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Review

Cyanogenesis in Plants[1]

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ABSTRACT

Several thousand plant species, including many economically important food plants, synthesize cyanogenic glycosides and cyanolipids. Upon tissue disruption, these natural products are hydrolyzed liberating the respiratory poison hydrogen cyanide. This phenomenon of cyanogenesis accounts for numerous cases of acute and chronic cyanide poisoning of animals including man. This article reviews information gathered during the past decade about the enzymology and molecular biology of cyanogenesis in higher plants. How compartmentation normally prevents the large-scale, suicidal release of HCN within the intact plant is discussed. A renewed interest in the physiology of these cyanogenic compounds has revealed that, in addition to providing protection for some species against herbivory, they may also serve as storage forms for reduced nitrogen.

Cyanogenesis, the ability of plants and other living organisms to release hydrogen cyanide, has been known for several centuries. Since its first description in plants in 1803, the phenomenon of cyanogenesis has been recognized in over 3000 species of higher plants distributed throughout 110 different families of ferns, gymnosperms, and both monocotyledonous and dicotyledonous angiosperms (1). However, only in approximately 300 plant species has the source of HCN been identified. In certain sapindaceous seeds, HCN may arise during cyanolipid hydrolysis. More frequently, HCN production in higher plants results from the catabolism of cyanogenic glycosides. The approximately 75 documented cyanogenic glycosides are all *O*-β-glycosidic derivatives of α-hydroxynitriles. Depending on their precursor amino acid, they may be aromatic, aliphatic, or cyclopentenoid in nature. Most are cyanogenic monosaccharides in which the unstable cyanohydrin moiety is stabilized by glycosidic linkage to a single sugar residue. Alternatively, in the cyanogenic disaccharides [*e.g.* (*R*)-amygdalin, (*R*)-vicianin, and linustatin] or trisaccharides (*e.g.* xeranthin), two or three sugar moieties, respectively, are involved in such stabilization. Sulfated, malonylated, and acylated derivatives of cyanogenic glycosides are also known.

Cyanogenesis is not exclusive to those plant species accumulating cyanolipids and cyanogenic glycosides. All higher plants probably form low levels of HCN as a coproduct of ethylene biosynthesis (11). This might explain why even 'acyanogenic' plants contain significant levels of the cyanide detoxifying enzyme β-cyanoalanine synthase. Cyanogenesis is also known in animals, but is restricted to the arthropods, notably to certain centipedes, millipedes, and insects. In fungi and bacteria, HCN may originate via oxidative decarboxylation of glycine.

Many economically important food plants are highly cyanogenic and have caused numerous cases of acute cyanide poisoning of animals including man. Additionally, in areas of the world where cyanogenic plants such as cassava and lima beans comprise the major item of the diet, chronic cyanide poisoning and associated pathological conditions still exist (17). It is highly desirable that the toxicity of cyanogenic plants to humans and livestock be reduced. This is achievable by: (a) selective breeding to produce low-cyanogen varieties, as was accomplished for almonds, (b) screening of natural populations for low-cyanogen varieties, (c) mutagenesis of protoplasts or cell cultures with subsequent regeneration of plants having desired mutant genotypes, or (d) genetic engineering. To facilitate these approaches, one must first acquire a working knowledge of the biochemistry, physiology, and molecular biology of cyanogenesis. This article provides an overview of recent publications which have advanced our understanding of these critical areas. Attention will be focused primarily on cyanogenic glycosides, but significant developments in the less studied field of cyanolipids are noted.

BIOSYNTHESIS OF CYANOGENIC GLYCOSIDES

Most cyanogenic glycosides are derived from the five hydrophobic protein amino acids tyrosine, phenylalanine, valine, leucine, and isoleucine. Cyclopentenoid cyanogens presumably originate from cyclopentenylglycine. A biosynthetic pathway originally proposed on the basis of *in vivo* tracer experiments has been confirmed by extensive studies with cell-free systems (4). Microsomal preparations from sorghum, arrow grass, linen flax, and *Trifolium repens* convert specific amino acids to their corresponding α-hydroxynitriles with *N*-hydroxyamino acids, aldoximes, and nitriles serving as intermediates (Fig. 1). The highly channeled nature of these pathway intermediates explains the failure of early labeling studies to detect them. The final step in glycoside biosynthesis is glycosylation of α-hydroxynitriles by soluble *O*-glycosyltransferases. These UDP-sugar dependent enzymes have been partially purified from sorghum, arrow grass, and black cherry.

The most elaborate studies on cyanogenic glycoside biosyn-

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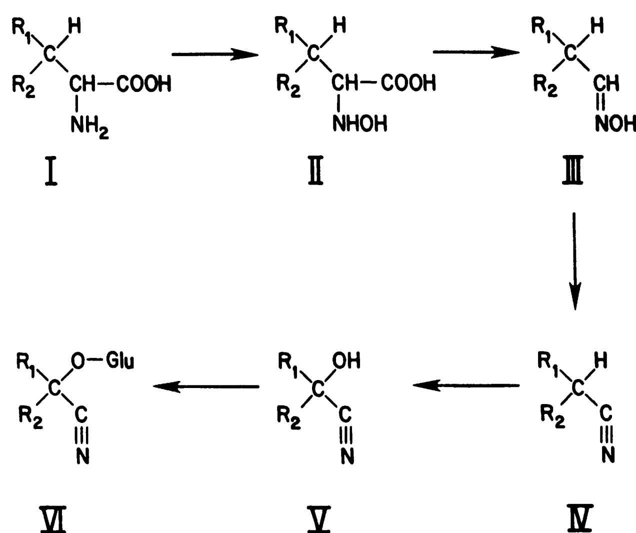

Figure 1. Proposed biosynthetic pathway for cyanogenic monosaccharides (VI) from L-amino acids (I), involving *N*-hydroxyamino acids (II), aldoximes (III), nitriles (IV), and α-hydroxynitriles (V) as intermediates.

thesis have focused upon the accumulation of dhurrin [*p*hydroxy-(*S*)-mandelonitrile-β-D-glucoside] by etiolated sorghum seedlings (4). Dhurrin constitutes 30% of the dry weight of shoot tips but is absent from the seed and roots. The codistribution of dhurrin and its synthesizing system in the upper portion of the shoot shows that production and storage sites are located within the same cells. The microsomal system was purified approximately 10-fold by sucrose density gradient centrifugation or gel filtration, but reconstitution experiments showed that further purification was thwarted by loss of essential components. Simultaneous measurement of tyrosine utilization, O_2 consumption, and NADPH oxidation revealed that three O_2-requiring hydroxylation steps are involved in the conversion of tyrosine to *p*-hydroxymandelonitrile *in vitro*. Monooxygenase activities catalyzing the *N*-hydroxylation of tyrosine and the stereospecific conversion of *p*-hydroxyphenylacetonitrile to *p*-hydroxymandelonitrile have been identified. Both enzymes require NADPH and O_2, but only the latter reaction is Cyt P450-dependent. The nature of the third hydroxylation step remains unknown.

The conversion of *N*-hydroxytyrosine into *p*-hydroxyphenylacetaldoxime is not well understood. Formally, this represents a two electron oxidative decarboxylation, but a requirement for an oxidized cofactor has yet to be demonstrated experimentally. Deuterium labeling studies have shown that the α-H atom of tyrosine is retained during this reaction, thus ruling out *p*-hydroxyphenylpyruvic acid oxime as intermediate (5). Earlier incorporation studies had already excluded *N*-hydroxytyramine. These data suggest that, if oxidation and decarboxylation occur as separate steps, 3-(*p*-hydroxyphenyl)-2-nitrosopropionic acid might be a likely intermediate.

Using reverse-phase HPLC, Halkier *et al.* (5) probed the stereochemistry of aldehyde oximes involved in cyanoglycoside biosynthesis. The oxidative decarboxylation of *N*-hydroxytyrosine gives rise initially to the (*E*) isomer of *p*-hydroxyphenylacetaldoxime, but it is the (*Z*) isomer which is preferentially utilized in subsequent steps. Isomerization is effected by a microsomal isomerase, which preferentially accepts the (*E*) isomer produced *in situ* over exogenously added compound. Although appearing as a simple dehydration, the conversion of this aldoxime to *p*-hydroxyphenylacetonitrile requires NADPH and displays photoreversible CO inhibition. By analogy to the proposed biosynthetic pathway for glucosinolates, hydroxylation to the corresponding *aci*-nitro compound has been suggested, a step which might explain the additional O_2 requirement in dhurrin biosynthesis. Purification of Cyt P450-dependent enzymes from detergent solubilized microsomes is in progress.

CATABOLISM OF CYANOGENIC GLYCOSIDES

Upon tissue disruption, cyanogenic glycoside degradation is initiated by cleavage of the carbohydrate moiety by one or more β-glycosidases, yielding the corresponding cyanohydrin. This intermediate may decompose either spontaneously or enzymically in the presence of an α-hydroxynitrile lyase to release HCN and an aldehyde or ketone. At first glance, the need for hydroxynitrile lyases appears puzzling, but it should be noted that, while nonenzymic decomposition procedes rapidly at alkaline pH, it is negligible below pH 5.5. The major role of α-hydroxynitrile lyases is presumably to accelerate release of HCN (and carbonyl compounds) in plant macerates, which commonly are slightly acidic (pH 5.0–6.5). This view is supported by mixed enzyme incubations in which various ratios of hydroxynitrile lyase to β-glucosidase were analyzed for rapidity of HCN evolution (22). A ratio of 2.4, close to the average found in seven *Hevea* varieties tested, accelerated the rate of acetone cyanohydrin dissociation 20-fold over nonenzymic rates. Noting that the efficacy of cyanogenesis as a defense mechanism against herbivory undoubtedly depends upon the rate of HCN release as well as the total amount liberated, Selmar *et al.* (22) proposed categorizing cyanogenic plants according to their ability for rapid or slow cyanogenesis.

Glycosidases Involved in Cyanogenic Glycoside Catabolism

In past years, the use of synthetic rather than natural substrates during the purification and characterization of relatively crude but highly active enzyme preparations contributed to the widely accepted view that plant β-glycosidases lack aglycone specificity (6). Since 1975, this viewpoint has been challenged by the isolation of β-glycosidases exhibiting pronounced specificities for their endogenous cyanogenic glycosides. Examples include β-glucosidases showing specificity toward taxiphyllin (*Triglochin maritima*), triglochinin (*Alocasia macrorrhiza, Triglochin maritima*), vicianin (*Davallia trichomanoides, Vicia angustifolia*), dhurrin (*Sorghum bicolor*), amygdalin (*Prunus serotina*), and prunasin (*P. serotina*) (16). In comparison, linamarases (EC 3.2.1.21) from flax (*Linum usitatissimum*) and *Hevea brasiliensis* show broader substrate specificities. β-Glycosidases involved in cyanogenesis possess acidic pH optima (pH 4.0–6.2) and, with few

exceptions, are glycoproteins having isoelectric points in the range pH 4 to 5.5. Despite wide variation in native molecular masses (40–600 kD), most have subunit molecular masses of 55 to 65 kD, suggesting a common structural relationship among these and other plant β-glycosidases. Multiple forms of cyanogenic β-glycosidases are commonly found. Whether such heterogeneity resides in their primary structures or carbohydrate side-chains or arises through partial proteolysis, protein-ampholyte interactions or aggregation-dissociation phenomena is largely unknown.

The hydrolysis of cyanogenic disaccharides may procede by either the 'sequential' or 'simultaneous' pathways depending on whether the two sugar residues are removed stepwise or as a disaccharide unit (Fig. 2). In the former case, the two hydrolytic steps are generally catalyzed by distinct β-glycosidases. Well-documented examples of the sequential mechanism are provided by the catabolism of amygdalin in *P. serotina* and of linustatin and neolinustatin in *L. usitatissimum* (2, 16). The hydrolyses of vicianin in squirrel's foot fern (*D. trichomanoides* Blume) and *V. angustifolia* and of linustatin in *H. brasilienis* occur via the simultaneous mechanism (16).

Involvement of α-Hydroxynitrile Lyases in Plant Cyanogenesis

α-Hydroxynitrile lyases catalyzing the dissociation of α-hydroxynitriles to HCN and an aldehyde or ketone have been extensively purified and characterized from plants accumulating aromatic and aliphatic cyanoglucosides (16). These enzymes generally show greatest, but not always exclusive, activity toward the endogenous cyanohydrins. While all known lyases possess slightly acidic pH optima and isoelectric points between pH 3.9 to 4.8, they appear to fall into at least three fundamentally distinct groups based on their FAD and carbohydrate contents (for review, see Poulton [16]). The first group contains (*R*)-(+)-mandelonitrile lyases (EC 4.1.2.10) from seeds of the Prunoideae and Maloideae. Requiring only 5 to 15-fold purification to reach homogeneity, these glycoproteins are clearly major seed constituents and may serve an additional role as storage proteins. Curiously, they possess FAD bound close to their catalytic sites although these enzymes do not catalyze a net oxidation-reduction reaction. The role of FAD has been a matter of considerable debate. In many species, mandelonitrile lyases exist as multiple forms, exhibiting minor differences in primary structure and carbohydrate side-chains. The nature and physiological significance of this microheterogeneity are poorly understood. A second group of hydroxynitrile lyases includes those isolated from vegetative tissues of sorghum, cassava, and flax. These proteins required 100- to 300-fold purification to attain homogeneity, and, where tested, they lacked FAD and carbohydrate. They usually possessed higher native molecular weights and were often prone to aggregation. Recently, a unique mandelonitrile lyase was characterized from *Ximenia americana* leaves (14). Like lyases from rosaceous species, it is glycoproteinous but it lacks FAD and is stereospecific for (*S*)-(−)-mandelonitrile. Whether FAD plays a direct role in determining the stereospecificity of mandelonitrile lyases remains unclear.

GENETICS AND MOLECULAR BIOLOGY OF CYANOGENESIS

Trifolium repens, *Lotus tenuis*, and *L. corniculatus* are among several plant species which are polymorphic for the cyanogenic character. In *T. repens*, HCN production is determined by alleles of two independently segregating loci (8). Only plants possessing at least one dominant functional allele of both genes liberate HCN when damaged. The locus *Ac*, which controls the presence or absence of the glycosides linamarin and lotaustralin, has yet to be fully understood. At least two steps are blocked in the conversion of amino acids to hydroxynitriles by microsomal preparations from *acac* plants. *In vivo* labeling experiments also suggest that *acac* plants lack the soluble glucosyltransferase. Far more is known at the molecular level about the locus *Li* which governs the presence or absence of the β-glucosidase linamarase. This stable, mannose-containing glycoprotein exists in active form as a homodimer of 62,000 M_r subunits. Homozygous *LiLi* plants have approximately twice as much linamarase activity in leaf extracts as *Lili* plants, while homozygous recessive plants (*lili*) show none. Synthesized in *Li* plants only during early stages of leaf development, linamarase has an apoplastic localization (10) and constitutes up to 5% of the leaf soluble proteins. *In vitro* translation of mRNA from a linamarase-positive *LiLi* plant yielded an immunoprecipitated linamarase polypeptide (59,000 M_r) which was modified to a 62,000 M_r product by dog pancreas microsomes. By contrast, plants homozygous for the 'null' allele (*lili*) synthesized neither translatable linamarase mRNA nor inactive but antigenically related linamarase protein.

More recent data clearly show that the *Li* locus affects the tissue level of linamarase mRNA (8). A cDNA clone

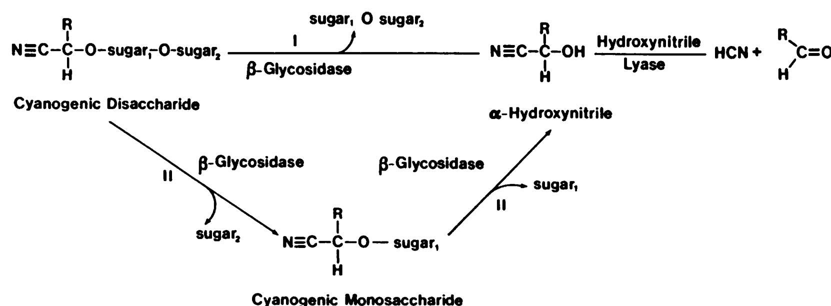

Figure 2. Simultaneous (I) and sequential (II) mechanisms for catabolism of cyanogenic disaccharides.

(pTRE36) has been isolated from linamarase mRNA, sequenced, and used to further characterize alleles of the *Li* locus. Consisting of 1726 bases, it encodes 512 amino acids and contains some 3′ noncoding sequence. However, codons for the 5′ noncoding sequence and the known N-terminus are lacking. Multiple lines of evidence indicated that the clone contains a cDNA of linamarase (8). Northern blot analysis of total RNA from developing leaves of *LiLi* plants showed hybridization of the complete insert of pTRE36 to a single major 2.15 kb RNA species. Low levels of mRNA homologous to TRE36 were found in *lili* leaves and *LiLi* roots, which lack linamarase activity. Leaves of heterozygous (*Lili*) plants contained intermediate levels, correlating with their intermediate linamarase content. Two possibilities exist for the low levels of mRNA homologous to pTRE36 found in *lili* leaves and *LiLi* roots despite their lack of detectable linamarase protein. First, the linamarase cDNA may show homology to mRNA(s) encoding the noncyanogenic β-glucosidase(s) known in white clover. Second, the low linamarase mRNA levels detected by Northern blots may give rise to protein amounts below the limit of detection of enzyme assay and immunoprecipitation methods.

Three genes with homology to the linamarase cDNA were detected in the white clover genome by Southern blot analysis of *Hin*dIII digested genomic DNA. Analysis of the cosegregation of linamarase activity (*Li allele*) and the presence of genomic restriction fragments identified the cDNA clone as structural information for linamarase. Whether the *Li* locus has a structural or *cis*-acting control function will be probed via transformation experiments.

While linamarases from white-clover, flax, cassava and *T. nigrescens* exhibit significant structural differences, preliminary studies utilizing the linamarase cDNA clone pTRE36 and affinity-purified antibodies raised against denatured white clover linamarase showed that some homology exists between them at both protein and DNA levels (8).

ROLE OF TISSUE AND SUBCELLULAR COMPARTMENTATION IN CYANOGENESIS

A common feature of cyanophoric plants is that cyanogenic glycoside hydrolysis occurs at a significant rate only after their tissues have been disrupted by herbivores, fungal attack, or mechanical means. Although other explanations are possible, it is generally assumed that the glycosides and their catabolic enzymes are separated in the intact plant by compartmentation at either tissue or subcellular levels (16). These possibilities have been extensively tested in a single organism, namely the leaves of 6-d-old light-grown sorghum seedlings (12). Somewhat unexpectedly, the authors demonstrated that the substrate and its catabolic enzymes were localized within different tissues. The cyanogenic glycoside dhurrin was sequestered in the vacuoles of epidermal cells, whereas the β-glycosidase and hydroxynitrile lyase were present almost entirely in the underlying mesophyll cells. These two enzymes were located in the chloroplasts and cytosol, respectively. It therefore seems likely that the large-scale hydrolysis of dhurrin, which probably provides a defense mechanism against herbivores by liberating HCN, occurs only after tissue disruption allows the mixing of contents of different tissues.

Available evidence from other plant species, however, favors compartmentation of components of the 'cyanide bomb' at the subcellular level. In cassava, cells throughout the entire root cross-section possess both cyanogens (principally linamarin) and linamarase (13). As in sorghum, highest glycoside levels are found in outer cell layers, again suggesting the involvement of cyanogens in defense against herbivores or pathogens, but the subcellular localizations of linamarin and linamarase remain unknown. In *Phaseolus lunatus*, the low recoveries of linamarin, linamarase, and hydroxynitrile lyase in leaf mesophyll protoplasts pointed to other tissues, perhaps the epidermis, as the principal site for these components (3). Although these data cannot unequivocally distinguish between an epidermal or mesophyll location, it seems certain that the *P. lunatus* linamarase is apoplastic. Leaf discs hydrolyzed externally supplied linamarin, and about one-third of the total linamarase activity was extractable by multiple infiltrations of the leaves. The *T. repens* linamarase was detected by immunocytofluorescence in cell walls, especially those of the epidermis, and in the cuticle (10). More recently, protoplast isolation and tissue filtration experiments with *Hevea* endosperm showed that linamarin and the hydroxynitrile lyase were intracellular but that linamarase occurred both intra- and extracellularly (19). The apoplastic distribution of most linamarases contrasts with the intracellular location of sorghum dhurrinase, a fact perhaps related to the nonglycoprotein character of the latter (16).

PHYSIOLOGICAL ROLES OF CYANOGENIC COMPOUNDS IN PLANTS

The physiological importance of cyanogenic compounds in plant metabolism is currently receiving renewed interest. As with other secondary products, cyanogenics were originally viewed as excretory substances (18), but their turnover (seasonal and even diurnal) argues strongly against this hypothesis. An alternative role as obligate precursors of protein synthesis (via β-cyanoalanine and asparagine) was abandoned in 1975 after discovery of glutamine-dependent asparagine synthetases. Given the well documented toxicity of HCN, a role in plant protection against herbivores, pathogens, and competitors is appealing. Much evidence, indeed, favors a defence function for cyanogenics against certain animals including insects (for reviews, see Jones [9] and Nahrstedt [15]). Species showing cyanogenic polymorphism have frequently been exploited to investigate such plant-herbivore interactions. In critically examining the literature, however, Hruska (7) found few studies incorporating adequate statistical analyses or having addressed herbivore feeding specificity. Since relatively few plants and herbivore species have so far been examined, more studies are clearly desirable. Future research should bear in mind that, in addition to HCN, the cyanogenic glycosides themselves and other degradation products (*i.e.* carbonyl compounds and β-cyanoalanine) may also be active as allelochemicals (9, 15).

In view of their widespread distribution in plants, might cyanogenic compounds not also play a primary role in undamaged or uninfected plants? Recent experiments have again raised the possibility that cyanogenic glycosides and cyanoli-

pids might serve as nitrogen storage compounds (20, 21). In *H. brasiliensis* seeds, the endosperm represents almost 85% of the seed dry matter and contains more than 90% of the cyanogenic glucoside linamarin. During germination and plantlet development, the cyanogenic potential of the entire seedling declines by 85% as cyanogenic compounds are metabolized to noncyanogenic substances. Negligible amounts of gaseous HCN are liberated during this process. Since highest levels of the cyanide detoxifying enzyme β-cyanoalanine synthase occur in young seedling tissues, Selmar *et al.* (21) proposed that linamarin is transported from the endosperm via the apoplast to the young, growing tissues for further catabolism. The lability of this glycoside to apoplastic and intracellular linamarase dictates the need for a protected transport form resistant to linamarase action. A suitable candidate would be the disaccharide linustatin, derived from linamarin by glucosylation. Moving safely via the apoplast and vascular system to target tissues, linustatin would be degraded there by a distinct disaccharidase to HCN. Detoxication of HCN to asparagine by β-cyanoalanine synthase would allow this nitrogen to reenter general metabolic pools. Much evidence supports this attractive hypothesis: (a) linustatin is not hydrolyzed by linamarase; (b) linustatin levels in *Hevea* seeds increase upon storage; (c) at that developmental stage when the linamarin content is decreasing, linustatin occurs in endosperm exudates, and increasing levels of β-cyanoalanine synthase and a linustatin-splitting disaccharidase are found in seedling tissues; and (d) linustatin is present in leaf nectary and phloem exudates. Whether linamarin mobilization and utilization occur in other cyanogenic species via this so-called 'linustatin pathway' is under investigation. Linustatin was detectable in all linamarin-containing genera so far analyzed, including *Manihot*, *Linum*, *Phaseolus*, *Dimorphotheca*, *Acacia*, *Trifolium*, and *Passiflora*. Furthermore, extracellular linamarase was seen in *Phaseolus lunatus*, *Trifolium repens*, *Manihot esculenta*, and *D. sinuata*. Cyanolipids may similarly function as storage compounds for reduced nitrogen (20). During development of *Ungnadia speciosa* (Sapindaceae) seedlings, their cyanolipids (400 μmol per seed) are completely metabolized without liberation of HCN to the atmosphere. Concurrently, cyanogenic glycosides are synthesized but reach levels equal to only one-fourth of the original cyanolipid content. This large decrease in cyanogenic potential points to major utilization of cyanolipids for synthesis of noncyanogenic compounds.

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