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STUDIES ON THE IDENTIFICATION, BIOSYNTHESIS AND METABOLISM OF A CYANOGENIC GLUCOSIDE IN *NANDINA DOMESTICA* THUNB.*

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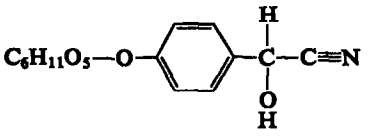
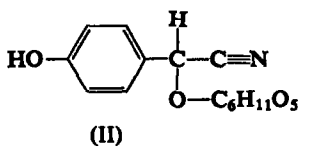
Abstract—The occurrence of a new cyanogenic glucoside, *p*-glucosyloxymandelonitrile, in young shoots of *Nandina domestica* Thunb. is reported. The amount of the cyanogen varies from 10–20 per cent of the dry weight in young shoots. The administration of ^{14}C labelled [^{14}C]tyrosine shows that this amino acid is effectively incorporated into the aldehyde and nitrile moieties. Though no intermediates have been established, experimental evidence indicates that the C_α–C_β bond of tyrosine remains intact during conversion. The nitrile moiety is further metabolized and is incorporated into the amide carbon of asparagine.

INTRODUCTION

PRELIMINARY investigations on the occurrence of HCN in different plant parts of *Nandina domestica* Thunb. were made by Dekker[1] who reported that a labile cyanogenic compound is present in this species. The cyanogen was assumed to be a glycoside of acetone cyanohydrin in view of the release of HCN and acetone when plant material was ground up. Similar observations were made by Plouvier.[2] However, experimental evidence obtained in this laboratory reveals the structure of the cyanogenic compound to be *p*-glucosyloxymandelonitrile (I). The similarity of the biosynthetic route to that for dhurrin (II) (*p*-hydroxymandelonitrile β-D-glucoside)[3] supports the structure assigned to this compound.



(I)



(II)

The presence of enormous quantities of the cyanogenic glucoside (3–6 per cent on a fresh weight basis or 10–20 per cent on a dry weight basis) in very young leaves and its virtual disappearance from older leaves, coupled with the role recently suggested[4] for cyanogenic compounds in asparagine biosynthesis in cyanophoric plants, provided us a good opportunity to study its *in vivo* degradation and the subsequent metabolism of the nitrile moiety.

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[1] J. DEKKER, *Pharm. Weekblad.* **43**, 942 (1906).

[2] V. PLOUVIER, *Bull. Sci. Pharmacol.* **49**, 150 (1942).

[3] J. KOUKOL, P. MILJANICH and E. E. CONN, *J. Biol. Chem.* **237**, 3223 (1962).

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RESULTS AND DISCUSSION

Elucidation of the Structure of the Cyanogenic Compound

Analyses of different plant parts, utilizing the endogenous enzymes, show HCN to be present, in some form, in large amounts in young tissues (Table 1). In young leaves the HCN content comprises 0·3–0·5 per cent of the fresh weight or 1·0–2·0 per cent of the dry weight. In older tissues, e.g. green leaves and fruits, smaller amounts are present.

Table 1. Cyanide content in different plant parts of *Nandina domestica*

Plant material	μmoles/g fresh weight
Young "red" leaves	100–180
Old green leaves	5–8
Flower buds	80–120
Red fruits	4–6

* Collected in the Fall of 1964.

The assumption of Dekker[1] and Plouvier[2] about the possible presence of an acetone cyanohydrin was examined by feeding the young shoots L-[U-^{14}C]valine, since this amino acid has been shown to be a precursor of the commonly occurring cyanogenic glucoside linamarin (a glucoside of acetone cyanohydrin) in a number of plants.[3] However, incorporation of activity into the HCN liberated by endogenous enzymes did not occur (Table 2) thereby indicating the absence of such an acetone cyanohydrin.

Table 2. Incorporation of valine and tyrosine into the cyanogen of *Nandina domestica*

Compound administered	Specific activity (μc/μmole)	Amount administered (μc)	Incorporation of activity into the cyanide carbon atom (%)
L-[U-^{14}C]Valine	205	1·0	0·01
L-[U-^{14}C]Tyrosine	189	1·0	4·0

In addition to the above evidence, the cyanogenic compound of *Nandina*, unlike linamarin, is unstable. Alcoholic (80%) plant extracts slowly lost cyanide on storage while steam distillation released HCN almost quantitatively after 15 min. Finnemore and Large[6] have reported the occurrence of an unstable cyanogenic glucoside in *Goodia latifolia*. Although not fully characterized, the authors did suggest that the compound might be a *p*-hydroxymandelonitrile with glucose attached to the phenolic hydroxyl group. The similarity in the reported instability of this compound to that from *Nandina* prompted us to feed L-[U-^{14}C]-tyrosine to *Nandina* shoots since tyrosine has been shown to be an effective precursor of a structurally related cyanogenic glucoside, dhurrin, found in *Sorghum vulgare*.[7, 8] The results

[3] G. W. Butler and E. E. Conn, *J. Biol. Chem.* **239**, 1674 (1964).
[6] H. Finnemore and D. K. Large, *Proc. Royal Soc. N.S.W.* **70**, 440 (1936).
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(Table 2) show that radioactivity is incorporated into the cyanogenic compound from tyrosine. This suggested that perhaps the cyanogenic compound in *Nandina* might in fact be similar to that postulated by Finnemore and Large for *Goodia latifolia.*

The instability of the cyanogenic compound, which will be elaborated on shortly, was such that even on chromatography appreciable decomposition occurred and consequently extensive purification of the compound was not possible. As a result of this the structure was elucidated by identification of the products of emulsin hydrolysis after partial purification by chromatography and by comparison of the properties of the compound with synthetic *p*-glucosyloxymandelonitrile. Cyanogenic glycosides on enzymic or dilute acid hydrolysis usually liberate hydrogen cyanide, a sugar or sugars and an aldehyde or ketone.

Plant extracts and partially purified extracts, after treatment with emulsin or dilute acid, yielded an ether extractable aldehyde with chromatographic mobilities on paper in three solvents similar to *p*-hydroxybenzaldehyde. The aldehyde produced also gave u.v. absorption spectra in ethanol and 0·1 N NaOH identical to those of *p*-hydroxybenzaldehyde. Final confirmation was obtained by preparing the 2,4-dinitrophenylhydrazone, m.p. 276° (literature m.p. 280°).

The sugar moiety liberated in the same way from partially purified material was shown to be glucose by its mobility on paper chromatograms in two solvents and also by the specific method used for its estimation (see experimental).

Estimation of the relative amounts of HCN, glucose and *p*-hydroxybenzaldehyde liberated on hydrolysis was complicated by the lability of the cyanogenic compound. When eluates from chromatograms of partially purified material were used a glucose to *p*-hydroxybenzaldehyde ratio of approximately 1:1 was obtained. The corresponding values for HCN were always low (Table 3) due to the lability of the cyanogenic compound. However, since

TABLE 3. HYDROLYSIS PRODUCTS OF THE CYANOGEN OF *Nandina domestica*

	Hydrolysis products (μmoles)		
	HCN	*p*-Hydroxybenzaldehyde	Glucose
Crude plant extracts	118	109	—
Partially purified material	16	89	86

chromatography had revealed the presence of only one major cyanogenic compound it can be assumed that the HCN liberated on hydrolysis of crude plant extracts is from this one compound. The relative amount of *p*-hydroxybenzaldehyde and HCN liberated from crude extracts (Table 3) indicates a ratio of approximately 1:1. These data are in agreement with a ratio of 1:1:1 for the glucose, *p*-hydroxybenzaldehyde and HCN liberated on hydrolysis. With such a 1:1:1 ratio the compound is likely to be a *p*-hydroxymandelonitrile with glucose attached to either the phenolic or the side chain hydroxyl group. The lability of the cyanogenic glucoside would indicate the siting of the glucose on the phenolic hydroxyl. This was confirmed by comparison of the properties of the natural compound with those of synthetic *p*-glucosyloxymandelonitrile (I) and *p*-hydroxymandelonitrile β-D-glucoside (Dhurrin, II). On paper chromatography with solvent 1 all these compounds showed similar mobility (about R_f 0·7); however, the natural compound and I showed appreciable loss of HCN whereas II was stable. Spraying of chromatograms with 0·1% 2,4-dinitrophenylhydrazine in 2N HCl revealed the presence of two carbonyl compounds from the *Nandina* cyanogen and I (R_f 0·7 and 0·85) but none from II. These compounds were respectively shown to be

the cyanogen which breaks down on such treatment and *p*-glucosyloxybenzaldehyde, a decomposition product of the cyanogen presumably formed on storage. Dhurrin gave one carbonyl spot (R_f 0·7) after treatment with emulsin. Neither the *Nandina* material nor I gave a colour on paper with 1·0% ferric chloride while II gave a purple colour. Finally, the u.v. absorption spectra of the three compounds were examined in 0·1 N NaOH (Fig. 1). The natural material and I exhibit a maximum absorption at 270 mμ identical to

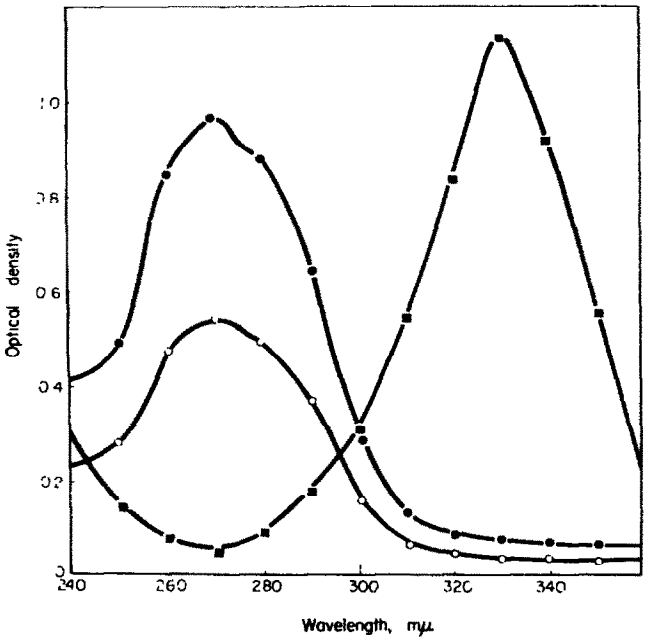

FIG. 1. ULTRAVIOLET ABSORPTION SPECTRA OF DHURRIN AND *p*-GLUCOSYLOXYMANDELONITRILE IN 0·1 N NaOH.
(■—■ dhurrin; ▲ - - ▲ synthetic *p*-glucosyloxymandelonitrile; ●—● compound from *Nandina domestica*.)

p-glucosyloxybenzaldehyde. However, II, after standing for about 30 min at room temperature, shows a maximum at 330 mμ as does *p*-hydroxybenzaldehyde. This may be interpreted as the hydrolysis of the compounds by the alkali to liberate *p*-glucosyloxybenzaldehyde from the *Nandina* compound and I, and *p*-hydroxybenzaldehyde from II.[20]

The above evidence establishes the structure of the cyanogenic glycoside of *Nandina domestica* as *p*-glucosyloxymandelonitrile.

Biosynthesis: Origin of Aldehyde and Nitrile Moieties

The results of experiments pertaining to the origin of the aglycone are shown in Table 4. It can be seen that while administration of [β-^{14}C]tyrosine resulted in the *p*-hydroxybenzaldehyde moiety being labelled [α-^{14}C]tyrosine yielded activity only in the nitrile portion. Radioactivity from [COOH-^{14}C]tyrosine was not incorporated into the glycoside at all.

Plants fed L-[U-^{14}C]tyrosine showed incorporation into both the *p*-hydroxybenzaldehyde and nitrile moieties. Determination of the specific activities of these compounds reveals the values to be approximately in the order of 7:1 (Table 5). In view of this observation and the

TABLE 4. ORIGIN OF ALDEHYDE AND NITRILE MOIETIES

Compound administered*	Specific activity ($\mu c/\mu mole$)	Uptake (%)	Incorporation radioactivity into *p*-Hydroxybenzaldehyde (%)	Incorporation radioactivity into HCN (%)
L-[COOH-^{14}C]Tyrosine	26·60	80	<0·01	<0·01
DL-[α-^{14}C]Tyrosine	1·36	91	0·1	34·84
DL-[β-^{14}C]Tyrosine	6·85	75	31·48	0·2

* 1·0 μc added in each case.

fact that [α-^{14}C]tyrosine and [β-^{14}C]tyrosine are incorporated into the nitrile and secondary alcoholic carbon atoms respectively, it seems reasonable to assume that the C_α–C_β bond of tyrosine remains intact during the conversion.

TABLE 5. INCORPORATION OF TYROSINE INTO THE ALDEHYDE AND NITRILE MOIETIES

Compound administered	Specific activity ($\mu c/\mu mole$)	Amount administered (μc)	Incorporation into *p*-hydroxybenzaldehyde (disintegrations/min/μmole)	Incorporation into HCN (disintegrations/min/μmole)
L-[U-^{14}C]Tyrosine	189	5	6420	750
L-[U-^{14}C]Tyrosine	189	1	3930	540

Metabolism: Incorporation of the Nitrile Moiety into Asparagine

It was observed that when H^{14}CN was fed to *Nandina* plants incorporation into asparagine took place with the activity being almost exclusively localized in the amide-carbon (Table 6). This suggested to us that a biosynthetic pathway involving the coupling of the cyanide to a three-carbon unit[9] operates in this plant.

In view of the large amount of cyanogenic glucoside present in young leaves and its disappearance in older ones, it is possible that slow production of HCN may occur from the glucoside and this HCN can in turn be utilized for asparagine biosynthesis. Since we now know that the aglycone of the cyanogenic glucoside in *Nandina* is derived from tyrosine, the

TABLE 6. DEGRADATION OF ^{14}C-ASPARAGINE BY THE BROMOSUCCINAMIDE METHOD

			Asparagine degraded*				
				Carboxyl-C		Amide-C	
Compound administered	Amount administered (μc)	Incorporation in asparagine (%)	Initial counts (disintegrations/min)	(disintegrations/min)	(%)	(disintegrations/min)	(%)
H^{14}CN	10	1.9	4560	140	(3)	4240	(93)
DL-[α-^{14}C]Tyrosine	10	1·5	5120	1500	(29)	3000	(59)
DL-[α-^{14}C]Tyrosine	2	11·5	11350	890	(8)	9750	(86)
DL-[β-^{14}C]Tyrosine	2	13·1	6970	520	(8)	570	(9)

* An aliquot of the asparagine isolated was used for the degradation studies.

[9] S. BLUMENTHAL-GOLDSCHMIDT, G. W. BUTLER and E. E. CONN, *Nature* **197**, 718 (1963).

validity of such a hypothesis can be tested by feeding [α-^{14}C]tyrosine. As shown in Table 6, about 60–90 per cent of the activity from this compound is located in the amide carbon atom of the asparagine.

As a control, DL-[β-^{14}C]tyrosine was administered to *Nandina* leaves and following a period of metabolism, the asparagine was isolated and degraded. As shown in Table 6, the asparagine contained approximately equal amounts of radioactivity in the carboxyl and amide carbon atoms and this accounted for only 17 per cent of the activity in the molecule. If tyrosine is degraded in higher plants by the route known to occur in animal tissues, namely via homogentisic acid and fumarylacetoacetic acid, then [α-^{14}C]tyrosine would give rise to carboxyl labelled acetic acid while [β-^{14}C]tyrosine would produce methyl-labelled acetate. These two forms of labelled acetate in turn could yield aspartic acid (and therefore asparagine) labelled respectively in carbon atoms 1 and 4 or predominantly in carbon atoms 2 and 3.

EXPERIMENTAL

The experiments were carried out with young shoots of *Nandina domestica* Thunb. (commonly known as Heavenly Bamboo). This plant is grown extensively as an ornamental in California and shoots were selected from plants growing on the University of California campus at Davis.

Almond emulsin was purchased from Calbiochem. Acetobromoglucose was purchased from K and K Laboratories. L-[U-^{14}C]Valine (205 μc/μmole), DL-[β-^{14}C]tyrosine (6·85 μc/μmole), L-[U-^{14}C]tyrosine (189 μc/μmole) and L-[COOH-^{14}C]tyrosine (26·6 μc/μmole) were purchased from New England Nuclear Corporation. DL-[α-^{14}C]Tyrosine (1·36 μc/μmole) and K^{14}CN (1·18 μc/μmole) were obtained from Volk Radiochemicals.

All radioactive compounds were administered in neutral solution to cut shoots (about 0·2–3·5 g fresh weight) except for K^{14}CN which was converted to H^{14}CN gas in an enclosed system.[9] Metabolism times were for 24–48 hr with continuous artificial illumination.

Plant extracts were made by freezing the plant material in liquid nitrogen, grinding in a mortar and transferring the resulting powder to boiling 80% ethanol for 5 min. The suspension was filtered and the filtrate evaporated to dryness under reduced pressure. The residue left on evaporation was dissolved in a known volume of water for analysis or chromatography.

Hydrogen cyanide was liberated from the cyanogenic glucoside by incubation in aqueous solution with emulsin for 2 hr in a closed system. CO_2-free air was then passed through the system and the HCN carried over in the air stream into 10 ml of 0·1 N NaOH. Aliquots of this trapping solution were assayed for cyanide by the method of Aldridge.[10] In initial experiments to determine the cyanide contents of different plant parts the powder prepared by grinding with nitrogen was mixed with water and aerated as before. The endogenous glucosidases in the plant effected liberation of the HCN. The aqueous solution, now depleted of cyanide, was extracted with diethyl ether. The ether extract was evaporated to dryness and the residue of *p*-hydroxybenzaldehyde estimated according to Friedemann and Haugen.[11] Alternatively the residue was dissolved in absolute ethanol and its u.v. spectrum measured, with and without the addition of NaOH solution, using a Cary 14 spectrophotometer.

The following solvent systems were used in the present study (all v/v, except 6 w/w): (1) *n*-butanol–pyridine–water (6:4:3); (2) butanol–ethanol–water (40:11·19); (3) ethanol–conc. ammonium hydroxide–water (80:5:15); (4) benzene–methanol (7:3); (5) butanol–acetic acid–water (12:3:5); and (6) phenol–water (8:2).

[10] W. N. ALDRIDGE, *Analyst* **69**, 262 (1944).
[11] T. E. FRIEDEMANN and G. E. HAUGEN, *J. Biol. Chem.* **147**, 415 (1943).

The cyanogenic glycoside was located on a narrow strip cut from the chromatograms by the picrate paper method[12] or by detection of reducing sugar by the silver nitrate dip method[13] after treatment with 0·1% emulsin solution. The cyanogenic compound was then eluted from the corresponding area on the remainder of the chromatogram with 50% ethanol.

The sugar moiety of the glycoside was assayed, after emulsin hydrolysis, by an enzymic method specific for the glucose. The method is based on the phosphorylation of glucose by ATP in the presence of yeast hexokinase and subsequent oxidation of the glucose-6-phosphate by NADP and glucose-6-phosphate dehydrogenase. The optical density of the NADPH formed is read at 340 mμ.[14]

All radioactivity measurements were made in Bray's solution[15] using a Packard Tricarb Scintillation Counter. [^{14}C]Benzoic acid was used as an internal standard. The specific activity of the 2,4-dinitrophenylhydrazone of the ether extracted *p*-[^{14}C]hydroxybenzaldehyde was determined by the method of Koukol *et al.*[3] The melting point was determined in a capillary melting point apparatus.

Methods for the chromatographic isolation of [^{14}C]asparagine, its hydrolysis to [^{14}C]-aspartic acid and the subsequent decarboxylation of both these compounds have been described in an earlier communication.[16] Asparagine was estimated by Moore and Stein's method.[17]

p-Glucosyloxymandelonitrile was synthesized as follows: Tetra-acetyl-*p*-glucosyloxybenzaldehyde was prepared according to the method of Robertson and Waters.[18] Deacetylation to *p*-glucosyloxybenzaldehyde was achieved by standing overnight at 5° with methanolic ammonia. Conversion to the cyanohydrin was then carried out with liquid hydrocyanic acid essentially by the method of Fischer.[19]

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