Oximes, Nitriles and 2-Hydroxynitriles as Precursors in the Biosynthesis of Cyanogenic Glucosides

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The biosynthesis of the cyanogenic glucosides, linamarin and prunasin, was investigated in linen-flax, peach and cherry-laurel shoots. It was shown that related 2-oximino acids, aldoximes, nitriles and 2-hydroxynitriles were generally good precursors of the aglycone moiety. Studies with double-labelled compounds confirmed the retention of the oximino nitrogen atom from 2-oximinoisovaleric acid and isobutyraldoxime in the biosynthesis of linamarin. A general pathway from amino acids to cyanogenic glucosides involving *N*-hydroxyamino acids, aldoximes, nitriles and 2-hydroxynitriles is proposed.

Several studies have shown that amino acids are good precursors for the biosynthesis of structurally related cyanogenic glycosides (Gander, 1962; Butler & Butler, 1960; Mentzer, Favre-Bonvin & Massias, 1963). It has been shown that the amino acid carboxyl group is lost and the amino nitrogen is oxidized to form the nitrile group of the cyanogenic glycoside (Butler & Conn, 1964; Uribe & Conn, 1966; Bleichert, Neish & Towers, 1966). In no case, however, was clear evidence presented to indicate the likely intermediates or pathway of biosynthesis. This paper describes a series of isotope-labelling experiments which indicate that aldoximes, nitriles, and 2-hydroxynitriles may be intermediates. Preliminary reports of parts of this work have been presented (Tapper, Conn & Butler, 1967; Hahlbrock, Tapper, Butler & Conn, 1968) or cited in review (Conn & Butler, 1969).

The cyanogenic glucosides investigated were linamarin (2-hydroxyisobutyronitrile β -D-glucopyranoside) from flax (*Linum usitatissimum* L.) and prunasin (D-mandelonitrile β -D-glucopyranoside) from peach (*Prunus persica*, Batsch) and cherry laurel (*Prunus laurocerasus* L.).

MATERIALS AND METHODS

Materials. L-[U-¹⁴C]Valine, L-[U-¹⁴C]phenylalanine, potassium [¹⁴C]cyanide and sodium [1-¹⁴C]isobutyrate were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Phenyl[1-¹⁴C]acetylhydroxamic acid and 2-oximino-3-phenyl[2-¹⁴C]propionic acid were generous gifts from Dr E. W. Underhill, Prairie Regional Laboratory, Saskatoon, Canada. [¹⁵N]Hydroxylamine hydrochloride of 95 atoms % excess was obtained from Dr E. E. Conn, University of California, Davis, Calif., U.S.A.

L-[¹⁵N]Valine of 45 atoms % excess was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

[U-¹⁴C]Isobutyraldoxime, [U-¹⁴C]isobutyraldehyde and 2-oximino[U-¹⁴C]isovaleric acid were prepared from L-[U-¹⁴C]valine as described by Tapper *et al.* (1967). [1-¹⁴C]Isobutyramide and [1-¹⁴C]isobutyronitrile were prepared from sodium [1-¹⁴C]isobutyrate as described by Hahlbrock *et al.* (1968).

[U-¹⁴C]Phenylacetaldoxime was prepared as follows. A solution of 1 mg of [U-¹⁴C]phenylalanine in 0.6 ml of 0.05 M-potassium phosphate buffer, pH 6.7, was treated with 2 mg of *N*-bromosuccinimide and 50 mg of succinimide and the mixture was shaken for 30 min at 5°C while being extracted with a total of 1 ml of diethyl ether in successive small portions. The ether extract containing [U-¹⁴C]phenylacetaldehyde was then added to 25 mg of hydroxylamine hydrochloride and 30 mg of NaHCO₃ dissolved in 200 μ l of water. The ether was removed with a stream of N₂ and approx. 20 mg of carrier phenylacetaldehyde added. The mixture was shaken for 40 min at 5°C to precipitate [U-¹⁴C]phenylacetaldoxime, the aqueous phase was removed, and the residue dried and recrystallized twice from diethyl ether and hexane, m.p., 96.5°C. The overall yield was low. The compound was shown to be radiochemically homogeneous by t.l.c.

[1-¹⁴C]Phenylacetonitrile was prepared by treating 11 mg of potassium [¹⁴C]cyanide with an excess of benzyl chloride (50 μ l) dissolved in 140 μ l of aq. 70% (v/v) ethanol. The mixture was heated at 75°C for 150 min in a sealed tube and the nitrile isolated by preparative g.l.c. (Tapper *et al.* 1967).

[¹⁵N]Isobutyraldoxime was prepared from isobutyraldehyde and [¹⁵N]hydroxylamine in the presence of NaHCO₃ solution. The oxime was extracted into diethyl ether and purified by preparative g.l.c. [¹⁵N]2-Oximinoisovaleric acid was prepared in a similar manner (Ahmad & Spenser, 1961) and purified by recrystallization twice from ether-hexane.

2-Hydroxy[1-¹⁴C]isobutyronitrile and [1-¹⁴C]mandelonitrile were administered to flax shoots and cherry-laurel

shoots respectively without being isolated from the reaction mixtures. The 2-hydroxy[1-¹⁴C]isobutyronitrile was prepared from 2.5 μ mol of potassium [¹⁴C]cyanide and 5 μ l of acetone dissolved in 10 μ l of water containing 5 μ mol of KH₂PO₄. The mixture was kept at room temperature for 1 h before being diluted to approx. 100 μ l with water and administered directly to 20 shoots in a closed 12 ml vessel. A control treatment without added acetone was performed. [1-¹⁴C]Mandelonitrile was prepared in a similar way to 2-hydroxy[1-¹⁴C]isobutyronitrile. A solution of 5 μ mol of KH₂PO₄ and 2 μ mol of potassium [¹⁴C]cyanide in 10 μ l of water was left with 2 μ l of freshly distilled benzaldehyde for 40 min at 5°C. The reaction mixture was transferred with 190 μ l of water to a 33 ml glass vessel containing three cherry-laurel shoots. A control treatment was included with the omission of benzaldehyde.

Emulsin obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. or β -glucosidase (sweet almonds) obtained from Seravac Laboratories, Cape Town, South Africa, was used for the hydrolysis of prunasin. The β -glucosidase, linamarase, was prepared from linseed meal by ethanol precipitation (Coop, 1940).

Plant material. Linen-flax seeds, variety Redwood, were germinated on moist cotton wool at 25°C in darkness for 4 days followed by 18 h exposure to artificial light (Butler & Conn, 1964). The top 2.5–3 cm portion of the shoots was cut under water to exclude air from the vascular system.

Cherry-laurel and peach shoots were selected with three or four expanding leaves.

Administration of labelled compounds. Compounds were administered to shoots in water or tris buffer and the shoots allowed to take up all the solution, followed by water as required. In certain cases where volatile compounds were administered, the shoots were enclosed in transparent vessels.

Flax shoots were generally used in lots of 20 and allowed 7 h to assimilate the compounds from 200 μ l of solution, under continuous artificial light of 20 000 lux from both fluorescent and incandescent sources. Where ¹⁵N-labelled compounds were administered, 160 seedling shoots were exposed in each treatment for 9 h with appropriately larger volumes of solution.

Peach and cherry-laurel shoots were used in lots of two, three or four and the administered compounds dissolved in 0.2–0.6 ml of solution. Some experiments with the peach and cherry-laurel shoots were of 44 h duration starting at midday with exposure to artificial light of 20 000 lux from mixed sources for 9 h followed successively by 6 h darkness, 18 h light, 6 h darkness and 5 h light. In other experiments the shoots were exposed to the artificial light continuously for 28 h.

Extraction. In general, linen-flax shoots were extracted twice in 25 ml or more of aq. 80% (v/v) ethanol by boiling for 15 or 20 min. Cherry-laurel and peach shoots were blended with appropriately larger volumes of 80% (v/v) ethanol. The combined extracts were concentrated under reduced pressure and dissolved in small volumes of water or aq. 20% (v/v) propanol and stored at –10°C.

Chromatography. All ethanolic extracts were examined by one- or two-dimensional chromatography either on Whatman 3MM paper or on thin layers of cellulose or on mixtures of cellulose and silica gel H (Bielecki & Turner,

1966). Solvents used were butanone–acetone–water^T (15:5:3, by vol.), propanol–water (7:3, v/v), and butan-1-ol saturated with water. Radioactive spots or zones were detected by radioautography or by scanning strips with radiochromatogram scanners. Cyanogenic glucosides were detected by enzymic release of HCN which catalyses the formation of a blue dye from *p*-nitrobenzaldehyde and *o*-dinitrobenzene under slightly alkaline conditions (Bennett & Tapper, 1968).

Analysis of cyanogenic glucosides. Linamarin was purified by paper chromatography or t.l.c. before analysis. Enzymic hydrolysis was carried out in small flasks fitted with centre wells. The linamarin dissolved in approx. 1 ml of 0.25 M-sodium phosphate buffer, pH 5.9, was hydrolysed with 0.5–1 ml of linamarase solution by shaking for at least 24 h at 30°C. The HCN produced by hydrolysis was absorbed in 0.5 ml of 1 M-NaOH in the centre wells and portions were analysed for cyanide (Aldridge, 1944). The specific radioactivity of linamarin was then calculated on the basis of the total radioactivity in linamarin and the amount of HCN produced by hydrolysis. Radioactivity was measured by liquid-scintillation counting.

As paper chromatography gave no evidence for any cyanogenic glycoside other than prunasin in the peach and cherry-laurel extracts, no preliminary separation was required. Portions of the extracts dissolved in sodium phosphate buffer, pH 5.9, were treated in small flasks with 1 mg of β -glucosidase, together with 125 μ g of chloramphenicol to prevent bacterial growth during the hydrolysis.

Benzaldehyde liberated from prunasin was trapped as the semicarbazone in centre wells containing 0.5 ml of 15% (w/v) semicarbazide hydrochloride and the HCN was trapped in cups suspended from the stoppers containing 0.5 ml of 1 M-NaOH. The flasks were shaken at 30°C for 48 h. The benzaldehyde semicarbazone was recrystallized two or three times from ethanol and the u.v. spectrum in ethanol was used to confirm the purity and determine the amount from the peak absorption at 283 nm. Both the quantity and the radioactivity of the liberated cyanide was measured. Two checks with recrystallized prunasin showed 95 and 98% recovery of HCN by this method.

The ¹⁵N analysis was performed by mass spectrometry (Sprinson & Rittenberg, 1949). Small samples of the [¹⁵N]2-oximinoisovaleric acid and [¹⁵N]isobutyraldoxime used for administration to linen-flax seedlings were digested by 3 ml of Kjeldahl digestion mixture in the presence of 10 mg of glucose, as an additional reducing compound. The NH₃ liberated by NaOH was steam distilled into 2% (w/v) boric acid and titrated with 5 mm-H₂SO₄. L-[¹⁵N]Valine was determined by an adaption of the method of Sobel, Hirschman & Besman (1945). Linamarin, isolated by paper chromatography, was treated with 1.5 M-NaOH for 30 min at 100°C to hydrolyse the nitrile moiety to NH₃. The NH₃ was then steam distilled and titrated as described above. Recovery of NH₃ was 95–100% of the theoretical yield from linamarin.

RESULTS

Administration of ¹⁴C-labelled compounds to linen-flax shoots. It has been shown that L-valine is an efficient precursor of linamarin. Table I gives

Table 1. Incorporation of ^{14}C -labelled compounds into linamarin

For experimental details see the text.

Expt. no.	Compound administered			Linamarin produced			
		Amount (μmol)	Sp. radio-activity ($\mu\text{Ci}/\text{mmol}$)	Amount (μmol)	Sp. radio-activity ($\mu\text{Ci}/\text{mmol}$)	Precursor converted (%)	Dilution of ^{14}C
1	L-[U- ^{14}C]Valine	1.2	910	12	17.1	23*	43*
	2-Oximino[U- ^{14}C]isovaleric acid	1.0	1150	11	7.6	9*	121*
2	L-[U- ^{14}C]Valine	3.3	300	15	13.5	25*	18*
	[U- ^{14}C]Isobutyraldoxime	3.4	390	15	18.5	21	21
	[U- ^{14}C]Isobutyraldehyde	1.3	1510	13	<1.0	<0.7	>1510
3	[1- ^{14}C]Isobutyronitrile	10.0	23.3	—	—	9	—
	[1- ^{14}C]Isobutyramide	7.3	46.5	—	—	<0.2	—
4	L-[U- ^{14}C]Valine	1.0	1210	13	17.6	23*	55*
	[1- ^{14}C]Isobutyronitrile	10.1	6.5	11	0.65	11	10
5	2-Hydroxy[1- ^{14}C]isobutyronitrile	2.5	1830	12	105	28	17.5
	Hydrogen [^{14}C]cyanide	2.5	1830	11	<2.5	<0.6	>700

* Corrected for an assumed loss of carboxyl carbon atom from precursor; see the text.

the results of experiments where a range of possible intermediates are compared with L-[U- ^{14}C]valine as precursors of linamarin. Butler & Butler (1960) showed that the administration of DL-[1- ^{14}C]valine gave insignificant label in linamarin, as would be expected if the carboxyl group is lost in the formation of the linamarin aglycone. For this reason the values for radioactivity of linamarin derived from L-[U- ^{14}C]valine and 2-oximino[U- ^{14}C]isovaleric acid have been adjusted by the factor 5/4 to calculate the percentage of precursor incorporated into linamarin and the dilution factor.

Table 1 shows that [U- ^{14}C]isobutyraldoxime and 2-hydroxy[1- ^{14}C]isobutyronitrile were incorporated into linamarin to a similar extent to L-[U- ^{14}C]valine, showing comparable dilution when approximately the same amounts were administered. This indicates that both compounds may be intermediates in linamarin biosynthesis. In each case linamarin was the major labelled product.

2-Oximino[U- ^{14}C]isovaleric acid and [1- ^{14}C]isobutyronitrile were incorporated to a lesser extent than L-[U- ^{14}C]valine although the dilution of the nitrile was not as great, presumably because more of the nitrile was administered. In view of the known non-enzymic conversion of several 2-oximino acids into nitriles by a concerted dehydration and decarboxylation (Ahmad & Spenser, 1961) it is possible, indeed likely, that some or all of the 2-oximino[U- ^{14}C]isovaleric acid was incorporated by way of a prior non-enzymic conversion into [U- ^{14}C]isobutyronitrile. Again linamarin was the major labelled product.

[U- ^{14}C]Isobutyraldehyde, [1- ^{14}C]isobutyramide and hydrogen [^{14}C]cyanide were not significantly incorporated into linamarin although radioauto-

graphs of chromatograms showed evidence for conversion of each of these compounds into other non-volatile compounds. In particular, hydrogen [^{14}C]cyanide extensively labelled compounds tentatively identified as asparagine and glutamine, and less radioactivity appeared in alanine, aspartic acid and some neutral or acidic compounds. Treatment of shoots with 2-hydroxy[1- ^{14}C]isobutyronitrile gave a similar pattern except that linamarin was strongly labelled. This pattern indicates that the solution of 2-hydroxy[1- ^{14}C]isobutyronitrile contained hydrogen cyanide, as would be expected since 2-hydroxynitriles dissociate in aqueous solutions. The incorporation of hydrogen cyanide into asparagine by various plant tissues is known (Conn & Butler, 1969).

Administration of ^{15}N , ^{14}C -labelled compounds to linen-flax shoots. Table 2 shows the results of an experiment where 20 μmol of double-labelled oximes or L-valine were administered to flax shoots. The results show that the ^{15}N label was diluted to approximately the same extent as the ^{14}C of the four aglycone carbon atoms in each treatment. If the C-N bond remained intact then a ratio of one would be expected for the dilutions of ^{15}N and ^{14}C . The relative dilutions obtained with 2-oximinoisovaleric acid and isobutyraldoxime are considered to lie within experimental error of unity. The C-N bond therefore remained essentially intact.

The relative dilution from double-labelled L-valine was just significantly greater than one. This indicates that some transamination of the L-valine may have occurred before the biosynthesis.

These results further indicate the possibility that isobutyraldoxime is an intermediate in linamarin

Table 2. Incorporation of ^{15}N -labelled oximes into linamarinFor experimental details see the text. 'Precursor converted' is calculated from the incorporation of ^{14}C into linamarin.

Compound administered	Linamarin produced				
	^{14}C sp. radioactivity ($\mu\text{Ci}/\text{mmol}$)	Atoms % excess of ^{15}N	Dilution of ^{14}C	Dilution of ^{15}N	Precursor converted (%)
L-[U- ^{14}C , ^{15}N]Valine	152	22.7	37.2*	46	13*
[U- ^{14}C , ^{15}N]2-Oximinisovaleric acid	30.1	22.1	38.5*	42	13*
[U- ^{14}C , ^{15}N]Isobutyraldoxime	187	22.1	20.5	18	24

* Corrected for an assumed loss of carboxyl carbon atom.

biosynthesis. They also exclude any significant incorporation of 2-oximinisovaleric acid by a pathway via valine involving transamination, as in that case a considerable relative dilution of the ^{15}N would have been expected.

Conversion of ^{14}C -labelled compounds into prunasin. Results similar to those described above were obtained with cherry-laurel and peach shoots. These results are given in Tables 3 and 4 respectively and show that phenylacetaldoxime, 2-oximino-3-phenylpropionic acid and phenylacetoneitrile were all better precursors of prunasin than L-phenylalanine, as demonstrated by the higher percentage of the compounds converted into prunasin and the lower dilution of ^{14}C . Again a non-enzymic conversion of 2-oximino-3-phenylpropionic acid into phenylacetoneitrile was possible in its incorporation into prunasin.

If the L-[U- ^{14}C]phenylalanine and [U- ^{14}C]phenylacetaldoxime were converted into prunasin without any cleavage of the phenyl ring from the side chain then the ratio of specific radioactivities of the recovered benzaldehyde and hydrogen cyanide should have been 7:1. The appropriate ratios in Tables 3 and 4 were considered to lie within the experimental error of 7, and therefore retention of the side chain was confirmed.

[1- ^{14}C]Phenylacethydroxamic acid was not an effective precursor of prunasin, thereby indicating that the formation of cyanogenic glycosides by rearrangement and glycosylation of hydroxamic acids was most unlikely.

An attempt was made to test DL-[1- ^{14}C]mandelonitrile as a precursor of prunasin in cherry-laurel shoots and compare the incorporation with that obtained from administering hydrogen [^{14}C]cyanide. The results in Table 3 show that hydrogen cyanide was incorporated into the nitrile moiety of prunasin to the small but significant extent of 4.0% of the amount administered. In the mandelonitrile experiment where the buffered [^{14}C]cyanide solution was given prior treatment with an excess of benzaldehyde the incorporation of radioactivity was increased to 6.5%. The very low specific radioactivity of the benzaldehyde from prunasin indicated that little randomization of label had occurred.

DISCUSSION

The results obtained from the experiments with linen-flax seedlings, peach shoots and cherry-laurel shoots, together with the findings of Kindl & Underhill (1968), support a general pathway of cyanogenic glycoside biosynthesis involving a sequence of reactions from amino acids through aldoximes, nitriles and 2-hydroxynitriles, as illustrated in Scheme 1.

Table 3. *Incorporation of ^{14}C -labelled compounds into prunasin in cherry-laurel shoots*

For experimental details see the text. In Expt. 1 the tissue was exposed for 44 h whereas in Expts. 2, 3, and 4 the tissue was exposed for 28 h

Expt. no.	Compound administered			Products from prunasin				
	Amount (μmol)	Sp. radio-activity ($\mu\text{Ci}/\text{mmol}$)	Tissue used (g fresh wt.)	HCN produced (μmol)	Precursor converted (%)	Benzaldehyde/ $\text{HCN } ^{14}\text{C}$ ratio	Dilution of ^{14}C	
1	L-[U- ^{14}C]Phenylalanine	1.07	3.4	326	16*	6.7	1940*	
2	[U- ^{14}C]Phenylacetaldoxime	1.2	3.4	314	43*	8.3	610*	
	L-[U- ^{14}C]Phenylalanine	2.14	3.8	278	8.3*	6.9	1550*	
3	2-Oximino-3-phenyl[2- ^{14}C]propionic acid	6.1	3.8	303	41†	<0.001	120†	
	L-[U- ^{14}C]Phenylalanine	2.14	2.9	260	14*	5.5	880*	
	[1- ^{14}C]Phenylacetoneitrile	3.14	2.9	250	63†	<0.0002	127†	
4	[1- ^{14}C]Phenylacetaldoxime	1.81	2.9	300	<0.3†	—	—	
	DL-[1- ^{14}C]Mandelonitrile	2.0	2.4	265	6.5†	0.007	2020†	
	Hydrogen [^{14}C]cyanide	2.0	2.4	280	4.0†	0.08	3630†	

* Based on conversion of $\text{C}_6\text{-C}_1$ moiety of precursor into benzaldehyde.

† Based on incorporation of radioactivity into hydrogen cyanide.

Table 4. *Incorporation of ^{14}C -labelled compounds into prunasin in peach shoots*

For experimental details see the text. In Expt. 1 the tissue was exposed for 44 h whereas in Expts. 2 and 3 the tissue was exposed for 28 h.

Expt. no.	Compound administered		Products from prunasin				
	Amount (μmol)	Sp. radio-activity ($\mu\text{Ci}/\text{mmol}$)	Tissue used (g fresh wt.)	HCN produced (μmol)	Precursor converted (%)	Benzaldehyde/ $\text{HCN } ^{14}\text{C}$ ratio	Dilution of ^{14}C
1	L-[U- ^{14}C]Phenylalanine	1.07	3.6	177	11*	6.9	1460*
	[U- ^{14}C]Phenylacetaldoxime	1.2	3.6	157	39*	7.3	340*
2	L-[U- ^{14}C]Phenylalanine	2.14	3.5	257	23*	7.4	520*
	2-Oximino-3-phenyl[2- ^{14}C]propionic acid	6.1	271	231	47†	<0.001	80†
	2-Oximino-3-phenyl[2- ^{14}C]propionic acid	2.58	271	251	62†	<0.001	160†
	[U- ^{14}C]Phenylacetaldoxime	2.08	23	230	29*	6.9	380*
3	L-[U- ^{14}C]Phenylalanine	2.14	1.7	38	7.6*	5.5	240*
	[1- ^{14}C]Phenylacetoneitrile	3.32	1.7	41	57†	<0.0001	21†

* Based on conversion of $\text{C}_6\text{-C}_1$ moiety of precursor into benzaldehyde.

† Based on incorporation of radioactivity into hydrogen cyanide.

Scheme 1. Proposed pathway for the biosynthesis of cyanogenic glucosides.

The experiments with linen-flax shoots and peach and cherry-laurel shoots demonstrated that suitable aldoximes, isobutyraldoxime and phenylacetaldoxime respectively, may be converted into cyanogenic glucosides. Isobutyraldoxime was converted into linamarin with the C-N bond intact, as would be expected if this compound is an intermediate derived from L-valine. Similarly, isobutyronitrile and phenylacetoneitrile were significantly incorporated into linamarin and prunasin respectively.

Although neither isobutyronitrile nor isobutyraldoxime were incorporated to a much greater extent than L-valine during the experiments, both were incorporated sufficiently to implicate them as intermediates. Factors that could decrease their incorporation were their volatility and possible toxic effect when administered. Both compounds are lipophilic and could well have a disruptive effect

on cell membranes and organelles at the administered concentrations.

The relatively high incorporation of 2-oximinoisovaleric acid and 2-oximino-3-phenylpropionic acid into linamarin and prunasin respectively is noteworthy. These compounds are not considered intermediates in the biosynthesis of structurally related glucosinolates (Kindl & Underhill, 1968; Tapper & Butler, 1967) although aldoximes, which are intermediates of glucosinolate biosynthesis, could in theory be derived from them by decarboxylation. It is reasonable to suppose that the observed incorporation of 2-oximino acids was via the nitriles. The non-enzymic conversion of a range of 2-oximino acids into nitriles has been reported (Ahmad & Spenser, 1961). The status of 2-oximino acids as intermediates in cyanogenic glycoside biosynthesis must therefore remain uncertain until evidence for a conversion into aldoximes is obtained or until the presence of enzymes for their formation and utilization is demonstrated.

With linen flax, good evidence was obtained to suggest that 2-hydroxyisobutyronitrile was also an intermediate. Confirmatory evidence has come from other experiments (Hahlbrock *et al.* 1968).

Results for incorporation of DL-mandelonitrile were less definitive. A notable feature was the significant incorporation of radioactivity from hydrogen cyanide into prunasin. This is attributed to a reaction between benzaldehyde derived from prunasin in the tissue and the administered hydrogen cyanide yielding a limited amount of labelled mandelonitrile. The labelled mandelonitrile could then be converted into prunasin without an overall net synthesis of prunasin.

The relatively low incorporation from the DL-[1-¹⁴C]mandelonitrile treatment may have been due to a number of factors. Mandelonitrile may not have been formed efficiently under the conditions used and some of the benzaldehyde may have been converted into unlabelled benzoin. Further, the mandelonitrile, excess of benzaldehyde, or any other compounds formed in the reaction, may have been toxic. It was observed that the bottom 3 mm of the shoots, which were immersed in the solution, showed 'browning' after about 30 min exposure. This effect cannot be attributed to a toxic effect of hydrogen cyanide alone, as the shoots treated with cyanide showed only modest 'browning' of the cut surface at the end of the experiment. Some of the incorporation observed may be attributed to incorporation of hydrogen [¹⁴C]cyanide similar to that already mentioned.

Experimental precedent for hydroxylation of the amino acid as a first step has come from studies on the biosynthesis of the antibiotic hadacin (Stevens & Emery, 1966). It was shown that this hydroxamic

acid derivative was formed by *N*-hydroxyglycine and a formyl donor and that *N*-hydroxyglycine could be obtained from glycine. Similarly Kindl & Underhill (1968) have indirect evidence for *N*-hydroxyphenylalanine formation in cell-free preparations of *Tropaeolum majus* L. This compound was shown to be converted into phenylacetaldoxime.

The final step in the biosynthesis of linamarin or prunasin and several closely related cyanogenic glucosides is probably the transfer of glucose to a 2-hydroxynitrile from a nucleoside 5'-diphospho-D-glucose by a mechanism of the general type for formation of glycosidic bonds. An enzyme capable of producing linamarin from UDP-glucose and 2-hydroxyisobutyronitrile has been purified from flax and its specificity studied (Hahlbrock & Conn, 1970).

Confirmation of the postulated intermediate roles of aldoximes, nitriles and 2-hydroxynitriles awaits the demonstration of the formation of the compounds *in vivo* and the characterization of specific enzymes for each reaction.

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REFERENCES

- Ahmad, A. & Spenser, I. D. (1961). *Can. J. Chem.* **39**, 1340.
- Aldridge, W. N. (1944). *Analyst, Lond.*, **69**, 262.
- Bennett, W. D. & Tapper, B. A. (1968). *J. Chromat.* **34**, 428.
- Bielecki, R. L. & Turner, N. A. (1966). *Analyt. Biochem.* **17**, 278.
- Bleichert, E. F., Neish, A. C. & Towers, G. H. N. (1966). In *Biosynthesis of Aromatic Compounds*, p. 119. Ed. by Billek, G. Oxford: Pergamon Press.
- Butler, G. W. & Butler, B. G. (1960). *Nature, Lond.*, **187**, 780.
- Butler, G. W. & Conn, E. E. (1964). *J. biol. Chem.* **239**, 1674.
- Conn, E. E. & Butler, G. W. (1969). In *Perspectives in Phytochemistry*, p. 47. Ed. by Harborne, J. B. & Swain, T. London: Academic Press.
- Coop, I. E. (1940). *N.Z. Jl. Sci. Technol.* **22**, 71 B.
- Gander, J. E. (1962). *J. biol. Chem.* **237**, 3229.
- Hahlbrock, K. & Conn, E. E. (1970). *J. biol. Chem.* **245**, 917.
- Hahlbrock, K., Tapper, B. A., Butler, G. W. & Conn, E. E. (1968). *Archs Biochem. Biophys.* **125**, 1013.
- Kindl, H. & Underhill, E. W. (1968). *Phytochemistry*, **7**, 745.
- Mentzer, C., Favre-Bonvin, J. & Massias, M. (1963). *Bull. Soc. Chim. biol.* **45**, 749.
- Sobel, A. E., Hirschman, A. & Besman, L. (1945). *J. biol. Chem.* **161**, 99.
- Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 707.
- Stevens, R. L. & Emery, T. F. (1966). *Biochemistry, Easton*, **5**, 74.
- Tapper, B. A. & Butler, G. W. (1967). *Archs Biochem. Biophys.* **120**, 719.
- Tapper, B. A., Conn, E. E. & Butler, G. W. (1967). *Archs Biochem. Biophys.* **119**, 593.
- Uribe, E. G. & Conn, E. E. (1966). *J. biol. Chem.* **241**, 92.