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# ALDOXIMES AND NITRILES AS INTERMEDIATES IN THE BIOSYNTHESIS OF CYANOGENIC GLUCOSIDES*

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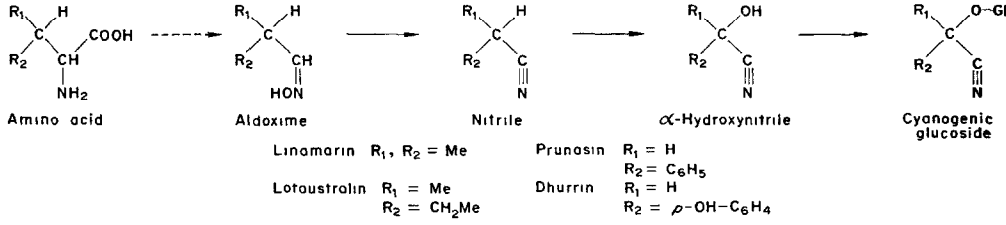
**Key Word Index**—*Sorghum vulgare*; Gramineae; sorghum; *p*-hydroxyphenylacetaldoxime; *p*-hydroxyphenylacetonitrile; cyanogenic glucoside; biosynthesis.

**Abstract**—Young sorghum shoots have been shown to convert tyrosine, *p*-hydroxyphenylacetaldoxime and *p*-hydroxyphenylacetonitrile to dhurrin, the cyanogenic glucoside characteristic of this plant. Evidence for the *in vivo* formation of *p*-hydroxyphenylacetaldoxime but not *p*-hydroxyphenylacetonitrile from tyrosine has been obtained from a 'trapping experiment.'

## INTRODUCTION

A BIOSYNTHETIC pathway for the formation of cyanogenic glucosides from naturally occurring amino acids has recently been proposed by Conn and Butler.[1] In this pathway (Scheme 1), amino acids (valine and isoleucine in flax and clover, phenylalanine in cherry laurel, and tyrosine in sorghum) are oxidatively decarboxylated to form aldoximes which are subsequently dehydrated, oxidized and glucosylated to form cyanogenic glucosides characteristic of the specific plant.




SCHEME 1. PROPOSED PATHWAY FOR THE BIOSYNTHESIS OF A CYANOGENIC GLUCOSIDE FROM ITS PRECURSOR AMINO ACID.

The evidence in support of this pathway consists primarily of *in vivo* feeding experiments in which the proposed precursor amino acids or intermediates of linamarin and prunasin biosynthesis (Scheme 1) were shown to be utilized by flax and cherry laurel shoots, respectively, for the biosynthesis of these compounds.[1,2] Evidence for the *in vivo* formation of isobutyraldoxime and isobutyronitrile from valine (proposed intermediates in the bio-

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[1] CONN, E. E. and BUTLER, G. W. (1969) *Perspectives in Phytochemistry* (HARBORNE, J. B. and SWAIN, T., eds.), Chap. 2, pp. 47–74, Academic Press, London.

[2] TAPPER, B. A. and BUTLER, G. W. (1971) *Biochem. J.* **124**, 935.

synthesis of linamarin, Scheme 1) has been obtained from a 'trapping' experiment.[3] Only in the case of the last step is there any evidence of an enzymatic nature. Enzymes from flax and sorghum which catalyze the glucosylation of acetone cyanohydrin and *p*-hydroxymandelonitrile to form linamarin and dhurrin, respectively, have been partially purified and their properties reported.[4,5]

The incorporation of tyrosine, *p*-hydroxyphenylacetaldoxime and *p*-hydroxyphenylacetonitrile into dhurrin by young sorghum shoots is reported here. In addition, evidence for the *in vivo* formation of *p*-hydroxyphenylacetaldoxime from tyrosine is reported.

## RESULTS AND DISCUSSION

Labelled *p*-hydroxyphenylacetaldoxime and *p*-hydroxyphenylacetonitrile, proposed intermediates in the biosynthesis of dhurrin in sorghum (Scheme 1), were fed to etiolated sorghum shoots and the incorporation of these compounds was compared with that of tyrosine (Table 1). *p*-Hydroxyphenylacetaldoxime was found to be efficiently converted (45%) to dhurrin compared to the incorporation of tyrosine (Table 1, Experiment A and B). *p*-Hydroxyphenylacetonitrile was also incorporated into dhurrin (Table 1, Experiment D), but the per cent incorporation of label from this compound into dhurrin was substantially less than the conversion of the oxime or amino acid. Differences in the amount of compound fed in each case do obscure this point. Nevertheless, the low incorporation of the nitrile observed was surprising since $^{14}$C-labelled isobutyronitrile and phenylacetonitrile are efficiently utilized (40–60% conversion) in the synthesis of linamarin and prunasin by flax and cherry laurel shoots, respectively.[2] This low incorporation of the nitrile in sorghum could have been due to the toxicity of this compound to sorghum shoots (see below).

The dhurrin synthesized from the labelled oxime and nitrile was purified chromatographically and the position of the labelling in the aglycone determined.[6] In both cases it was expected that the nitrile carbon atom of the aglycone would have been the only atom labelled, and in fact 97% of the label from 1-$^{14}$C-*p*-hydroxyphenylacetaldoxime and 95% of the label from 1-$^{14}$C-*p*-hydroxyphenylacetonitrile was found in this carbon atom.

TABLE 1. INCORPORATION OF TYROSINE, *p*-HYDROXYPHENYLACETALDOXIME AND *p*-HYDROXYPHENYLACETONITRILE INTO DHURRIN

| Feeding experiment | Precursor | Duration of feeding experiment (hr) | Radioactivity administered (dpm) | Dhurrin specific activity ($\mu$Ci/$\mu$mol) | Dilution factor | Total dhurrin ($\mu$mol) | Incorporation into dhurrin (%) |
|---|---|---|---|---|---|---|---|
| A | [U-$^{14}$C]-tyrosine (475 $\mu$Ci/$\mu$mol) | 8 | $0\,994 \times 10^{6}$ | $2\,62 \times 10^{-3}$ | $1\,61 \times 10^{5}$* | 22 2 | 13 3* |
| B | 1-$^{14}$C-*p*-hydroxyphenylacetaldoxime (1 005 $\mu$Ci/$\mu$mol) | 8 | $1\,19 \times 10^{6}$ | $1\,27 \times 10^{-2}$ | 78 | 25 5 | 44 6 |
| C | [U-$^{14}$C]-tyrosine (407 $\mu$Ci/$\mu$mol) | 9 | $1\,63 \times 10^{6}$ | $4\,34 \times 10^{-3}$ | $8\,36 \times 10^{4}$* | 19 0 | 14 1* |
| D | 1-$^{14}$C-*p*-Hydroxyphenylacetonitrile (0 464 $\mu$Ci/$\mu$mol) | 9 | $2\,11 \times 10^{6}$ | $3\,51 \times 10^{-3}$ | $1\,30 \times 10^{2}$ | 18 8 | 6 9 |

* Corrected for the presumed loss of carboxyl carbon atom tyrosine as $CO_2$.

[3] TAPPER, B A. and BUTLER, G. W. (1972) *Phytochemistry* **11**, 1041.
[4] HAHLBROCK, K. and CONN, E. E. (1970) *J. Biol. Chem.* **245**, 917.
[5] REAY, P. F. and CONN, E. E. (1969) *Federation Proc* **28**, 540; and manuscript in preparation.
[6] REAY, P. F. and CONN, E. E. (1971) *Phytochemistry* **9**, 1825.

The feeding experiments in Table 1 have demonstrated that *p*-hydroxyphenylacetaldoxime and *p*-hydroxyphenylacetonitrile can be utilized in the synthesis of dhurrin as proposed in Scheme 1. It is not known whether these compounds are in fact synthesized *in vivo* from tyrosine. Evidence on this point was obtained from 'trapping' experiments, the results of which are presented in Tables 2 and 3. It can be seen that feeding unlabelled *p*-hydroxyphenylacetaldoxime together with labelled tyrosine (Table 2, Experiment F) resulted in a decreased incorporation into dhurrin, an increased dilution of label and a decreased specific activity of the dhurrin isolated, when compared to feeding tyrosine alone (Table 2, Experiment E.) Similar results were obtained in Experiments G and H (Table 2) using *p*-hydroxyphenylacetonitrile as the 'trap'. In the case of the *p*-hydroxyphenylacetaldoxime 'trapping' experiment, label from the $^{14}$C-tyrosine administered was found to be incorporated into the oxime (Table 3, Experiments E and F) following the separation of these compounds by the two chromatographic systems indicated in Table 3. This conversion of labelled tyrosine to labelled *p*-hydroxyphenylacetaldoxime was confirmed by adding unlabelled carrier oxime to the extract, reisolating the oxime by preparative TLC, and crystallizing it from diethyl-ether-hexane. After two recrystallizations, the specific gravity of the isolated *p*-hydroxyphenylacetaldoxime was essentially constant (81·1 and 79·2 dpm/mg, respectively). Melting points of 118–119° were recorded for both samples (lit. 119°).[8]

TABLE 2. INCORPORATION OF LABELLED TYROSINE INTO DHURRIN IN THE PRESENCE AND ABSENCE OF EITHER UNLABELLED *p*-HYDROXYPHENYLACETALDOXIME OR *p*-HYDROXYPHENYLACETONITRILE

| Feeding experiment | Pretreatment conditions* | Compounds administered† | Radioactivity administered (dpm) | Dhurrin specific activity ($\mu$Ci/$\mu$mol) | Dilution factors§ | Total dhurrin ($\mu$mol) | Incorporation into dhurrin§ (%) |
|---|---|---|---|---|---|---|---|
| E | None | [U-$^{14}$C]-tyrosine (475 $\mu$Ci/$\mu$mol) | $1\,05 \times 10^{6}$ | $4\,31 \times 10^{-3}$ | $9\,79 \times 10^{4}$ | 11 2 | 27 |
| F | *p*-Hydroxyphenylacetaldoxime (15 2 $\mu$mol)‡ | [U-$^{14}$C]-tyrosine (475 $\mu$Ci/$\mu$mol) + *p*-Hydroxyphenylacetaldoxime (15 2 $\mu$mol) | $0\,886 \times 10^{6}$ | $1\,10 \times 10^{-3}$ | $3\,82 \times 10^{5}$ | 10 4 | 7 4 |
| G | None | [U-$^{14}$C]-Tyrosine (475 $\mu$Ci/$\mu$mol) | $1\,12 \times 10^{6}$ | $3\,31 \times 10^{-3}$ | $1\,24 \times 10^{5}$ | 15 1 | 27 |
| H | *p*-Hydroxyphenylacetonitrile (16 9 $\mu$mol) | [U-$^{14}$C]-Tyrosine (475 $\mu$Ci/$\mu$mol) + *p*-hydroxyphenylacetonitrile (16 9 $\mu$mol) | $1\,11 \times 10^{6}$ | $0\,225 \times 10^{-3}$ | $1\,88 \times 10^{6}$ | 15 8 | 3 7 |

* Experiment F for 1·25 hr, Experiment H for 2 hr.
† Experiments E and F for 8 hr, Experiments G and H for 5 hr.
‡ 86% of the oxime was taken up. The remaining 14% was estimated colorimetrically.[7]
§ Corrected for the presumed loss of the carboxyl carbon atom of tyrosine as $CO_2$.

In the nitrile 'trapping' experiment, no incorporation of $^{14}$C-label from tyrosine into the nitrile was detected (Table 3, Experiments G and H). This is probably accountable to the observed toxicity of the nitrile to the sorghum shoots. Considerable 'browning' of the shoots was noticed during the pre-feeding period, and at the end of the experiment most of the shoots had lost all turgidity. This toxic effect of the nitrile probably explains not only the

[7] SHUKLA P. S. and MAHADEVAN, S. (1968) *Arch. Biochem. Biophys.* **125,** 873.
[8] KINDL, H. and SCHIEFER, S. (1969) *Monatsch. Chem.* **106,** 1773.

very low incorporation of radioactivity from $^{14}$C-labelled tyrosine into dhurrin in the trapping experiments (Table 2, Experiment H) but also the relatively poor incorporation of radioactivity from $^{14}$C-labelled nitrile itself (Table 1, Experiment D). An unsuccessful attempt was made to see if a pool of labelled *p*-hydroxyphenylacetonitrile could be detected following the administration of $^{14}$C-tyrosine alone.

TABLE 3. CHROMATOGRAPHIC SEPARATION OF EXTRACTS

| Compound | $R_f$ | Experiment E (dpm) | | Experiment F (dpm) | | Experiment G (dpm) | Experiment H (dpm) |
|---|---|---|---|---|---|---|---|
| *Formamide paper** (1/40th aliquot of extract chromatographed) | | | | | | | |
| Tyrosine + dhurrin | 0 | 13 229 | | 14 195 | | 9386 | 11 549 |
| *p*-Hydroxyphenylacetaldoxime | 0 16 | 22 | | 669 | | 77 | 288 |
| *p*-Hydroxyphenylacetonitrile + *p*-hydroxybenzaldehyde | 0 48 | 49 | | 107 | | 11 | 90 |
| | | 1/200 | 1/80 | 1/200 | 1/80 | 1/200 | 1/200 |
| *TLC-silica gel*† (1/80th and 1/200th aliquots of extract chromatographed as indicated) | | | | | | | |
| Tyrosine + dhurrin | 0 | 3405 | 8259 | 3479 | 8563 | 1934 | 2233 |
| *p*-Hydroxyphenylacetaldoxime | 0 13 | 6 | 11 | 186 | 515 | 13 | 122 |
| *p*-Hydroxybenzaldehyde | 0 21 | 9 | 25 | 10 | 33 | 0 | 10 |
| *p*-Hydroxyphenylacetonitrile | 0 31 | 0 | 0 | 1 | 38 | 12 | 11 |

* Ascending development with solvent 1. Compounds were detected by UV.

† Ascending development with solvent 1 using Bakerflex-IBF sheets. Compounds were detected by UV.

The results reported here support the postulated intermediate roles of aldoximes and nitriles in the biosynthesis of cyanogenic glucosides. Currently, we are attempting to demonstrate and characterize the specific enzymes responsible for the *in vitro* conversions of oximes to nitriles and nitriles to cyanohydrins.

## EXPERIMENTAL

*Chemical syntheses.* 1-$^{14}$C-*p-hydroxyphenylacetonitrile.* 4-Benzyloxybenzyl chloride[9] (0·024 mmol) and $^{14}$C-potassium cyanide (0·01 mmol, 15·0 mCi/mmol) were mixed in dimethylsulphoxide (1·0 ml) and heated at 100° for 10 min. Sodium cyanide (0·016 mmol) was then added and heating continued for a further 2·5 hr with occasional stirring. The mixture was saturated with NaCl and extracted with $Et_2O$ (5 × 3 ml). This extract was washed with $H_2O$, evaporated to dryness and the residue dissolved in MeOH (1·5 ml). 10% Pd–C catalyst (2 mg) was added and a slow stream of $H_2$ passed through the solution for 6 hr. After the removal of the catalyst, the solution was taken to dryness and carrier *p*-hydroxyphenylacetonitrile (2 mg) added with 10 ml of $Et_2O$. This solution was extracted with 0·1 N NaOH (2 × 5 ml), and the aqueous phase acidified and extracted with $Et_2O$ (3 × 5 ml). The 1-$^{14}$C-*p*-hydroxyphenylacetonitrile was purified by successive PC using solvents 2 and 4. Radiochemical yield 5·3% specific activity 0·562 μCi/μmol.

1-$^{14}$C-*p-hydroxyphenylacetaldoxime.* 1-$^{14}$C-*p*-Hydroxy-ω-nitrostyrene (prepared from 200 μCi, 271 μmol $^{14}$C-nitromethane)[10] was dissolved in a small amount of pyridine and hydrogenated (2400 g/cm$^2$) for 40 min using a Pd–C catalyst. The catalyst was removed by filtration and washed with 6 N HCl. The filtrate was extracted with $Et_2O$, and the product purified by TLC chromatography (silica-gel HF-254) using solvent 1. Radiochemical yield 15·2%, 1·005 μCi/μmol.

*p-Hydroxyphenylacetaldoxime* was prepared from *p*-hydroxyphenylacetaldehyde[11] and hydroxylamine hydrochloride.[12] M.p. 116–118°. 10–15% yield. NMR data on the product showed that it contained 97% of the *trans* and 3% of the *cis* isomer.[13]

*Administration of precursors.* Compounds in (2% EtOH) were fed to 20–30 etiolated 3-day-old excised sorghum shoots in the light for various times. Additional 2% EtOH was added as required.

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[11] ROBBINS, J. H. (1966) *Arch. Biochem. Biophys.* **114**, 576.
[12] VOGEL, A. H. (1956) *Textbook of Practical Organic Chemistry*, 3rd Edn, p. 345, Wiley, New York.
[13] KARABATSOS, G. J. and TALLER, R. A. (1968) *Tetrahedron* **24**, 3347.

*Extraction.* The tissue was ground in a mortar with sand and hot 80% EtOH. The suspension was heated to boiling, filtered and the residue washed with solvent. The filtrate was taken to dryness and the residue dissolved in a known vol. of 80% EtOH.

*Dhurrin concentrations* in the crude extracts were determined by two methods, either spectrophotometrically following alkaline degradation to *p*-hydroxybenzaldehyde[14] or by colorimetric determination of the cyanide released following enzymatic degradation.[6]

*Determination of labelling* Radioactivity of samples on paper, thin layer sheets or in solution was determined in Triton-toluene scintillation fluid[15] to which a total of 1 ml $H_2O$ was added. Quench corrections were made using $^{14}$C-benzoic acid as an internal standard or by the use of an external standard. Radioactivity of NaCN samples was measured in Bray's solution[16] with $^{14}C$-$Na_2CO_3$ as an internal standard.

*Chromatographic separations.* The following solvents were used: (a) EtOAc–$C_6H_6$ (1:5), (b) *n*-BuOH–$H_2O$ (50.9), (c) 2-butanone–$Me_2CO$–$H_2O$ (15:5:3), (d) *n*-BuOH–$NH_4OH$ (4:1), and (e) *n*-BuOH–propionic acid–$H_2O$ (62:31:43). Formamide papers were prepared as described by Kindl and Underhill.[17]

*Incorporation of label into dhurrin* was determined following the descending PC separation of an aliquot of the plant extract in solvent 3. Dhurrin was detected by the method of Anet and Reynolds.[18]

*Specific activities of dhurrin* were determined as previously described,[6] following chromatographic purification in solvents 2, 3 and 5. The values for the dilution factor and % incorporation of radioactivity from [U-$^{14}$C]tyrosine into dhurrin were corrected for the presumed loss of the carboxyl carbon atom of the amino acid as $CO_2$.

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