Cyanogenic Glycosides

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Approximately 1000 plant species representing 90 families and at least 250 genera have been reported to be cyanogenic. Several dozen species have been studied in greater detail, in some instances because of their economic significance, and 11 cyanogenic glycosides have been identified. The majority of these are formed by the plant from one of four amino acids (valine, isoleucine, phenylalanine, and tyrosine) by means of a biosynthetic pathway involving oximes, nitriles, and α-hydroxynitriles (cyanohydrins).

The cyanogenic glycosides are toxic because they yield hydrogen cyanide (HCN) when enzymically degraded. Enzymatic hydrolysis results when the plant tissue is crushed or otherwise disrupted, but hydrolysis by the digestive enzymes of an animal feeding on cyanophoric plants may also occur. Plant tissues containing cyanogens may be rendered less toxic by extraction or by maceration and dehydration to remove the volatile HCN.

The cyanogenic glycosides are compounds which liberate HCN, one or more molecules of sugar, and, with one exception (Rimington, 1935), an aldehyde or ketone on treatment with dilute acid or the appropriate hydrolytic enzymes. These compounds have a wide distribution among the higher plants (Dilleman, 1958) but are also found in insects (Blum and Woodring, 1962; Eisner *et al.*, 1963; Jones *et al.*, 1962) and fungi (see Tschiersch, 1967, for review). If the concentration of glycoside in a cyanophoric plant is sufficiently high, the plant will be toxic to animals owing to the production of hydrocyanic acid (HCN) when the plant is ingested. Hydrocyanic or prussic acid is a highly toxic material, 200 mg. being a lethal dose for an adult human; an atmosphere containing 200 p.p.m. will result in death within a few minutes. HCN is an effective inhibitor of many metalloenzymes, but cytochrome oxidase, the terminal oxidase of aerobic organisms, is the primary site of action. Cyanide poisoning therefore results in death due to oxygen starvation at the cellular level.

The ability of plants to produce cyanide from a parent compound was first reported in 1803 when Schrader (1803), a German pharmacist, recognized the odor of HCN produced by the cherry laurel and other plants. This ability of plants to produce HCN, known as cyanogenesis, is exhibited by at least 1000 species representing approximately 90 families and at least 250 genera (Dilleman, 1958; Hegnauer, 1963). Although the parent substance in a majority of these cyanophoric plants has not been identified, it is probably one of the 12 cyanogenic glycosides whose structures have been described (Table I).

STRUCTURE AND DISTRIBUTION

Amygdalin (Figure 1), which is probably the most familiar of the cyanogenic glycosides and is found in many members of the *Rosaceae*, is a β-glycoside of D(−) mandelonitrile or benzaldehyde cyanohydrin. On hydrolysis, this glycoside yields 2 moles of glucose and one mole each of benzaldehyde and HCN. Amygdalin was the subject of many chemical studies during the 19th Century (see Robinson, 1930, for review). Wöhler and

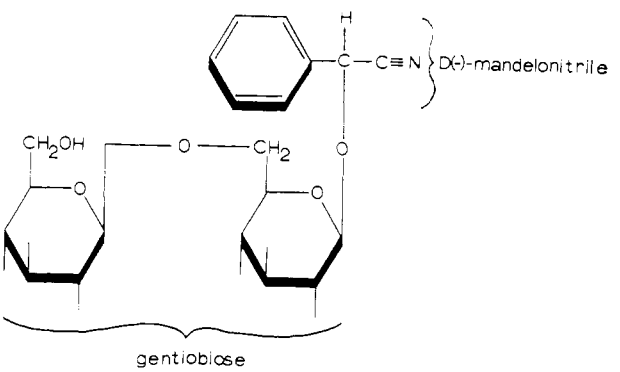

Figure 1. Amygdalin

Liebig (1837) reported that alkaline hydrolysis yielded ammonia and the glycoside of the corresponding carboxylic acid, amygdalinic acid. These authors first described the action of an enzyme system, called emulsin, in almonds, which decomposed amygdalin into its components. Emulsin contains a β-glucosidase, among other enzymes, that is responsible for hydrolyzing the β-linkage. Over the years, a positive action of this enzyme has been taken as evidence of the glycoside having the β rather than α-linkage.

Emil Fischer (1895) investigated the action of enzymes from different sources on amygdalin and reported that a preparation from yeast catalyzed the hydrolysis between the two sugars to form glucose and a cyanogenic glucoside containing only one sugar molecule. This compound was subsequently found to occur naturally in *Cerasus padus* (Herissey, 1907), other prunus species, and *Photinia serrulata* Lindl. (Herissey, 1912), and was given the name prunasin. An enzyme in snails (Giaja, 1919) acts on amygdalin to cleave the linkage between the cyanohydrin and sugar moieties and form gentiobiose. The structure of amygdalin was finally considered established in 1924 when Campbell and Haworth (1924) synthesized the cyanogen.

Table I lists several glycosides which contain mandelonitrile as the aglycone. The asymmetric carbon atom at the point of attachment of the glucose provides the possibility of stereoisomerism, and the diastereomer of prunasin containing L(+) mandelonitrile is known as sambunigrin

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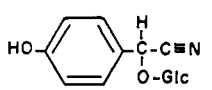
Table I. Cyanogenic Glycosides of Known Structure[a]

Glycoside	Sugar	Aglycone	Occurrence
Amygdalin	Gentiobiose	D-Mandelonitrile	*Prunus* Sp.
Prunasin	D-Glucose	D-Mandelonitrile	*Prunus* Sp., Many *Rosaceae* *Eucalyptus* Sp.
Sambunigrin	D-Glucose	L-Mandelonitrile	*Sambucus nigra*, *Acacia* Sp. (Australin)
Prulaurasin	D-Glucose	DL-Mandelonitrile[b]	
Vicianin	Vicianose	D-Mandelonitrile	*Vicia angustifolia* L. and other Vicia
Dhurrin	D-Glucose	L-*p*-Hydroxymandelonitrile	*Sorghum* Sp.
Taxiphyllin	D-Glucose	D-*p*-Hydroxymandelonitrile	*Taxus* Sp.
Zierin	D-Glucose	*m*-Hydroxymandelonitrile	*Zieria laevigata* Sm.
Linamarin	D-Glucose	α-Hydroxyisobutyronitrile (acetone cyanohydrin)	*Linum usitatissimum*, L.; *Phaseolus lunatus*, L.; *Trifolium repens*. L. *Lotus* Sp., *Manihot* Sp., *Dimorphotheca* Sp.
Lotaustralin	D-Glucose	α-Hydroxy-α Methyl Butyronitrile (methylethyl ketone cyanohydrin)	(See Linamarin)
Acacipetalin	D-Glucose	β-Dimethyl-α-Hydroxyacrylonitrile	*Acacia* Sp. (South African)
Gynocardin[c]	D-Glucose	Gynocardinonitrile	*Gynocardia odorata*. R.Br. *Pangium edule*. Reinw.

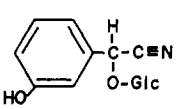
[a] See Dilleman (1958) for references.
[b] Prulaurasin is undoubtedly an artifact of isolation (Plouvier, 1935).
[c] Gynocardin structure proposed by Coburn and Long (1966).

after the plant *Sambucus nigra* L., in which it was first detected (Bourquelot and Danjou, 1905; Guignard, 1905). An equimolar mixture of the two compounds known as prulaurasin was originally believed to occur naturally (Herissey, 1906) but subsequent work showed the mixture to be an artefact of isolation (Plouvier, 1935). Vicianin, found in some members of the vetch family (Bertrand, 1906) is related to amygdalin in that the sugar component is a disaccharide vicianose, consisting of one molecule of glucose and one of arabinose.

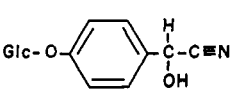
Several cyanogenic glycosides (Figure 2) are derivatives of hydroxymandelonitrile and therefore yield one of the hydroxybenzaldehydes on hydrolysis. The *Sorghum* genera contains the glucoside dhurrin, the aglycone of which is *p*-hydroxymandelonitrile (Dunstan and Henry, 1902). A positional isomer of dhurrin is the compound zierin, which is found in an Australian rutaceous shrub, *Zieria laevigata* S. (Finnemore and Cooper, 1936). Still another isomer of dhurrin is *p*-glucosyloxymandelonitrile, the labile cyanogenic glycoside of *Nandina domestica* Thunb., the ornamental shrub known as heavenly bamboo (Abrol *et al.*, 1966). In this compound, the glucose is attached to the aromatic hydroxyl, and the compound is unstable, dissociating to form *p*-glucosyloxybenzaldehyde and HCN. Recent evidence supports the view that the labile cyanogen in *Thallictrum aquilegifolium* L. also has this structure (Sharples and Stoker, 1969). There is also the possibility



DHURRIN



ZIERIN



p-GLUCOSYLOXY-MANDELONITRILE

Figure 2. Hydroxymandelonitrile glucosides

of a second glucose molecule being attached to the aliphatic hydroxyl position in this compound and such a diglucoside has been reported by Young and Hamilton (1966).

Studies on the conformation (Towers *et al.*, 1964) of dhurrin became possible when methods were developed (Mao *et al.*, 1965) for isolation of substantial quantities of this compound as a crystalline product. The conformation of the group of atoms attached to the asymmetric carbon atom of the cyanohydrin moiety and the C_1 of glucose in dhurrin and its diastereomer taxiphyllin are shown in Figure 3. The diastereomer which contains D-*p*-hydroxymandelonitrile was isolated by Towers *et al.* (1964) from the Canadian yew, and the proton magnetic resonance spectrum of its pentaacetate was compared with that of dhurrin. These spectral data, together with the molar rotations observed, permitted Towers *et al.* (1964) to represent dhurrin as R′=CN and R″ as H, and taxiphyllin therefore as R′=H and R″ as $^{-}$CN.

Two other cyanogenic glycosides which are widely distributed (Butler, 1965) in the higher plant kingdom are linamarin and lotaustralin, the glucosides of acetone cyanohydrin and methylethylketone cyanohydrin. An interesting aspect of these compounds is that they usually coexist in the same plant, although they may be present in widely differing amounts. Thus, the linen flax plant contains a 50-50 mixture of linamarin and lotaustralin while *Lotus arenarius* Brot. contains the two compounds in a ratio of 99 to 1. On the other hand, the ratio of linamarin to lotaustralin in *Lotus tenuis* L. is 4 to 96.

Gynocardin, the cyanogenetic glucoside of *Gynocardia odorata* R. Br. (Power and Lees, 1905) has been the subject of a recent structural study by Coburn and Long (1966). While the configuration of all the groups has not yet been established, the structure proposed by Coburn and Long is given in Figure 4. The biosynthetic origin of gynocardin, which is also believed to be the cyanogen of *Panguim edule* Reinw. seeds (De Jong, 1911), should prove to be a particularly interesting problem when one considers the biosynthesis of the cyanogenic glycosides described later.

There is extensive literature on toxic plants which describes many reports of poisoning both of humans and

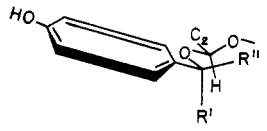


Figure 3. The conformation of groups or atoms attached to the asymmetric carbon atom of the aglycone of dhurrin and taxiphyllin

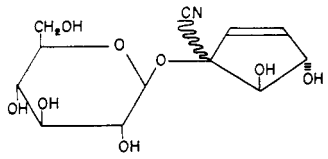


Figure 4. Gynocardin after Coburn and Lang (1966)

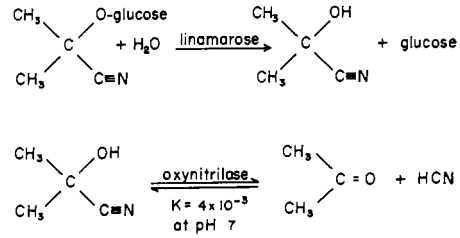


Figure 5. The enzymatic degradation of linamarin

livestock (see Kingsbury, 1964; Watt and Breyer-Brandwijk, 1962). Cyanogenic glycosides are only one example of the toxic compounds that plants may possess, and a critical analysis of recent cases of human poisoning by plants would probably disclose that cyanogenic glycosides were not responsible. On the other hand, the cyanide content of animal forage crops such as sorghum, New Zealand clover, and lima beans used as cattle feed has frequently been responsible for livestock poisoning. Plant breeding has been utilized to obtain strains of these species having low cyanide content that can be safely used.

Natural populations of *Trifolium repens* L. and *Lotus corniculatus* L. contain both cyanogenic and noncyanogenic plants. Genetic analysis of these species has disclosed that two genes determine the cyanophoric property of the cyanogenic specimens. Thus, a gene dominant for production of the glucoside and another dominant for the β-glucosidase which hydrolyzes the glucoside have been recognized. The four phenotypes exist in nature and of course may be obtained by breeding. Each can be distinguished by appropriate tests (Daday, 1965; Corkill, 1942, 1952).

It is difficult to generalize regarding which part of a cyanophoric plant will contain the cyanogenic glycoside. Leaves frequently contain the highest concentration but cyanogens may be formed in almost any part. The young, vigorously growing, green leaves of the sorghum plant are a rich cyanide source; the concentration of HCN may reach 0.2% in such leaves. On the other hand, sorghum is a common material used for ensilage, and the dried and partially decayed leaves are clearly no longer toxic. This is due to the rapid breakdown of the cyanogenic glucoside by endogenous enzymes as the plant ferments in the silo. Interestingly enough, the seed of the sorghum plant which is widely consumed as food by humans in India and as an animal feed in this country is not toxic because the seed contains no detectable amount of the cyanogenic glucoside. On germination, however, the dark grown seedling may reach a concentration of 3 to 5% dhurrin on a dry weight basis within a period of three to four days.

Members of the cherry family have been cited as responsible for much loss of livestock in the United States (Kingsbury, 1964). The leaves and fruit stones of this family, which includes the apricot, peach, cherry, and almond, contain one or more of the mandelonitrile glycosides. At least one instance of human poisoning is recorded in the case of children who ate large amounts of western choke cherries without removing the stone (Pijoan, 1942). Almonds may be divided into bitter and sweet varieties. The former contains amygdalin in significant quantities; consequently, commercial almond production in the United States is confined to the sweet variety.

Another plant in which there are varietal differences is the lima bean (*Phaseolus lunatus* L.). The uncultivated native bean, found in Central and South America is smaller and highly cyanophoric, producing as much as 0.3% HCN on hydrolysis. Plant breeders have been successful in breeding low cyanide varieties of this species and there is an interesting correlation between the amount of cyanogen—in this case, primarily linamarin—in the seed and the color and size of the bean. Highly colored beans have been shown to have a higher linamarin content whereas those grown for human consumption are white, large, nearly flat, and noncyanophoric. Again the literature cites instances of humans being poisoned by the tropical lima bean when they were mistaken for navy beans.

Several ornamental plants—heavenly bamboo, *Nandina domestica*, and the ornamental yew (Taxus species), previously cited—are cyanophoric. The cherry laurel (*Prunus laurocerasus* L.), extensively used in laurel hedges, is especially rich in prunasin. The concentration in cherry laurel leaves may run as high as 75 μmoles per gram fresh weight corresponding to about 1% HCN on a dry weight basis. Squashed leaves are commonly used by collectors to kill butterflies and moths. The berries of *Nandina domestica* can contain up to 150 μmoles per gram. Obviously, if any significant quantity of these materials is consumed by a human, particularly a child, rapid poisoning will occur. Fortunately, the plants mentioned usually have other bitter components which prevent their being consumed in any large amount.

One extremely important cyanogenic plant that furnishes the basic food for millions of people is the cassava or manioc (*Manihot esculenta* Cranz). The root of the plant is edible and is the source of commercial tapioca. Persons employing this plant as a major source of carbohydrate will scrape or grate the roots and then soak the tissue in a bag to leach out the cyanogen, which is linamarin (Clapp *et al.*, 1966; Dunstan *et al.*, 1906). After drying and sifting the tissue, a flour is obtained which can be made into thin cakes known as cassava bread. During the second World War, an army pamphlet on edible tropical plants warned service men that the bitter varieties of the cassava were indeed highly toxic and could be made edible only if cooked. Bitter and sweet varieties, which are poisonous and nonpoisonous, respectively, are known.

The botanist has been interested in the significance of the cyanogenic glucosides as a taxonomic tool (Alston and Turner, 1963), but the literature on cyanogenicity in plants is of limited value, primarily because most references state only that HCN is produced. If the cyanogenic glycoside had been identified in each case, the taxonomic value of the observation would have been greatly increased. Closely related if not identical species may differ greatly in this single property of cyanogenicity. Thus, common vetch (*Vicia sativa* L.) does not contain a cyanogenic

glucoside whereas *Vicia angustifolia* L., which many botanists consider as a variety of *V. sativa*, is the plant in which the cyanogen vicianin occurs. The distribution of cyanophoric plants (1000 species in some 90 families and 250 genera) is indeed large and may be even more widespread than presently reported because of the relatively insensitive chemical tests that were used by botanists in the past. Today there are more sensitive colorimetric methods available, and a new effort in this regard may show that these compounds are even more widely distributed than is now believed.

ENZYMIC DEGRADATION OF CYANOGENIC GLYCOSIDES

With few if any exceptions, cyanogenic plants contain an enzyme system which is capable of converting the cyanogenic glycoside to sugar, HCN, and an aldehyde or ketone. The crude preparation of enzymes from almonds, known as emulsin, has long been recognized to carry out the complete degradation of amygdalin and other glycosides into the three components mentioned. This process, however, may be broken down into two separate enzymatic steps as illustrated for the cyanogen linamarin in Figure 5. Initially, there is an enzymatic hydrolysis by a β-glucosidase to yield the corresponding cyanohydrin and sugar. This is followed by the dissociation of the cyanohydrin to yield the corresponding aldehyde or ketone and HCN, and this reaction is catalyzed by an enzyme known as oxynitrilase or hydroxynitrile lyase.

Only recently have the cyanogenic glucosidases been purified and studied. In 1965 Butler *et al.* partially purified the β-glucosidase in flax which catalyzes the hydrolysis of the aliphatic glucosides linamarin and lotaustralin. The partially purified flax glucosidase catalyzes the hydrolysis of prunasin as well as linamarin and lotaustralin, but does not act on amygdalin. These workers also obtained evidence for another enzyme in the same plant which would hydrolyze amygdalin. Clearly, these two β-glucosidases exhibit specificity for the type of sugar bound in glycosidic linkage. Butler *et al.* (1965) also confirmed the earlier finding of Finnemore and Cooper (1938), that almond emulsin was essentially inactive on linamarin and lotaustralin. Although the inability of emulsin to catalyze the hydrolysis of linamarin had been considered as evidence that the flax glucoside was an α-glucoside, Butler *et al.* (1965) presented data (infra red spectra) confirming the assignment of the β-configuration. More recently, Clapp *et al.* (1966) have conclusively shown linamarin to be a β-glucoside. In the absence of evidence to the contrary, lotaustralin may also be considered a β-glucoside.

Mao and Anderson (1967) have purified the β-glucosidase in sorghum which catalyzes the hydrolysis of dhurrin and found that it has rather broad specificity with respect to its substrate. Thus, taxiphyllin, the diastereomer of dhurrin, is hydrolyzed about 50% more rapidly than is dhurrin, and prunasin, which lacks the aromatic hydroxyl, is hydrolyzed even more rapidly. Finally, Haisman and Knight (1967) have examined the enzymes present in almond emulsin and concluded that three enzymes are present. There is one β-glucosidase, which converts amygdalin to prunasin, another which hydrolyzes prunasin to mandelonitrile and glucose, and a third enzyme that catalyzes the dissociation of mandelonitrile to benzaldehyde and HCN.

The enzyme in sorghum, known as oxynitrilase or hydroxynitrile lyase, catalyzes the cyanohydrin equilibrium

Figure 6. The reversible dissociation of *p*-hydroxymandelonitrile

between *p*-hydroxybenzaldehyde, HCN, and *p*-hydroxymandelonitrile (Figure 6). This enzyme, which has been isolated from young sorghum seedlings and purified to a homogeneous state by Seely *et al.* (1966), exhibits a high degree of substrate specificity. Thus, the maximum rate of dissociation of *p*-hydroxymandelonitrile is observed to be about 140 times as large as that of mandelonitrile, which differs only by lacking the *para*-hydroxy group. Vanillin cyanohydrin, which has a *meta*-*O*-CH_3 group as well as the *para*-hydroxy group, is only one-seventh as rapidly attacked by the enzyme, and isovanillin cyanohydrin, in which these groups are reversed (and the *para*-hydroxyl group therefore absent), is acted upon at a rate only $^1/_{1000}$ of that of *p*-hydroxymandelonitrile dissociation. Clearly, the sorghum enzyme requires the *para*-hydroxy substitution for significant activity.

Pfeil and his associates (Becker *et al.*, 1963) have extensively purified the oxynitrilase of almonds as did Seely *et al.* (1966). The almond enzyme is reported by these workers (Becker and Pfeil, 1966) to have a much broader substrate specificity in that it will react with the cyanohydrins of a large number of aromatic, aliphatic and heterocyclic aldehydes. This type of enzyme was first reported by Rosenthaler (1908) who was interested in the asymmetric synthesis which this enzyme catalyzed. Rosenthaler reported that almond preparations could catalyze the formation of an optically active cyanohydrin and devised an assay based on the optical rotation of the product formed. A correction has to be made for the nonenzymic reaction between aldehyde and HCN that yields the racemic mixture of the two cyanohydrins. This is the analytical method which Pfeil used in his studies, but it is also possible to follow the reaction by measuring the absorption of ultra-violet light by the aromatic aldehyde that is formed on dissociation of the cyanohydrin. The absorption coefficient of the aldehyde is much greater than that of the parent cyanohydrin.

One particularly interesting aspect of this work concerns the requirement of the almond hydroxynitrile lyase for a prosthetic group, namely flavin adenine dinucleotide (FAD). Becker and Pfeil (1966) have shown that their purified preparations of the almond enzyme contain one mole of FAD per equivalent molecular weight of 75,000. They have further demonstrated the reversible dissociation of FAD from this enzyme by precipitation with ammonium sulfate at acidic pH, and the lack of enzymatic activity in the absence of the flavin. While Seely *et al.* (1966) were able to confirm that the almond enzyme isolated from almonds also contained flavin, they were not able to accomplish the reversible dissociation of the flavin. More important, however, the sorghum enzyme, which is homogeneous by the usual physical chemical criteria, lacks a flavin prosthetic group and yet retains full activity. It is difficult to see why a flavin should participate as a cofactor in the cyanohydrin equilibrium since the reaction does not involve oxidation-reduction. Since the sorghum enzyme does not have this prosthetic group, further work

must be performed to answer this interesting question. Recently, Stevens and Strobel (1968) have reported a fungal hydroxynitrile lyase which catalyzes the reversible dissociation of the cyanohydrins of acetone and methylethylketone, but which is inactive with aromatic cyanohydrins.

BIOSYNTHESIS OF CYANOGENIC GLYCOSIDES

The extensive amount of work on the biosynthesis of cyanogenic glycosides in recent years has been recently reviewed (Conn and Butler, 1969). Research in several laboratories has shown that four of the known cyanogenic glucosides are derived from closely related amino acids (Figure 7). Thus, tyrosine is known to be effectively converted into dhurrin in the sorghum plant (Conn and Akazawa, 1958; Gander, 1958) and similarly, tyrosine is converted into taxiphyllin in shoots of the yew tree (Bleichert *et al.* 1966). Phenylalanine has been shown to be converted to prunasin in cherry laurel leaves (Mentzer and Favre-Bonvin, 1961) and in young peach seedlings (Ben-Yehoshua and Conn, 1964). Butler and Butler (1960) first showed the conversion of valine to linamarin in New Zealand clover as well as the conversion of isoleucine to lotaustralin. A similar conversion of these amino acids to linamarin and lotaustralin in flax seedlings was shown by Butler and Conn (1964).

In each of these biosyntheses, there is a loss of the carboxyl carbon and an oxidation of the amino carbon and nitrogen atoms to the level of a nitrile. These relationships are indicated in Figure 8, where the origins of the nitrile carbon and nitrogen atoms and the glycosidic carbon

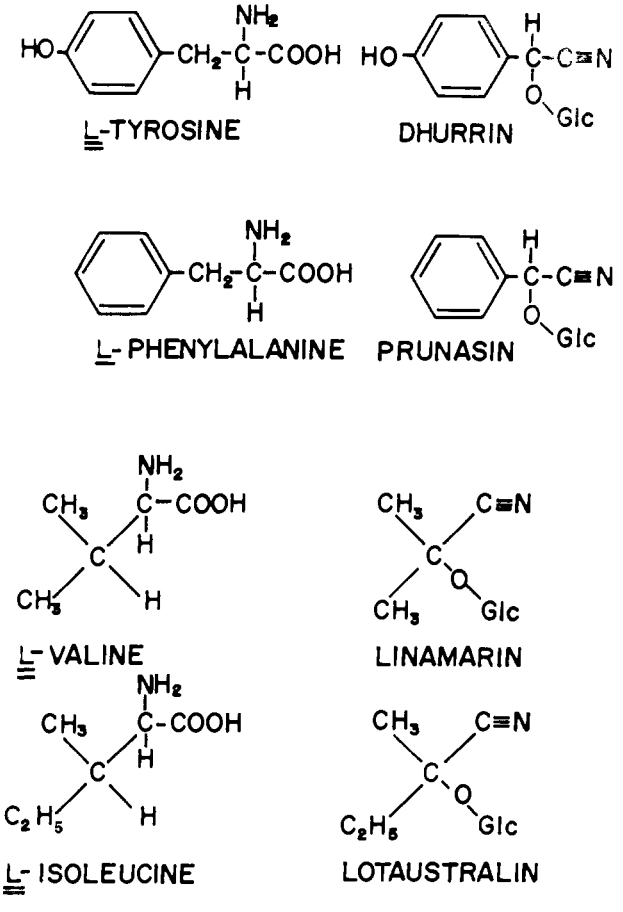


Figure 7. The amino acid precursors of four cyanogenic glucosides

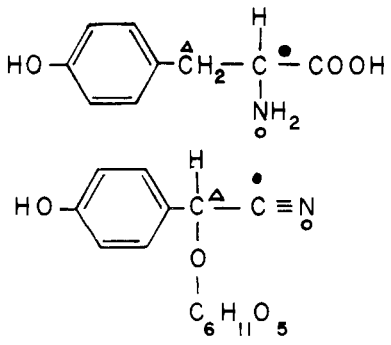


Figure 8. The origin of the glucosidic carbon and nitrile atoms of dhurrin

atom from the precursor amino acid are shown. Using tyrosine doubly labeled with ^{14}C in the alpha carbon and ^{15}N in the amino group, Uribe and Conn (1966) showed that the nitrile group of dhurrin was derived directly from the precursor amino acid (Table II). Similar data were obtained for the conversion of tyrosine to taxiphyllin in yew shoots (Bleichert *et al.*, 1966) and the conversion of doubly labeled valine to linamarin in flax seedlings (Butler and Conn, 1964). These data indicated that all intermediates between the amino acid and the cyanogenic glycoside were nitrogenous in nature.

Another experiment that placed limitations on the nature of the intermediates between the precursor amino acid and cyanogenic glucoside was performed by Koukol *et al.*, (1962). To determine whether the bond between the C—2 and C—3 atoms of tyrosine is broken as tyrosine is converted to dhurrin, sorghum seedlings were fed tyrosine labeled in the 2- and the 3-positions in known ratios. The ratio of the specific activities of *p*-hydroxybenzaldehyde and HCN obtained on hydrolysis of the biosynthesized dhurrin was determined and found to be maintained in three separate experiments. Therefore, it was concluded that the carbon-carbon bond between α- and β-carbon atoms is not cleaved as the cyanogenic glucoside is formed. These results ruled out the possibility of the alpha carbon of tyrosine being in some manner converted to HCN, the ring and β-carbon atoms of the amino acid giving rise to *p*-hydroxybenzaldehyde, and these two moieties condensing to yield a cyanohydrin that could be glucosylated. In the biosynthesis of taxiphyllin from tyrosine in *Taxus*, Bleichert *et al.* (1966) similarly have shown that the bond between C—2 and C—3 remains intact.

Some of the difficulties in this research may be better appreciated when the experimental approach is described. Dry sorghum seed contains no dhurrin, but etiolated seedlings that are three to five days old may contain 3 to 5% dhurrin on a dry weight basis (Akazawa *et al.*, 1960).

Table II. Dilution of ^{14}C and ^{15}N on Incorporation of L-Tyrosine-^{14}C-^{15}N into Dhurrin-^{14}C-^{15}N

	^{15}N Concentration (Apxs)		^{14}C Concentration (C.P.M./μatom C)	
Expt.	Tyr	Dhurrin	Tyr-α-C	Dhurrin-CN
1	50.03	1.17	20,800	610
2	50.03	1.18	42,800	1220

	Dilution of		Ratio ^{14}C:^{15}N	
Expt.	^{14}C	^{15}N	Calcd.	Found
1	34	43	1.00	1.26
2	35	42	2.00	2.40

Data taken from Table I in Uribe and Conn (1966).

Similarly, flax seed has a very low content (0.1% dry wt.) of cyanogenic glucoside, but the young green seedling may contain 5% dry wt. of linamarin plus lotaustralin (Butler and Conn, 1964). Such seedlings may be supplied possible precursors at a time when glycoside synthesis is rapidly occurring and the glycoside produced should be labeled. In typical experiments, 20 μmoles of labeled tyrosine fed to 1 gram of sorghum seedlings will be converted to the extent of 15 to 20% in a period of 24 hours. There is, therefore, a very efficient conversion, and one might reasonably expect to detect one or more intermediates between the amino acid and the cyanogen.

When attempts were made in different laboratories to detect possible intermediates in this biosynthetic pathway, they were unsuccessful. Thus, experiments were carried out with plants in the dark or in the presence of metabolic inhibitors (Gander, 1960, 1962) in such a way that energy sources within the plants would be depleted, or that there might be an inadequate supply of glucose for synthesis of the glucoside. Anaerobic conditions were also applied to inhibit any oxidation-reduction reactions, but in none of these experiments was it possible to detect significant quantities of any intermediate which could subsequently be identified.

Other approaches involved the use of competition experiments, in which valine was given to flax seedlings and suspected intermediates were simultaneously fed to see whether the conversion of valine to linamarin was affected. Experiments of this sort (Butler and Conn, 1964) yielded little useful information. In early experiments (Koukol *et al.*, 1962), $H^{14}CN$ was administered to sorghum plants to see whether HCN produced from some source might react with an aldehyde to form its cyanohydrin, which in turn could be glucosylated and yield dhurrin. This method of synthesis would in effect be a reversal of the degradation outlined in Figure 5. There was no evidence for such a method of synthesis, and indeed cyanide was shown to be extensively metabolized in plants but not into the cyanogenic glucosides. Cyanide metabolism in higher plants has recently been reviewed (Blumenthal *et al.*, 1968; Tschiersch, 1967).

The work of Ahmad and Spenser (1961) on the decarboxylation of ketoximes suggested a different possibility by which a biologically significant nitrile could be formed *in vivo*. This reaction, which is broken down into two steps for reasons to become apparent, is shown in Figure 9. In this reaction, the oxime of a keto acid, a ketoxime, is decarboxylated to yield an aldoxime, and this is subsequently dehydrated to yield a nitrile. While the over-all reaction can be written as a concerted process, it was possible that either a ketoxime or an aldoxime might be a precursor of the cyanogenic glucosides. To test this possibility, the oximes of keto-valine and isobutyraldehyde labeled with ^{14}C were synthesized and compared with valine as precursors of linamarin in flax seedlings (Tapper *et al.*, 1967). The results, summarized in Table III, show that the oxime of α-keto-isovaleric acid-U-^{14}C (keto-

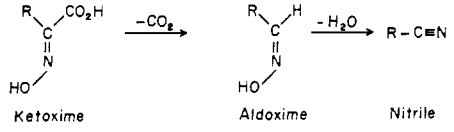


Figure 9. The decarboxylation and dehydration of an α-ketoxime

Table III. Conversion of Oximes to Linamarin

Expt.	Compound Administered	Amount, μmoles	Sp. Act., μC/mmole	Linamarin Dilution Factor	Linamarin %^{14}C Converted
1	L-Valine-U-^{14}C[a]	1.2	730	43	23
	α-Keto-isovaleric[a] acid oxime-U-^{14}C	1.0	920	120	9
2	L-Valine-U-^{14}C[a]	3.3	240	18	25
	Isobutyraldoxime-U-^{14}C	3.4	390	21	21
	Isobutyraldehyde-U-^{14}C	1.3	1510	1510	0.7

[a] Specific activity corrected for loss of carboxyl carbon atom. Data are taken from Table I in Tapper *et al.* (1967).

valine) was converted about one half as effectively to linamarin as was valine-U-^{14}C. On the other hand, isobutyraldoxime-U-^{14}C was converted equally as well to linamarin as was the valine. Similar data were soon obtained for the conversion of the oxime of phenylpyruvic acid (keto-phenylalanine), except that the ketoxime in this case was five times more effectively converted to prunasin in cherry laurel leaves than was phenylalanine (Tapper, 1968). Likewise the oxime of phenylacetaldehyde was about three times as effectively converted as was phenylalanine.

These results thus indicated that aldoximes were effective precursors, and it became necessary to consider whether the hydroxyl group attached to glucose in the cyanogenic glucoside was next introduced or whether the dehydration of the oxime to form the nitrile first occurred. The two possibilities are shown in Figure 10 in the case of linamarin biosynthesis. Because of the ease of synthesis of labelled isobutyronitrile and acetone cyanohydrin, these compounds were tested first.

Table IV shows that both isobutyronitrile-1-^{14}C and α-hydroxyisobutyronitrile-1-^{14}C (acetone cyanohydrin) are effective precursors of linamarin (Hahlbrock *et al.*, 1968). Similar data are available for prunasin biosynthesis in that phenylacetonitrile and α-hydroxyphenylacetonitrile are effectively incorporated into prunasin without randomization of the radioactivity (Hahlbrock *et al.*, 1968).

These results suggest a possible pathway which is summarized in Figure 11. Here, an amino acid, valine in the case of linamarin and phenylalanine in the case of prunasin, is converted to a cyanogenic glucoside by a pathway involving the aldoxime, nitrile, and α-hydroxynitrile. At least one intermediate may be expected between the amino acid

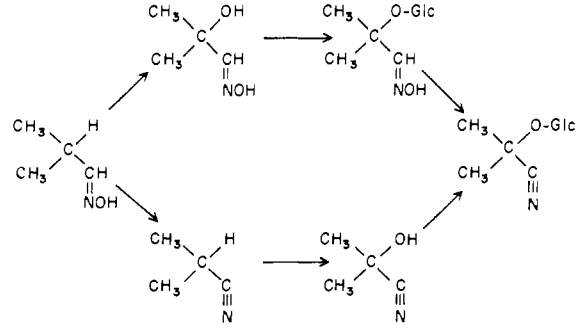


Figure 10. Two possible routes for conversion of isobutyraldoxime to linamarin

Table IV. Conversion of Nitriles to Linamarin

Expt.	Compound Administered	Linamarin: Dilution Factor	Linamarin: %^{14}C Converted	%^{14}C as CN: Found	%^{14}C as CN: Calcd.
1	L-Valine-U-^{14}C[a]	55	23		
	Isobutyronitrile-1-^{14}C	10	11		
	α-Hydroxyisobutyronitrile-1-^{14}C	17	28		
2	L-Valine-U-^{14}C[a]	7×10^4	28	26	25
	α-Hydroxyisobutyronitrile-1-^{14}C	2.4	8	100	100

[a] Corrected for loss of carboxyl carbon atom. Data are those of Expts. I and II in Table I in Hahlbrock *et al.* (1968).

and the aldoxime, and recent work by Kindl and Underhill (1968) indicates that this compound can be, in the biosynthesis of glucosinolate compounds (mustard oil glycosides), the *N*-hydroxyamino acid.

To have observed the effective conversion of aldoximes, nitriles, and cyanohydrins to cyanogenic glycosides is not to have established that these compounds are true intermediates in the pathway. This can only be established when enzymes catalyzing the individual steps that are suggested here can be detected, purified, and characterized. Nevertheless, there is other substantial information suggesting that this pathway is correct. For example, when radioactive valine is fed to young flax plants and nonradioactive isobutyraldoxime is administered simultaneously, it is possible after a period of metabolism to re-isolate the aldoxime from the plants and observe that it is radioactive. Similarly, it is possible to render the nitrile radioactive upon administration of radioactive amino acid and nonradioactive nitrile in excess amounts. Such experiments, which are referred to as "trapping experiments," are indicative that the compounds which have acquired radioactivity under these conditions are true biosynthetic intermediates.

Evidence in support of the biosynthetic outline proposed for cyanogenic glycosides (Figure 11) is found in recent work by Hahlbrock and Conn (1969). An enzyme in flax seedlings which will catalyze a reaction between acetone cyanohydrin and UDP-glucose to form linamarin (Reaction 1) has been detected and partially purified.

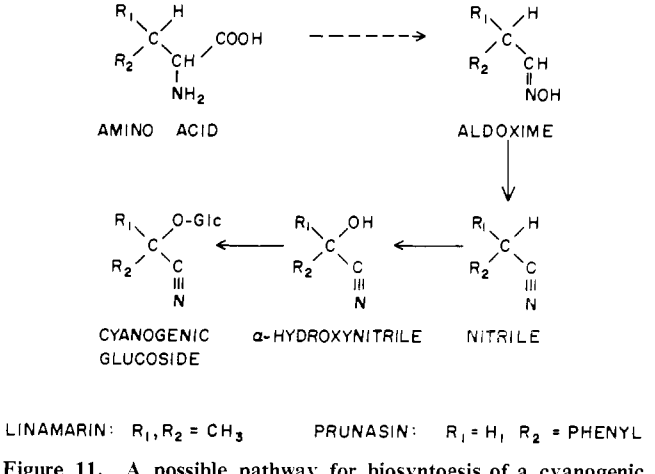


Figure 11. A possible pathway for biosyntoesis of a cyanogenic glycoside from its precursor amino acid

Acetone cyanohydrin + UDP-glucose → linamarin + UDP (1)

The enzyme, which has been purified approximately 120-fold from acetone powders of flax seedlings, is specific for the glucosyl donor UDP-glucose and will not react with ADP-glucose, CDP-glucose, GDP-glucose, IDP-glucose, or TDP-glucose. Similarly, the enzyme at this stage of purity possesses considerable specificity for the cyanohydrins with which it will react (Figure 12). Thus it catalyzes maximum reaction rates with ketone cyanohydrins that possess two, three, and four methyl or methylene carbon atoms, but as the number of carbon atoms in the cyanohydrin is further increased or decreased there is a rapid decrease in the rate of glucosylation of these compounds. The ketone cyanohydrins possessing two and three methyl or methylene carbon atoms are acetone and butanone, and linamarin and lotaustralin are the glucosides formed from these substrates. The enzyme catalyzes little or no reaction with the cyanohydrins of benzaldehyde and *p*-hydroxybenzaldehyde.

Plants glucosylate compounds possessing hydroxyl groups in a nonspecific manner. While nonspecific glucosylation is a property of the intact plant, the glucosyl transferase just described does not appear to be the enzyme involved. This fact is indicated when aromatic hydroxy compounds, for example, can not serve as substrate. On the contrary, the enzyme just described is believed to catalyze this significant and final reaction in cyanoglucoside biosynthesis. Enzymes that catalyze the formation of the cyanohydrin from its indicated precursors, the aldoximes and nitriles, need to be sought and studied. Clearly, before the pathway presented in Figure 11 can be considered as established, it will be necessary to detect the enzymes in that pathway and observe that they exhibit a high degree of specificity.

CONCLUSION

No discussion of cyanogenetic glycosides will be complete without some comment on their role in the plant kingdom. Robinson (1930) has reviewed the early suggestions, which included the cyanogens being a nitrogen reserve and precursors for protein synthesis, excretory waste products, and protective substances. While such suggestions are not easily subjected to experimentation, modern knowledge of protein synthesis has eliminated the

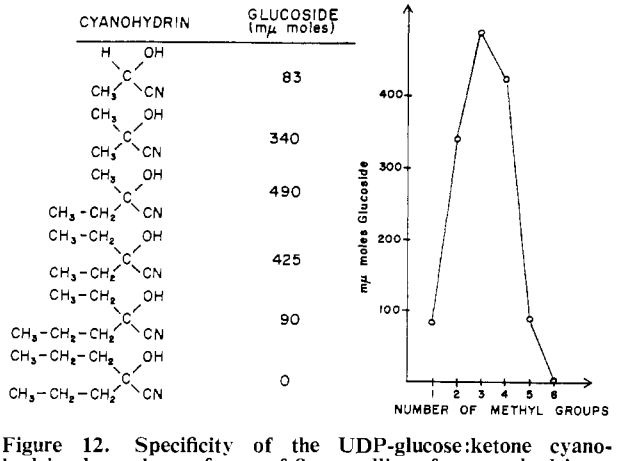


Figure 12. Specificity of the UDP-glucose:ketone cyanohydrin glucosyl transferase of flax seedlings for cyanohydrins

glycosides from being direct precursors of proteins as proposed. The suggestion that the cyanogenic glycosides are excretory products has been frequently criticized, but recent work on the assimilation of HCN by plants does cast a somewhat different light on these criticisms. Since HCN is efficiently converted to the amide carbon atom of asparagine by a large number of plants (Blumenthal *et al.*, 1968; Tschiersch, 1964) the cyanogenic glycosides and HCN could be intermediates in a scheme to conserve the nitrogen atom rather than dispose of it (Abrol and Conn, 1966).

Because the cyanogenic glycosides are not ubiquitous in nature, they must be classified as secondary plant products, and no primary metabolic or other physiological role seems likely. Rather, it seems more plausible that during evolution some plants acquired the biochemical ability to synthesize this fascinating group of compounds, and this ability has been maintained because of the survival or protective value which these substances confer upon the plant. This function for secondary plant products has been discussed by Fraenkel (1959), and research relating directly to cyanogenetic glycosides has been published (Jones, 1966). A protective function of this sort does not rule out a possible metabolic role recently suggested for these compounds and HCN in the biosynthesis of β-cyanoalanine and other lathyrism factors (Blumenthal *et al.*, 1968; Tschiersch, 1966).

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