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Chronic Cassava Toxicity

Proceedings of an interdisciplinary workshop
London, England, 29-30 January 1973

Editors: Barry Nestel and Reginald MacIntyre

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Contents

Cyanogenic Glycosides: Their Occurrence, Biosynthesis, and Function

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Abstract Cyanogenic glycosides are widely distributed among plants and in two classes of animals (Myripoda and Insecta). The structure and distribution of some cyanogenic glycosides are discussed, in particular the structure of linamarin and lotaustralin which occur in cassava (*Manihot* spp.). The biosynthesis and functions of these compounds are discussed, as well as their possible role in the etiology of tropical ataxic neuropathy and goitre.

Résumé Les glycosides cyanogènes sont largement répandus parmi les plantes et dans deux classes d'animaux (Myripoda et Insecta). L'auteur examine la structure et la distribution de quelques glycosides cyanogènes, plus particulièrement la structure de la linamarine et de la lotaustraline, qui se trouvent dans le manioc (*Manihot* sp.). Il discute de la biosynthèse et des fonctions de ces composés, de même que leur rôle possible dans l'étiologie de la neuropathie ataxique et du goitre dans les régions tropicales.

THE cyanogenic glycosides may be defined chemically as glycosides of α-hydroxynitriles (cyanohydrins). They have a wide distribution among the higher plants but also occur in some ferns, and two classes of animals (Myripoda and Insecta). Cyanogenic glycosides will release prussic or hydrocyanic acid (HCN) upon treatment with dilute acids, usually at elevated temperatures. However, the phenomenon of "cyanogenesis," the production of HCN from these compounds, is usually due to the action of enzymes present in the tissues of cyanophoric plants. The action of the enzymes is initiated by crushing or otherwise destroying the cellular structure of the plant.

The present scientific interest in these compounds arises from at least three different areas. Firstly, the toxicity of many cyanophoric plants can be directly attributed to their ability to produce a high level of HCN, a potent inhibitor of cellular respiration. The tubers of cassava (*Manihot* spp.) and the leaves of sorghum and cherry laurel can produce from 25 to 250 mg HCN/100 g of fresh tissue. These and other plants have been responsible for many cases of acute cyanide poisoning of animals including man (Kingsbury 1964; Montgomery 1969). Secondly, the unusual chemical structure of cyanogenic glycosides has attracted the interest of organic chemists for more than a century. More recently, biochemists have concentrated on the metabolism of these compounds in the plants in which they are found. Recent reviews emphasizing both the chemical features (Eyjolfsson 1970) and the metabolism of the cyanogenic glycosides (Conn 1969; Conn and Butler 1969; Conn 1973) have appeared. Thirdly, considerable interest has centred on the possible role of two of these compounds in the etiology of tropical ataxic neuropathy and goitre (Montgomery 1969). Indeed, it is this subject which has provided the impetus for this meeting.

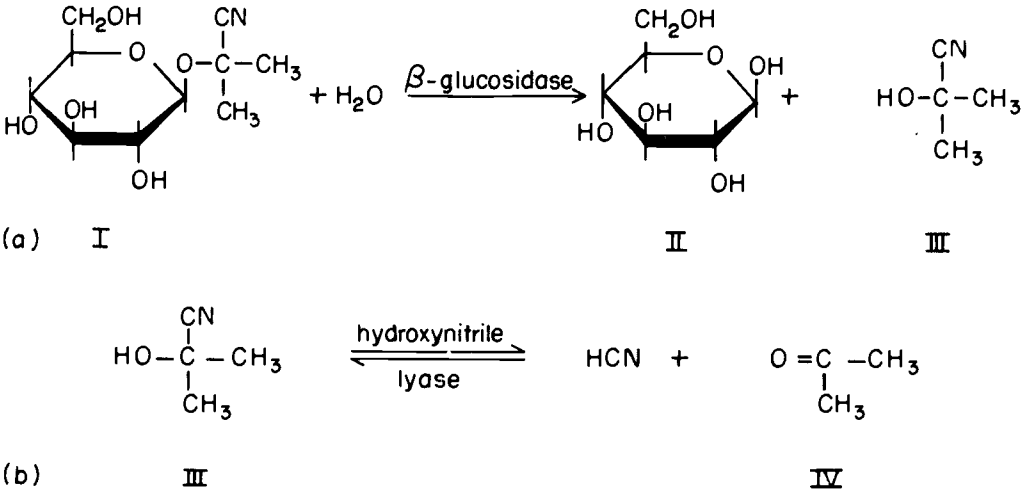

FIG. 1. The mechanism of enzymatic decomposition of linamarin.

Structure and Distribution

Figure 1 shows the structure of linamarin (I), one of the two cyanogenic glucosides that occur in cassava. Also represented is the process by which HCN can be produced in this plant from linamarin. In step *a*, the β-glucosidic bond linking β-(D)-glucose(II) to 2-hydroxyisobutyronitrile (acetone cyanohydrin) (III) is hydrolyzed by the endogenous β-glucosidase (linamarase) to form those two compounds. In step *b*, the hydroxynitrile dissociates to form acetone (IV) and HCN. While this process can and does readily occur non-enzymically, enzymes catalyzing this type of reaction (hydroxynitrile lyases) are known and have been studied in *Sorghum vulgare* (Seely et al. 1966) and the Rosaceae (Gerstner et al. 1968). The presence of such an enzyme in higher plants such as cassava that contain linamarin (and lotaustralin) may therefore be predicted.

The process represented in Fig. 1 is the one usually employed to determine if a specific plant is cyanogenic. The HCN released upon crushing or otherwise destroying the plant tissue can be detected by sensitive, qualitative, colorimetric tests; Eyjolfsson (1970) may be consulted for descriptions for three such tests. In addition, the recognition of the characteristic odor of acetone (from linamarin) or benzaldehyde (from amygdalin, prunasin, sambunigrin, and vicianin) has served to indicate tentatively the presence of cyanogenic glycosides in many species.

It should perhaps be noted that the production of HCN according to Fig. 1 is dependent not only on the presence of the parent cyanogenic glycoside but also on the enzymes that accomplish its decomposition. It is well established that varieties of *Lotus corniculatus* and *Trifolium repens* may lack either or both of these factors and, as discussed elsewhere, these capabilities are under genetic control. There are indications in the literature that other plants such as certain acacias (Finnemore and Gledhill 1928) may produce the glucoside but lack the enzyme(s) which degrade it. The fact that a single species may possess both bitter and sweet varieties may be an indication that cyanogenesis is under genetic control in that species (Jones 1972).

Table 1 lists 5 of the 20 known cyanogenic glucosides, some of the plants in which they occur, and the products formed on hydrolysis. These cyanogens have been chosen to illustrate several points regarding the chemistry and distribution of these compounds. First, in the case of linamarin and lotaustralin, it may be pointed out that, with one exception, these two cyanogens always appear together in the same species. Butler (1965) examined 20 species reported to contain linamarin or lotaustralin and, with the exception of *Hevea brasiliensis*, showed that both compounds were present, albeit in widely varying ratios. Thus in cassava, linamarin accounted for 96% of the cyanogenic material and lotaustralin therefore only 4%. On the other hand, the two compounds

TABLE 1. Some cyanogenic glycosides.

Glycoside	Some plant sources	Hydrolysis products
Linamarin	*Dimorphotheca berberiae* (Compositae); several *Manihot* sp., *Hevea brasiliensis*, *Cnidoscolus texanus* (Euphorbiaceae); *Linum* sp. (Linaceae); *Papaver nudicaule* (Papaveraceae); many sp. of *Lotus*, *Phaseolus lunatus* and *Trifolium repens* (Papilionaceae)	D-glucose + HCN + acetone
Lotaustralin	Occurs with linamarin	D-glucose + HCN + 2-Butanone
Prunasin	*Eremophila maculata* (Myoporaceae); *Eucalyptus cladocalyx* (Myrtaceae); *Cystopteris fragilis*, *Pteridium aquilinum* (Polypodiaceae); species of *Cydonia*, *Eriobotrya*, *Prunus*, *Pyrus* and other genera (Rosaceae); *Jamesia americana* (Saxifragaceae); *Linaria* sp. (Scrophulariaceae)	D-glucose + HCN + Benzaldehyde
Dhurrin	*Sorghum vulgare*, *Bambusa arundinacea*, *Zea mays* (Gramineae)	D-glucose + HCN + *p*-Hydroxybenzaldehyde
Zierin	*Zieria laevigata* (Rutaceae)	D-glucose + HCN + *m*-Hydroxybenzaldehyde

are present in the ratio of 55 :45 in linen flax, a plant in which the biosynthesis of these compounds has been thoroughly examined (Tapper and Butler 1971). The simultaneous occurrence of these two compounds in a single species is attributed to the existence of a set of biosynthetic enzymes that can act on both valine and isoleucine converting these amino acids to linamarin and lotaustralin respectively (Hahlbrock and Conn 1971).

Another noteworthy feature of linamarin and lotaustralin is their relatively broad distribution in the plant kingdom, having been demonstrated in the following families of higher plants: Compositae, Euphorbiaceae, Linaceae, Papaveraceae, and Papilionaceae (Eyjolfsson 1970; Hegnauer 1971). A similar wide distribution has been observed for prunasin in six families (Myoporaceae, Myrtaceae, Polypodiaceae, Rosaceae, Saxifragaceae, and Scrophulariaceae). On the other hand, sambunigrin, vicianin, and amygdalin, which are closely related in chemical structure to prunasin, have been reported in only three (Caprifoliaceae, Mimosaceae, and Oleaceae), two (Polypodiaceae and Papilionaceae), and one (Rosaceae) families respectively. With the exception of these compounds, the more common distribution pattern is that a particular cyanogenic compound will occur in only one or two families (e.g. dhurrin only in the Gramineae) and in several instances the cyanogen will have been unequivocally demonstrated and characterized only in a single species (e.g. zierin in *Zieria laevigata*, family Rutaceae).

Conversely, it is generally true that, with few exceptions, only one characteristic glycoside will occur in a given family. Thus the Gramineae appear to contain only dhurrin and the Compositae only linamarin (and lotaustralin). However, the Polypodiaceae contain both prunasin (in *Cystopteris fragilis* Bernli) and vicianin (in three species of the genus *Davallia*). It can be pointed out that prunasin and vicianin are chemically similar, especially when one considers the biosynthetic origin of their aglycones from phenylalanine. Similarly the Rosaceae contain both amygdalin and prunasin. In this family these two closely related compounds are frequently observed in the same species, the amygdalin occurring in the seeds and prunasin in the leaves or other vegetative tissue. One intriguing family is the Mimosaceae where a single genus *Acacia* contains two quite dissimilar cyanogens. Sambunigrin, which gives rise to benzaldehyde on hydrolysis, has been isolated from *A. glaucescens*, an Australian species (Finnemore and Cox 1928) while acacipetalin possessing an aliphatic aglycone has been isolated from a South African species *A. sieberiana* (Rimington 1935). In like manner, the family Papilionaceae contains vicianin in the genus *Vivia* while linamarin (and lotaustralin) occur in the genera *Lotus*, *Phaseolus*, and *Trifolium*.

The preceding remarks on the occurrence of cyanogenic glycosides needs to be considered in the following context. The task of classifying a plant as cyanogenic is fraught with uncertainty. For example, the quantity of HCN that will be released for a given amount of plant tissue is extremely variable ranging from trace amounts to as much as 500 mg from 100 g (fresh weight) of leaves of *Nandina domestica* (Abrol et al. 1966). The part of the plant containing the cyanogen varies, and should therefore be examined in any careful test. Thus, young vigorously growing tissue (leaves, hypocotyls) frequently contain the cyanogens but they are also found in seeds and stems (e.g. in the Rosaceae) and in tubers (in cassava). Finally, the amount of cyanogen in a given plant, like the content of many other secondary plant products (Fluck 1963), may vary greatly with soil, climate, and the geographical location as well as the age of the plant (Seifert 1955). Nevertheless, the number of plants known to produce HCN approaches 1000 species representing more than 70 families and 250 genera (see Hegnauer 1971 for general review). In spite of this, the number of species in which known cyanogenic compounds have been carefully identified number fewer than 50 species representing only 20 families. There obviously is a great need to identify the cyanogenic substance(s) in many of the remaining families to provide information of maximum value to the chemotaxonomist who would use these compounds as a tool.

Other common structural features of the cyanogenic glucosides can be recognized in part from information given in Table 1. For example, the sugar produced from a majority of the glycosides on hydrolysis is D-glucose. Moreover, where carefully determined, the glucose is present in the pyranosyl form with the β-configuration. The majority of the cyanogens, therefore, are *O*-β-(D)-glucopyranosides. The exceptions are amygdalin, vicianin, and lucumin (Eyjolfsson 1970, 1971) which are disaccharides that have a second sugar (glucose, arabinose, and xylose respectively) linked to the glucose that is bound in turn to the aglycone.

Table 1 also indicates that either an aldehyde or ketone is formed on hydrolysis of cyanogenic glycosides. These compounds are produced when the α-hydroxynitrile that constitutes the aglycone of the glycoside dissociates to form HCN. Indeed the chemical structure of the aglycone has been the basis on which Robinson (1930), Dilleman (1958), and Eyjolfsson (1970) have organized their classification of these compounds.

With the recent work on the metabolism of the cyanogenic glycosides, it is also possible to classify these compounds on the basis of the biosynthetic origin of their aglycones (Conn 1973). Thus, the aglycones of 13 of the 20 known glycosides are formed, or may be assumed to be formed, from five proteinaceous amino acids. These are the three branch-chain amino acids, valine, isoleucine, and leucine, and the aromatic amino acids phenylalanine and tyrosine. The aglycones of the other seven cyanogens do not appear to be formed, at least directly, from proteinaceous amino acids. However, Conn (1973 in press) suggests that five of these could arise from L-2-cyclopentene-1-glycine, an amino acid which has not yet been shown to occur naturally. This method of classification of the cyanogens does not differ in principle from that used earlier, but does serve to raise certain questions. Why, for example, should some but not all of the cyanogenic glycosides be derived from protein amino acids? Then too, why should only the five amino acids cited serve as precursors of the aglycones of the cyanogens? Does this suggest that one will find the other proteinaceous amino acids serving as precursors when the structures of the cyanogenic compounds in sufficiently diverse plant material are examined?

Biosynthesis

The biosynthesis of the cyanogenic glycosides has been recently reviewed (Conn 1969, 1973). Research in several laboratories has demonstrated a precursor–product relationship between four proteinaceous amino acids and the aglycones of several cyanogenic glycosides. This relationship is illustrated in Fig. 2 for four of the glycosides. The evidence consisted of feeding these amino acids labelled in one or more carbon atoms with ^{14}C to cyanogenic plants (or parts thereof) and measuring the incorporation of isotope into the aglycone of the glycoside after a period of metabolism.

Taking dhurrin as a specific example (Fig. 3), work in several laboratories has shown that the α-carbon of tyrosine becomes the nitrile carbon of the glycoside while the β-carbon of the amino acid becomes the aglycone carbon that bears the glucosyl group (see Conn and Butler 1969 for review). The nitrogen atom of the aglycone is

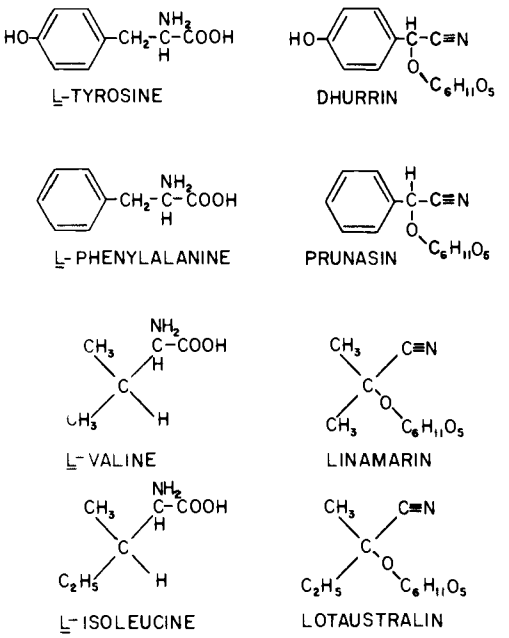

FIG. 2. The precursor–product relationship between certain amino acids and certain cyanogenic glycosides.

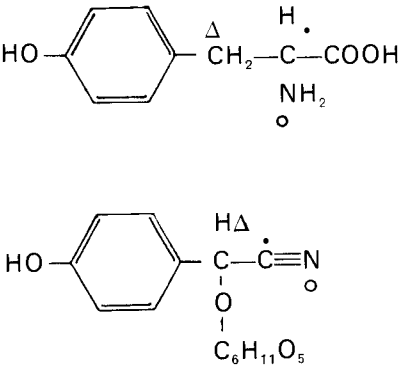

FIG. 3. The origin of certain atoms of dhurrin.

derived from the amino nitrogen of the amino acid. In the case of dhurrin (Uribe and Conn 1966), linamarin (Butler and Conn 1964), and taxiphyllin (Bleichert et al. 1966), double-labelled experiments involving the use of amino acids labelled with ^{14}C in the α-carbon and ^{15}N in the amino group have shown that, as the amino acid is converted to the glucoside, there is little change in the ratio of specific activities of the two isotopes. This has been taken as evidence that the bond between those two atoms is not severed during the conversion and therefore that all intermediates in the biosynthetic pathway must be nitrogenous in nature. Further, in the case of dhurrin, tyrosine double-labelled in the α- and β-carbon atoms was administered and the ratio of specific activities of the corresponding atoms in the aglycone determined (Koukol et al. 1962). As there was no significant difference in the isotope ratio for those two atoms, it was concluded that the covalent bond linking the α–β carbons was not severed during the biosynthesis.

These observations required a biosynthetic pathway in which the carboxyl carbon of the precursor amino acid is lost while the α-carbon is oxidized to the level of a nitrile (a 4-electron oxidation) and the nitrogen atom is retained. In addition, the β-carbon undergoes a 2-electron oxidation (a hydroxylation, Zilg et al. 1972) to form the hydroxyl group to which the sugar is attached. These requirements and the known nonenzymic conversion of 2-oximino acids into nitriles by a concerted dehydration and decarboxylation led to the testing of ^{14}C-labelled oximes and nitriles as possible precursors of the cyanogenic glycosides in flax and sorghum (Conn and Butler 1969). The data in Table 2 were some of the earliest to show that the incorporation of isotope from labelled aldoxime, nitrile, and α-hydroxynitrile was remarkably efficient when compared with the precursor amino acid. To account for these observations the biosynthetic pathway (Fig. 4) involving these compounds as intermediates was postulated (Hahlbrock et al. 1968). It should perhaps be pointed out that the sequence of compounds in the pathway was not determined by the relative efficiency with which these compounds were incorporated since they all were about equally effective

TABLE 2. Conversion of oximes and nitriles to linamarin in linen flax. (Data from Tapper et al. 1967 and Hahlbrock et al. 1968.)

Compound administered	% converted to linamarin
Experiment 1	
L-[U-^{14}C]-valine	25
[U-^{14}C]-isobutyraldoxime	21
[U-^{14}C]-isobutyraldehyde	0.7
Experiment 2	
L-[U-^{14}C]-valine	23
[1-^{14}C]-isobutyronitrile	11
2-hydroxy-[1-^{14}C]-isobutyronitrile	28

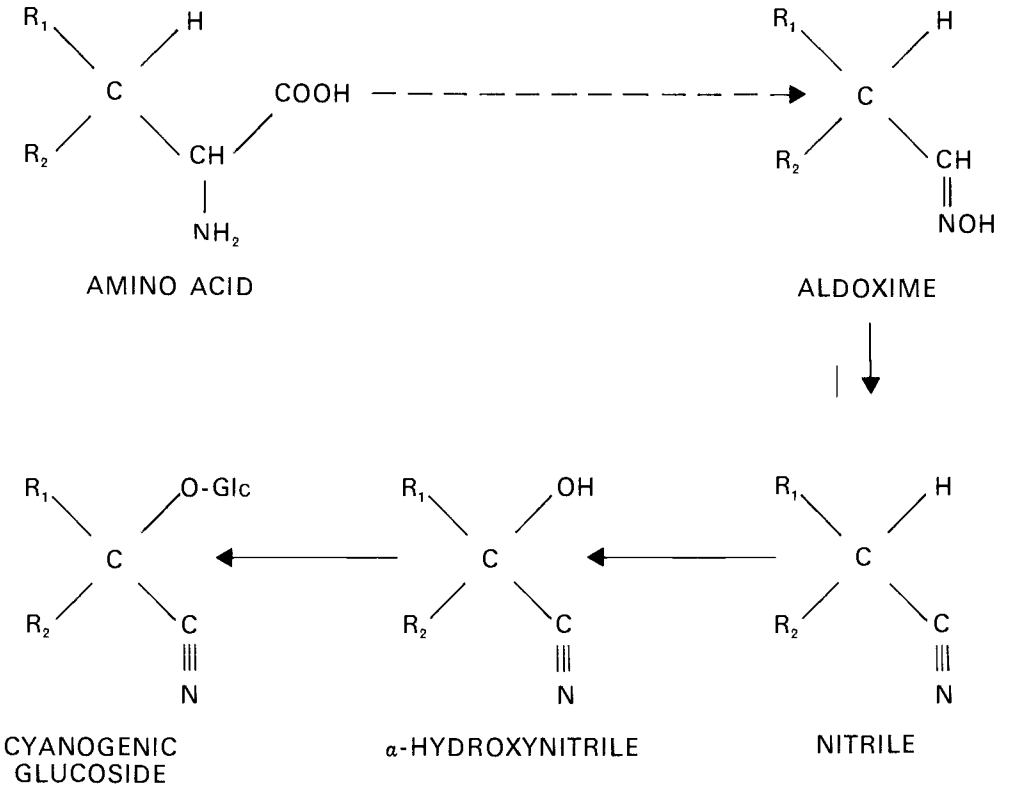

FIG. 4. A possible pathway for biosynthesis of a cyanogenic glycoside from its precursor amino acid. For linamarin, $R_1 = R_2 = CH_3-$, and the amino acid is valine. For lotaustralin, $R_1 = C_2H_5-$, $R_2 = CH_3-$, and the amino acid is isoleucine. For prunasin, R_1 = phenyl, $R_2 = H-$, and the amino acid is phenylalanine. For dhurrin, R_1 = *p*-hydroxyphenyl, R_2 = H, and the amino acid is tyrosine.

when compared with the precursor amino acid. Instead, the sequence was based primarily on the known fact that oximes are readily dehydrated to nitriles and that the cyanohydrin could be glucosylated enzymically.

One might reasonably expect that the postulated intermediates shown in Fig. 4 should be detectable in extracts of untreated cyanophoric plants. This, however, with one exception, has not been possible. The failure to detect these compounds obviously might be due to the analytical methods employed not being sufficiently sensitive to detect very small amounts. An alternative possibility, of course, is that the intermediates remain bound on the surface of the enzyme systems(s) that carry out the biosynthesis.

There is, however, other evidence which supports the existence of the pathway shown in Fig. 4. The first consists of evidence of the sort presented in Table 2 showing that the appropriate aldoximes, nitriles, and α-hydroxynitriles when labelled with ^{14}C and administered to cyanophoric plants are effectively incorporated into the cyanogenic glucoside. Data of this sort have been published (Tapper and Butler 1971) for the formation of linamarin and prunasin in linen flax and cherry laurel, respectively, and for the formation of dhurrin in sorghum (Conn 1973). While such data are clearly indicative of these compounds truly being intermediates, they are subject to the criticism that these compounds may, when fed to the plant, be incorporated due to their closely resembling, but not actually being, true intermediates.

A second type of evidence consists of "trapping" experiments in which the suspected intermediate (aldoxime or nitrile) is administered together with the ^{14}C-labelled precursor amino acid to the appropriate plant. After a period of metabolism, the intermediate is then reisolated from the plant, purified, and examined for radioactivity. In those

instances where radioactivity is found in the intermediate being examined in the trapping experiment, one can conclude that the intermediate can be formed from the precursor amino acid and therefore participate in the postulated pathway. Such data have been published in the case of linamarin biosynthesis (Tapper and Butler 1972) and are available for dhurrin biosynthesis (Farnden et al. 1972).

To the biochemist, the most satisfactory evidence would be the isolation and characterization of the individual enzymes catalyzing the reactions of the biosynthetic pathway. Progress of this sort is being made since the isolation and characterization of an enzyme that synthesizes linamarin and (R)-lotaustralin from 2-hydroxyisobutyronitrile and 2-hydroxy-2-methylbutyronitrile, respectively, was described (Hahlbrock and Conn 1970). Similarly, the isolation and characterization of an enzyme in sorghum that produces dhurrin from the corresponding α-hydroxynitrile has been achieved (Reay and Conn 1968). Both enzymes utilize uridine diphosphate glucose [UDPG] as the glucosylating agent and the type reaction may be represented as:

α-hydroxynitrile + UDPG → UDP + cyanogenic glucoside.

Interesting stereochemical aspects of these reactions have been studied and reviewed elsewhere (Conn 1973). Work is underway to detect and characterize other reactions of the pathway suggested in Fig. 4 and, in the case of the conversion of *p*-hydroxyphenylacetaldoxime to *p*-hydroxyphenylacetonitrile, a particulate enzyme that catalyzes this reaction in sorghum has been detected (Farnden et al. 1972). Its properties are presently under investigation in this laboratory.

A major area of uncertainty in the pathway (Fig. 4) is the reaction(s) by which the amino acid is converted to the aldoxime. This conversion, which involves a 4-electron oxidative decarboxylation of the precursor amino acid may well be identical to the initial portion of the pathway leading from amino acids to glucosinolate compounds (Underhill and Wetter 1966). In the case of these compounds, Kindl and Underhill (1968) have postulated that the corresponding *N*-hydroxyamino acid is an intermediate and have provided some evidence in support of this suggestion. However, this type of compound has by no means been demonstrated as an intermediate in the case of the cyanogenic glucosides and much additional work is required to establish the role, if any, of the *N*-hydroxyamino acid in the biosynthesis of cyanogenic glycosides.

Function of Cyanogenic Glycosides

No discussion of cyanogenic glycosides is complete without some comment on their role in the plant. Robinson (1930) reviewed the early suggestions which included the cyanogens being nitrogen reserves and precursors for protein synthesis, excretory waste products, and protective substances. Our present knowledge of protein synthesis clearly eliminates the first of these suggestions. Then, too, the suggestion that the cyanogenic glycosides are inert waste products that must be excreted has frequently been criticized. The now fairly extensive literature on the metabolic activity of these compounds and the assimilation of HCN by plants (Conn and Butler 1969) provides basis for further criticism of this second suggestion.

Because the cyanogenic glycosides are not ubiquitous in nature they must be classified as secondary plant products. Therefore, no primary metabolic or physiological role seems likely, although at one point a role in asparagine biosynthesis appeared promising (Castric et al. 1972). 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 has been discussed by Fraenkel (1959) for secondary plant products in general and by Jones (1972) for cyanogenic glycosides in particular. A protective function of this sort does not rule out a role for one of these compounds (vicianin) being the source of HCN that in turn can be used in the biosynthesis of β-cyanoalanine and other lathyrism factors (Tschiersch 1966). As more research is performed on the cyanogenic glycosides, we may learn more about the specific roles that they may play in individual species.

Acknowledgment

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