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EVIDENCE FOR TWO PHENOLIC GLUCOSIDES DERIVED FROM L-TYROSINE IN SORGHUM SEEDLINGS*

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Abstract—Evidence has been presented for the presence of a phenolic substance which has not been previously reported in sorghum seedlings. The substance is similar to *p*-hydroxymandelonitrile-β-glucose in that it is derived from D-glucose and L-tyrosine. However, glucose is not released when the material is treated with β-glucosidase although hydrolysis with 1 N H_2SO_4 for 15 min at 100° C releases glucose and an aromatic compound which reduces silver ions under alkaline conditions. The phenolic material is also different from *p*-hydroxymandelonitrile-β-glucose in that it reacts with acidic 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone. The maximum absorption of the 2,4-dinitrophenylhydrazone is at 370 mμ. When seedlings metabolized DL-tyrosine-2-^{14}C the hydrazone isolated contained ^{14}C. Furthermore the relative specific activity of the 2,4-dinitrophenylhydrazone derived from this phenolic substance was approximately the same when the seedlings metabolized DL-tyrosine-2-^{14}C, DL-tyrosine-3-^{14}C, L-tyrosine-U-^{14}C or D-glucose-U-^{14}C. It is suggested the substance may be a precursor of *p*-hydroxymandelonitrile-β-glucose.

INTRODUCTION

PREVIOUS investigations from this laboratory[1,2] and in Conn's laboratory[3,4] have shown that L-tyrosine is an excellent precursor of the cyanogenic glucoside *p*-hydroxymandelonitrile-β-glucose (dhurrin) found in *Sorghum vulgare*. These investigations[2] have demonstrated that the α-carbon of tyrosine appeared as the nitrile carbon of the glucoside, and that no ^{14}C from carboxyl labelled DL-tyrosine was incorporated into the glucoside. Koukol *et al.*[4] presented evidence showing that the bond between carbon 2 and carbon 3 of tyrosine remained intact during the biogenesis of the cyanogenic glucoside.

This paper reports evidence for a new phenolic glucoside derived from tyrosine and glucose, and it is proposed that it may be a precursor of *p*-hydroxymandelonitrile-β-glucose.

RESULTS

Figure 1 is a line drawing showing the distribution of radioactivity on chromatograms of alcoholic extracts of sorghum seedlings which had metabolized either DL-tyrosine-2-^{14}C or D-glucose-^{14}C for two days. Previously it has been reported[2] that *p*-hydroxymandelonitrile-β-glucose migrates in the R_f 0·8 region of the chromatogram. The presence of a double peak in this region of the chromatogram may be due to the presence of two closely related substances, both derived from L-tyrosine and D-glucose, which migrate in this solvent at a similar

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[1] J. E. GANDER, *Plant Physiol.* **35**, 767 (1960).
[2] J. E. GANDER, *J. Biol. Chem.* **237**, 3229 (1962).
[3] T. AKAZAWA, P. MILJANICH and E. E. CONN, *Plant Physiol.* **35**, 535 (1960).
[4] J. KOUKOL, P. MILJANICH and E. E. CONN, *J. Biol. Chem.* **237**, 3223 (1962).

rate, or alternatively, they may represent tautomeric forms of the glucoside. The compounds from the glucoside area were found to react with 2,4-dinitrophenylhydrazine in 2 N HCl. Accordingly they were eluted from the paper with 80% ethanol, and after concentrating were treated with the acidic reagent either before (A) or after (B) a preliminary hydrolysis with β-glucosidase (see Methods).

Table 1 shows the specific activity of HCN and the 2,4-dinitrophenylhydrazone isolated by procedure B in the course of an experiment in which sorghum seedlings metabolized either

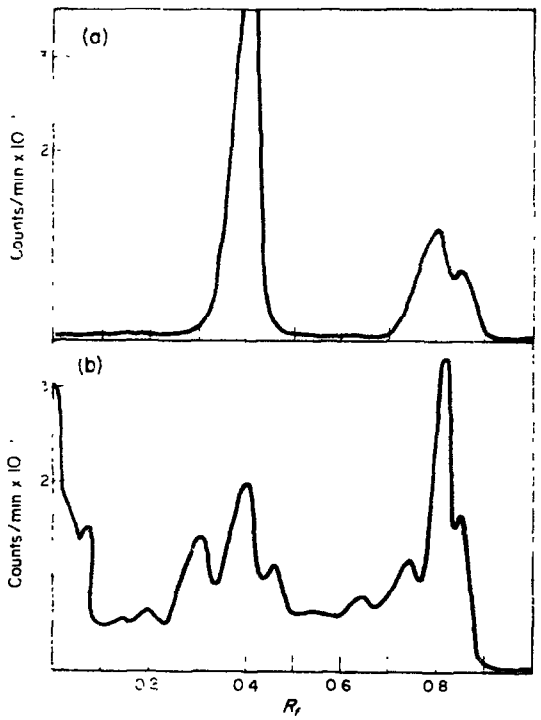

FIG. 1. The roots of the ten 3-day-old sorghum seedlings were placed in 15 ml of a solution containing 10 μmoles of L-tyrosine and either (*a*) 5 μc of DL-tyrosine-2-^{14}C or (*b*) 10 μc of D-glucose-^{14}C. The solution was aerated in the light for 40 h. The shoots were macerated in boiling 80% ethanol, the extract was evaporated to dryness and the residue taken up in a small volume of H_2O, deposited in strips upon Whatman 3 MM paper and chromatographed in butanol:pyridine:H_2O (6:4:3). After chromatography the chromatogram was dried and radioactivity estimated continuously with a 4π chromatogram scanner with a 0.15 cm window.

DL-tyrosine-2-^{14}C, DL-tyrosine-3-^{14}C or a 1:1 mixture of both. This table shows first that a 2,4-dinitrophenylhydrazone containing radioactivity can be isolated when the seedlings metabolize DL-tyrosine-2-^{14}C, and secondly that the specific activity of the 2,4-dinitrophenylhydrazone isolated from seedlings metabolizing DL-tyrosine-3-^{14}C was greater than the specific activity of the nitrile carbon when the seedlings metabolized DL-tyrosine-2-^{14}C. However, it must be assumed that the extinction coefficients for the two compounds are the same. These results are incompatible with the assumption that all the radioactive material eluted from the glucoside area of the chromatogram was derived only from *p*-hydroxymandelonitrile-β-glucose.

TABLE 1. INCORPORATION OF ^{14}C FROM DL-TYROSINE-2-^{14}C AND DL-TYROSINE-3-^{14}C INTO HCN AND ALSO INTO A 2,4-DINITROPHENYLHYDRAZONE ISOLATED FROM THE PHENOLIC GLUCOSIDE

Labelled compound metabolized	Initial specific activity (counts/min per μmole)	Specific activity	
		2,4-dinitrophenyl-hydrazone (counts/min per μmole)	HCN (counts/min per μmole)
DL-Tyrosine-2-^{14}C	65,000	690	3520
DL-Tyrosine-3-^{14}C	76,000	8940	<160
DL-Tyrosine-2-^{14}C + DL-Tyrosine-3-^{14}C	70,000	5940	1920

The seedlings were treated as described in Fig. 1. In the experiment containing both DL-tyrosine-2-^{14}C and DL-tyrosine-3-^{14}C, 2·5 μc of each of the labelled compounds were used. In all other experiments 5 μc of labelled material were used. The glycoside portion of the chromatogram was eluted with 80% ethanol at 30°, the solvent removed by flash evaporation and the residue dissolved in 2 ml of H_2O. Two mg of β-glucosidase were added to the sample and the specific activity of HCN was measured as previously described.[2] The specific activity of the 2,4-dinitrophenylhydrazones dissolved in absolute ethanol was measured after the addition of an equal volume of 2 N HCl saturated with 2,4-dinitrophenylhydrazine to the solution containing the glucoside(s), followed by precipitation and washing of the hydrazone in H_2O. An extinction coefficient for the 2,4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde of $3·8 \times 10^4$ was used.

Table 2 shows the results of an experiment in which the glucoside isolated from seedlings allowed to metabolize either DL-tyrosine-2-^{14}C, DL-tyrosine-3-^{14}C or L-tyrosine-U-^{14}C was treated with 2,4-dinitrophenylhydrazine either without prior treatment with β-glucosidase (procedure A) or after treatment with β-glucosidase (procedure B). A radioactive 2,4-dinitrophenylhydrazone was isolated by either treatment, but the specific activity of the 2,4-dinitrophenylhydrazone isolated from the glucoside obtained when the seedlings metabolized

TABLE 2. INCORPORATION OF ^{14}C FROM TYROSINE ^{14}C INTO THE 2,4-DINITROPHENYLHYDRAZONE(S) ISOLATED FROM PHENOLIC GLUCOSIDE(S)

Labelled compound	Experiment no.	β-glucosidase treatment	Total μmoles of hydrazone	Specific activity of hydrazone(s) (counts/min per μmole)	Dilution of specific activity (−/+)
DL-Tyrosine-2-^{14}C	1	−	0·571	1890	5·7
		+	5·49	330	
	2	−	0·331	4295	
		+	7·21	358	11·9
DL-Tyrosine-3-^{14}C	1	−	0·193	5795	
		+	6·23	3360	1·7
	2	−	0·432	3270	
		+	6·25	1550	2·1
L-Tyrosine-U-^{14}C	3	−	0·41	8100	
		+	5·8	4360	1·9

The specific activity of the 2,4-dinitrophenylhydrazones was obtained by procedures A and B as described in Methods.

Dilution of specific activity (last column) refers to the ratio of relative specific activities obtained for hydrazones isolated prior to β-glucosidase treatment divided by that obtained after β-glucosidase treatment. The extinction coefficient for the 2,4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde was used in both instances.

DL-tyrosine-2-^{14}C was approximately 5–10-fold higher than that found after treatment with β-glucosidase. Similar experiments with DL-tyrosine-3-^{14}C or L-tyrosine-U-^{14}C showed only a two-fold difference in specific activity. The smaller total number of counts in the hydrazone isolated from the glucoside derived from DL-tyrosine-2-^{14}C that had not been treated with β-glucosidase probably reflects a larger proportional loss of hydrazone during precipitation and washing the approximately 0·4 μmoles of hydrazone as compared to a similar washing procedure on 10-fold that quantity of hydrazone obtained when the glucoside was first treated with β-glucosidase. The increase in 2,4-dinitrophenylhydrazone resulted from the release of *p*-hydroxybenzaldehyde during treatment of the glucoside with β-glucosidase. This would account for the difference in total counts isolated and would suggest that the ratio of the quantity of two hydrazones was approximately equivalent to the dilution factor. This would be strictly true only if no ^{14}C from DL-tyrosine-2-^{14}C became incorporated into the aldehydic

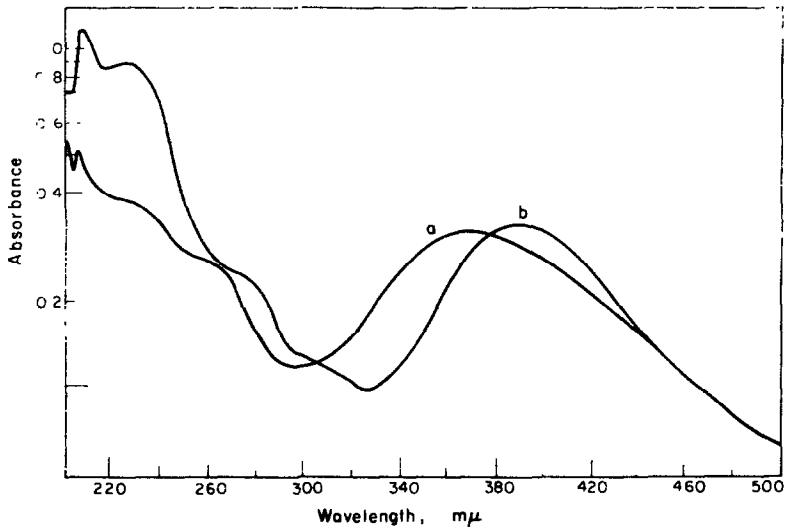

FIG. 2. The 2,4-dinitrophenylhydrazones were prepared from material eluted from the R_f 0·72–0·90 portion of the chromatogram by addition of an equal volume of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl to the eluate either prior to (*a*) or after (*b*) treatment with β-glucosidase

carbon of *p*-hydroxybenzaldehyde and if the extinction coefficient of the two hydrazones was identical.

The data from the DL-tyrosine-3-^{14}C and L-tyrosine-U-^{14}C experiment are compatible with this explanation since, after a correction for loss of sample in the washing procedure is taken into account, there appears to be approximately 10-fold more total radioactivity in the hydrazones isolated after β-glucosidase treatment than was obtained prior to treatment. In these experiments both hydrazones would be expected to contain ^{14}C while in the experiments with DL-tyrosine-2-^{14}C no ^{14}C should be incorporated into the *p*-hydroxybenzaldehyde moiety derived from the cyanogenic glucoside. The table also shows that the specific activity of the hydrazones isolated by the two procedures showed a dilution factor of approximately 2 when the hydrazones were prepared from experiments in which the plants metabolized either DL-tyrosine-3-^{14}C or L-tyrosine-U-^{14}C. Furthermore, the hydrazone isolated from the glucoside containing ^{14}C from either DL-tyrosine-2-^{14}C, DL-tyrosine-3-^{14}C or L-tyrosine-U-^{14}C without prior treatment with β-glucosidase each contained, with the exception of experiment

1 with DL-tyrosine-2-^{14}C, approximately the same specific activity. This observation suggests that the hydrazone isolated was derived from a compound which contains 8 of the carbon atoms of tyrosine.

Information relative to the structure of the hydrazone may be obtained from spectral data (See Discussion). Figure 2 shows a comparison of the spectra of the 2,4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde to the hydrazone isolated by treatment of the glucosides with acidic 2,4-dinitrophenylhydrazine. This figure shows that the spectra are different with a 20 mμ shift in maximum for the intermediate hydrazone.

Similar experiments were carried out with D-glucose-U-^{14}C as the source of ^{14}C and L-tyrosine was added to the medium in order to keep to a minimum the ^{14}C incorporated from D-glucose into the aglycone moiety. Table 3 shows the results of such an experiment in which the seedlings were allowed to metabolize either D-glucose-U-^{14}C or DL-tyrosine-2-^{14}C. The data show that similar dilutions were obtained for the hydrazones when the seedlings metabolized either DL-tyrosine-2-^{14}C or D-glucose-U-^{14}C. Furthermore, the total radioactivity in the hydrazones derived from the glucoside containing radioactivity from D-glucose-U-^{14}C

TABLE 3. INFLUENCE OF β-GLUCOSIDASE TREATMENT OF PHENOLIC GLUCOSIDE(S) UPON THE SPECIFIC ACTIVITY OF THE 2,4-DINITROPHENYLHYDRAZONE(S) ISOLATED

Labelled compound	β-glucosidase treatment	μmoles of hydrazone	Specific activity of hydrazone counts/min per μmole)	Dilution (−/+)
DL-Tyrosine-2-^{14}C	+	7·21	358	12·0
	−	0·33	4300	
D-Glucose-^{14}C	+	6·00	357	12·7
	−	0·24	4550	

The sorghum seedlings were treated as described in Fig. 1, and the specific activity of the 2,4-dinitrophenylhydrazones was estimated as described in Table 2.

was approximately the same, again taking into account a greater per cent loss during washing the smaller quantity of hydrazone.

These results suggest that the substance which reacts with acidic 2,4-dinitrophenylhydrazine contains carbon atoms derived from D-glucose as well as 8 carbons from tyrosine. Thus it appears that β-glucosidase does not remove the ^{14}C derived from glucose in this substance.

An experiment was conducted to determine if glucose would be released by acid hydrolysis from material obtained from the glucoside area of the chromatogram after treatment of the glucoside with β-glucosidase.

The glucoside from 1 gm of seedlings was isolated in the usual manner and treated with 10 mg of β-glucosidase for 60 min at 35°. The reaction was stopped by heating at 100° for 10 min. The protein was removed by centrifugation and the supernatant solution chromatographed in butanol:pyridine:H_2O (6:4:3). Large quantities of glucose and *p*-hydroxybenzaldehyde were detected upon the chromatogram. In addition there appeared to be a small quantity of material migrating at R_f 0·8, which reacted with diazotized sulfanilic acid but not readily with 2,4-dinitrophenylhydrazine. This material was eluted with 80% ethanol, the solvent removed, and the material subjected to hydrolysis in 1 N H_2SO_4 at 100° for 15 min.

The acidic solution was neutralized with $BaCO_3$, the $BaSO_4$ removed and the supernatant solution subjected to chromatography. The chromatogram was treated with $AgNO_3$ in acetone followed by development with alcoholic NaOH and $Na_2S_2O_3$[5]. Two spots were obtained, one corresponding to glucose and the other migrating at R_f 0·8. Another portion of the unhydrolyzed sample was further treated with β-glucosidase and assayed for HCN. Only a trace could be detected. These results suggest that sorghum seedlings contain a glucoside which is resistant to β-glucosidase treatment.

DISCUSSION

These experiments suggest sorghum seedlings contain two glucosides whose aglycon moieties are derived from L-tyrosine. The new glucoside appears to be different from *p*-hydroxymandelonitrile-β-glucose in that it reacts with acidic 2,4-dinitrophenylhydrazine before treatment of the glucosides with β-glucosidase. After treatment of the glucosides with β-glucosidase the new glucoside retains both the glucosyl moiety and the *p*-hydroxyphenylethyl carbon skeleton of tyrosine. It has previously been shown [2] that no ^{14}C from DL-tyrosine-1-^{14}C is incorporated into the glucoside region of the chromatogram when extracts of sorghum seedlings that had metabolized DL-tyrosine-1-^{14}C were chromatographed.

Jones *et al.*[6] have shown that the greater the extent of conjugation the longer the wave length of the maximum absorption of the 2,4-dinitrophenylhydrazone. The spectrum of the new 2,4-dinitrophenylhydrazone shows an absorption maximum at 370 mμ which is 20 mμ shorter than the hydrazone of *p*-hydroxybenzaldehyde.[4] These data suggest that the new hydrazone probably contains fewer then four conjugated double bonds derived from *p*-hydroxyphenylethyl portion of the 2,4-dinitrophenylhydrazone. These data do not allow assignment of structure of the new phenolic glucoside.

Preliminary investigations have shown that incubation of a mixture of the two phenolic glucosides (2120 counts/min/reaction mixture) derived from DL-tyrosine-2-^{14}C with a particulate preparation from etiolated sorghum seedlings and reduced nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and ATP results in the isolation of approximately one-fourth of the ^{14}C as ^{14}C-2,4-dinitrophenylhydrazone. This represented an increase of more than 200 counts/min/reaction mixture when compared to the zero time control or controls in which ATP or reduced nicotinamide adenine phosphate had been omitted. No increase in precipitable ^{14}C-2,4-dinitrophenylhydrazone when compared to zero time controls, was observed if the phenolic glucosides were preincubated with the enzyme preparation prior to addition of the other components of the reaction mixture.

This evidence is compatible with *in vivo* data suggesting that the two phenolic glucosides may be metabolically related.

METHODS

Sorghum seedlings were germinated and grown, and the glucoside isolated as previously described.[2] In preliminary experiments 2,4-dinitrophenylhydrazine was shown to react with material eluted from the glucoside area of the chromatogram. 2, 4-Dinitrophenylhydrazones were prepared by either of two procedures. In both procedures the paper cut from the glucoside area of the chromatogram was shaken with 80% ethanol, the ethanolic extract was filtered

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[6] L. A. JONES, J. C. HOLMES and R. B. WELIGMAN, *Anal. Chem.* **28**, 191 (1956).

and the solvent was removed on a flash evaporator at 30° C. The glucosides were redissolved in a minimum of water and the sample was divided into two equal parts. In procedure A, an equal volume of a 2 N HCl solution saturated with 2,4-dinitrophenylhydrazine was added to one-half of the aqueous glucoside solution and the solution left overnight at 0–3°. In prodecure B, the other half of the glucoside solution was first incubated for 50 min with 2 mg per ml of β-glucosidase, and then the reaction mixture heated for 3 min at 100°. The protein was removed by centrifugation, and an equal volume of acidic 2,4-dinitrophenylhydrazine solution was added to the supernatant solution which was again held at 0–3° overnight. The 2,4-dinitrophenylhydrazones were centrifuged and were twice suspended in water and sedimented by centrifugation. They were then dissolved in absolute ethanol and their spectra obtained on a Beckman DB spectrophotometer. The absorption at 390 mμ was estimated. It was found that the 2-4-dinitrophenylhydrazone of *p*-hydroxybenzaldehyde has an extinction coefficient of $3{\cdot}8 \times 10^4$. The radioactivity in the 2,4-dinitrophenylhydrazones was estimated on infinitely thin samples in a Nuclear Chicago Model 181A gas flow counter.

The HCN was estimated in 0·1 M NaOH by the procedure of Aldridge,[7] and the radioactivity was measured in a Tri-Carb scintillation counter with toluene and ethanol (60:40) as the solvent and 2,5-diphenyloxazole and 1,4-bis-2(5-phenyloxazolyl)benzene as the scintillators. The procedure gave 42 per cent counting efficiency with this instrument.

The chromatograms were scanned with an Atomic Associates Scanogram II 4π scanner attached to a Texas Instruments Company recorder, and an Atomic Associates Model 423A rate-meter.

L-Tyrosine-U-^{14}C, DL-tyrosine-3-^{14}C, DL-tyrosine-2-^{14}C and D-glucose-U-^{14}C were purchased from Nuclear Chicago and New England Nuclear Corporation. β-Glucosidase prepared from almonds was obtained from Sigma Chemical Company. All other chemicals used were reagent grade.

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