The column was eluted with a logarithmic gradient of acetic acid generated by adding dropwise 2 N acetic acid to a mixing flask containing 362 ml H_2O, initially. The elution volume of tyrosine from this column was 50–60 ml. The concentration of tyrosine was calculated using its extinction coefficient of $1{\cdot}34 \times 10^3$ at 274·5 nm.

[17] W. D. Loomis and J. Battaile, *Phytochem.* **5**, 423 (1966).
[18] R. A. Anderson and J. A. Sowers, *Phytochem.* **7**, 293 (1968).
[19] W. Steck, *Phytochem.* **7**, 1711 (1968).