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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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The Genetics of Cyanogenesis

MONICA A. HUGHES

Department of Plant Science
University of Newcastle-upon-Tyne, England

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Abstract Part of the variation in the levels of hydrogen cyanide (HCN) production, which exists between cyanogenic plants, is genetically controlled in *Sorghum* species, *Lotus* species, and *Trifolium repens*. In *Sorghum* no discrete class for HCN production can be recognised in a population of individuals showing variation in levels of HCN. The genetic control of HCN production in *Sorghum* species is considered multigenic. In *Lotus* and *Trifolium* another form of variation exists in which discrete classes can be recognised. In *Trifolium repens* the locus *Ac* controls the presence or absence of the cyanogenic glucosides, linamarin and lotaustralin, and the locus *Li* controls the presence or absence of the enzyme which hydrolyses these glucosides. Plants possessing only nonfunctional alleles of at least one of the loci are acyanogenic. Biochemical and genetical studies on the cyanogenic system of *Trifolium repens* have shown the presence of inherited variation in the level of hydrolytic enzyme present in plants possessing functional *Li* alleles. Using this variation, together with immunological studies, it has not been possible, as yet, to demonstrate that the *Li* locus is a structural gene for this enzyme. Comparison of the linamarin biosynthetic pathway between *Trifolium* plants which produce the cyanoglucosides and plants which do not, has suggested that the *Ac* locus may control more than one step in this biosynthetic pathway.

Résumé Une partie des variations du niveau d'acide cyanhydrique (HCN) observées chez certaines plantes cyanogènes sont sous contrôle génétique chez les espèces de *Sorghum*, de *Lotus* et chez *Trifolium repens*. Dans une population d'individus du genre *Sorghum* montrant des variations de niveau de HCN, on ne peut déceler de classes discontinues quant à la production de HCN. On est généralement d'avis que la production de HCN par les espèces de *Sorghum* est contrôlée par plusieurs gènes. Par ailleurs, il existe chez *Lotus* et *Trifolium* une autre forme de variations qui peuvent être groupées en classes discontinues. Chez *Trifolium repens*, le locus *Ac* contrôle la présence ou l'absence des glucosides cyanogènes, linamarine et lotaustraline, alors que le locus *Li* contrôle la présence ou l'absence de l'enzyme hydrolysant ces glucosides. Les plantes qui ne possèdent que des allèles non fonctionnels d'au moins un des loci sont acyogènes. Des études biochimiques et génétiques sur le système cyanogène de *Trifolium repens* démontrent une variation héréditaire du niveau de l'enzyme hydrolytique présent chez les plantes possédant des allèles *Li* fonctionnels. En se basant sur cette variation et sur des études immunologiques, il a été impossible, à venir jusqu'ici, de démontrer que le locus *Li* est un gène structural contrôlant cet enzyme. Une comparaison de la voie biosynthétique de la linamarine entre les plants de *Trifolium* qui produisent les cyanoglucosides et ceux qui ne les produisent pas suggère que le locus *Ac* peut contrôler plus d'une étape de cette voie biosynthétique.

ALL the cyanogenic plant species which have been investigated in any detail show variation in the amount of hydrocyanic acid (HCN) produced. This variation reflects variation in both the production of cyanoglucosides themselves and the enzymes which degrade them. Variation occurs within individual plants, or genotypes, depending on the tissue analysed, the age of the tissue, and the cultural conditions of the plant (Sinha and Nair 1968; Gillingham et al. 1969; Roger and Frykolm 1937). Variation in HCN production also occurs between individual plants, and it is this variation which has been studied by geneticists and plant breeders. Since individual plants show variation depending on cultural conditions, part of the variation between plants must be due to environmental differences. However, in those species studied, some of the interplant variation is due to the genotype of the plants and is therefore genetically controlled.

The grass species *Sorghum sudanense* (sudangrass) and *S. bicolor* produce the cyanoglucoside, dhurrin. This glucoside is broken down by two enzymes, β-glucosidase and oxynitrilase, to give glucose, *p*-hydroxybenzaldehyde, and HCN. In order for a plant to produce HCN it must be able to synthesise dhurrin and the enzymes which degrade dhurrin. Both these grasses and the hybrid, sorghum-sudangrass, are used as animal feed and plant breeders have been working for a number of years to produce low-HCN strains.

The variation in HCN content of these grasses is continuous, that is it is not possible to recognise any discrete class of individuals within a variable population of plants. A number of studies of the inheritance of HCN production in sudangrass and sorghum-sudangrass hybrids have been made, without the same results (Nass 1972). Authors differ in their conclusions about the number of genes involved and the dominance relationships of these genes. For example, Snyder (1950) concluded that in sudangrass there was one pair of genes determining high versus low cyanide content and that low HCN production was dominant over high HCN production. Barnett and Caviness (1968), on the other hand, showed that in sorghum-sudangrass hybrids the inheritance of HCN production was multigenic, with high HCN production partially dominant to low HCN production.

There has been some interest in finding an association between low HCN production and other simply inherited characters in sorghum. In sorghum-sudangrass hybrids, Carlson (1958) found low HCN production associated with the following loci: green striped-1, golden-2, yellow leaf tip, and single seeded spikelets. Pleiotropy seemed a more likely explanation of this phenomenon than linkage.

In three plant species, *Trifolium repens* (white clover), *Lotus tenuis*, and *L. corniculatus* (birdsfoot trefoil), another form of variation exists in which discrete classes can be recognised, namely plants which produce HCN when damaged and plants which produce no HCN when damaged. Continuous variation in HCN production also exists in those plants capable of releasing HCN (Jones 1962; Corkill 1942), so that these plants may also be arbitrarily classified high, low, or intermediate for HCN production. These three species produce two structurally related cyanoglucosides, linamarin and lotaustralin, and HCN release occurs when these are hydrolysed by the enzyme, linamarase, to produce glucose and an unstable aglycone, which undergoes further decomposition to give HCN and either acetone or methylethyl ketone.

In white clover the discrete form of variation is controlled by two genes (Corkill 1942). The presence or absence of both the glucosides is governed by alleles of a single gene, designated *Ac*, whereas the presence or absence of the enzyme linamarase is governed by alleles of another, independently inherited, gene (*Li*). Only plants which possess dominant functional alleles of both genes liberate HCN when damaged. However, the presence of a functional allele of only one of these genes may be shown by adding either a glucoside or enzyme extract to the test material. The inheritance of cyanogenesis is diploid in white clover. The genetic system in *Lotus* is very similar except that *L. corniculatus* is an autotetraploid and the inheritance of HCN production is tetrasomic.

Biochemical aspects of plant breeding have gained increasing prominence over the past few years, although our understanding of the genetic control of metabolism in plants is still rudimentary. The difficulties of studying gene action in higher plants have been well documented (Nelson 1967), but these studies are essential if we are to avoid the obvious danger of extrapolating information gained from microorganisms without the means of testing such hypotheses. My interest in cyanogenesis has arisen as the result of an attempt to use the cyanogenic polymorphism of white

TABLE 1. Specific activities (as *micromoles substrate hydrolysed per milligram of protein in 10 min*) of extracts of white clover plants.

Plant	Genotype	No. samples	Mean specific activity (±SE)		
			Linamarin–lotaustralin	*p*-Nitrophenyl β-D-glucoside	*p*-Nitrophenyl β-D-galactoside
CS	*LiLi*	3	2.58 ± 0.23	3.44 ± 0.16	2.07 ± 0.10
C2	*LiLi*	6	2.35 ± 0.16	3.55 ± 0.18	1.94 ± 0.08
C3	*Lili*	3	0.60 ± 0.02	1.18 ± 0.03	0.65 ± 0.04
C4	*Lili*	3	0.93 ± 0.04	1.60 ± 0.01	0.86 ± 0.03
C5	*Lili*	3	0.64 ± 0.04	1.14 ± 0.04	0.64 ± 0.02
C7	*Lili*	3	1.20 ± 0.06	1.99 ± 0.06	1.07 ± 0.04
C10	*Lili*	3	0.87 ± 0.01	1.52 ± 0.03	0.87 ± 0.02
C11	*Lili*	3	0.20 ± 0.01	0.57 ± 0.02	0.37 ± 0.03
C13	*Lili*	3	0.89 ± 0.02	1.69 ± 0.03	0.91 ± 0.02
C14	*Lili*	3	1.31 ± 0.03	2.21 ± 0.03	1.26 ± 0.05
D4	*lili*	3	0	0.16 ± 0.01	0.19 ± 0.003
D9	*lili*	6	0	0.31 ± 0.01	0.21 ± 0.01

clover in a detailed study of gene action in higher plants. Specifically, we have attempted to define the nature of the *Ac* and the *Li* loci in white clover.

Experimental

The *Li* locus controlling linamarase activity was the first choice because of the direct relationship between gene and enzyme, established in micro-organisms. The *Li* locus may represent the gene specifying the structural information for the enzyme, linamarase, or it may control the amount of enzyme synthesised in the plant. Mixing experiments have shown that the loss of linamarase activity in plants homozygous for the nonfunctional allele is not due to the production of enzyme inhibitors in this genotype (Hughes 1970). The system is slightly complicated, because the *Li* locus controls a number of enzyme activities and it is not clear how many distinct enzyme proteins are involved in these activities.

Two approaches, one genetic the other immunological, have been used in this study. Immunological studies provided no evidence for a structural gene (Hughes and Maher 1973) since no inactive protein, immunologically related to the normal enzyme (cross-reacting material), was found in plants containing only nonfunctional alleles (*lili*). This is perhaps surprising because *lili* plants have some residual enzyme activity and the antigen–antibody complex of the normal enzyme (from *LiLi* plants) retains enzyme activity.

Table 1 (Maher and Hughes 1973) shows the mean specific activity of extracts from several white clover plants measured against three substrates. This table shows that the *lili* genotype having two nonfunctional alleles has no linamarase activity but has low activity towards *p*-nitrophenyl β-D-glucoside (PNPG) and *p*-nitrophenyl β-D-glucoside (PNPGAL). The lack of dominance of functional *Li* alleles is also indicated in Table 1, since the plants (CS and C2) homozygous for functional alleles have higher enzyme activities compared with the heterozygous plants. (This was confirmed by further breeding work.)

The rationale of the approach was as follows: the stability of enzymes to heat and certain inhibitors is a character which depends on the structure of enzyme protein. Differences in these characters may therefore be taken as evidence of differences in protein structure. Crosses are made between a plant possessing an altered linamarase and being heterozygous at the *Li* locus and plants possessing only recessive, nonfunctional alleles at the *Li* locus. The progeny from these crosses are scored for the presence of linamarase and for the properties of the enzyme. If the *Li* locus specifies the structure of linamarase, all individuals in the progeny containing a functional *Li* allele will also possess an enzyme having the modified characters of the heterozygous parent.

One of the heterozygous plants (C11), shown in Table 1, differed markedly from the other heterozygous plants in having a much lower specific

TABLE 2. Specific activities (*as micromoles substrate hydrolysed per milligram of protein in 10 min*) of extracts of the progeny from crosses involving C7, C11, and C14.

Parents	Genotype of progeny	No. progeny analyzed	Mean specific activity (±SE)		
			Linamarin–lotaustralin	*p*-Nitrophenyl β-D-glucoside	*p*-Nitrophenyl β-D-galactoside
C7 × D4	*Lili*	20	1.52 ± 0.06	2.23 ± 0.06	1.35 ± 0.04
	lili	20	0	0.27 ± 0.01	0.18 ± 0.01
C7 × D9	*Lili*	20	1.24 ± 0.05	1.89 ± 0.05	1.12 ± 0.03
	lili	10	0	0.27 ± 0.01	0.18 ± 0.01
C11 × D4	*Lili*	18	0.22 ± 0.01	0.51 ± 0.03	0.32 ± 0.002
	lili	10	0	0.15 ± 0.01	0.17 ± 0.01
C11 × D9	*Lili*	18	0.29 ± 0.01	0.65 ± 0.02	0.40 ± 0.01
	lili	10	0	0.22 ± 0.01	0.17 ± 0.01
C14 × D4	*Lili*	16	1.22 ± 0.05	1.84 ± 0.06	1.08 ± 0.04
	lili	9	0	0.24 ± 0.02	0.17 ± 0.02
C14 × D9	*Lili*	18	1.21 ± 0.12	1.78 ± 0.07	1.04 ± 0.04
	lili	20	0	0.28 ± 0.01	0.17 ± 0.01

activity toward all three substrates. The effect of individual *Li* and *li* alleles was made by making crosses between C11 and the two *lili* plants given in Table 1. Similar crosses were made between two other heterozygous plants (C7 and C14) and the *lili* plants. Measurements of enzyme activity were then made on the progeny of these crosses, and the results are given in Table 2. The levels of activity of all the heterozygous progeny from the C11 crosses were distinguishable from the activities of all the heterozygous progeny from the remaining four crosses. This is shown in Table 2 by the difference in mean specific activities and the low standard error of means for these crosses. This analysis of the *Lili* heterozygous progeny supports the conclusion that the functional allele of C11 is controlling the activity of either a reduced level of, or a low activity form of, the enzyme(s) responsible for the hydrolysis of linamarin, lotaustralin, PNPG, and PNPGAL.

Three characters were investigated to test for the presence of an altered enzyme protein in the low activity plant C11 and two *lili* plants. No evidence for an altered enzyme was found in C11, but the plants (D4 and D9) which contained only non-functional alleles were shown to possess a different β-glucosidase. However, this low-activity enzyme may also exist in plants having the functional alleles, where its presence would be masked by the more active linamarase. Subsequent work (Hughes and Ayre unpublished data) on cultured cells of white clover (which are not cyanogenic) has demonstrated this enzyme in *LiLi* genotypes, and so this work also provides no evidence for a structural gene at the *Li* locus.

Discussion

The function of the locus (*Ac*) controlling cyanoglucoside production has been investigated (Hughes and Conn unpublished data) in a study designed to determine which step in the biosynthesis of linamarin and lotaustralin is affected by this gene. Figure 1 represents the proposed pathway for the biosynthesis of linamarin. The glucosyltransferase enzyme, which catalyses the final step (4) has been purified from linen flax (Hahlbrock and Conn 1970) but the isolation of the other enzymes involved has yet to be achieved and this meant that the direct measurement of enzyme activity could not be made. The presence of *N*-hydroxyvaline in this pathway has not been established and in this paper the conversion of valine to isobutyraldoxime is considered as a single step. Since Hahlbrock and Conn (1971) have shown that the biosynthesis of both linamarin and lotaustralin is catalysed by the same enzymes the study was limited to a comparison of the biosynthesis of linamarin between plants which contain the cyanoglucosides and plants which do not.

Table 3 shows the results of feeding U-^{14}C valine to shoots of white clover plants. The plants S100/1 and S100/10 produced cyanoglucosides but no linamarase, and the plants DWW/1 and DWW/3

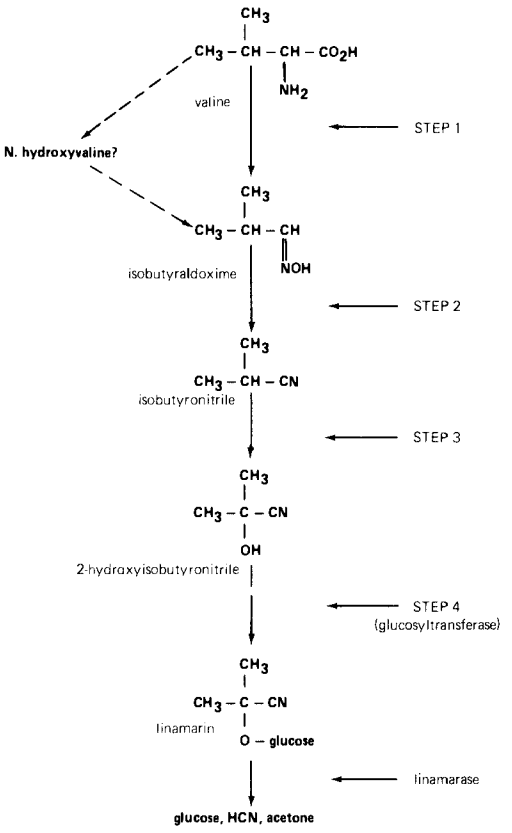

FIG. 1. The biosynthesis of linamarin (from Tapper and Butler 1971).

TABLE 3. Incorporation of ^{14}C-valine into linamarin in white clover.

Plant	HCN content[a]	Fresh weight (g)	U-^{14}C valine administered (μci)	Converted to linamarin (%)
S100/1	2.60	0.6	0.92	6.6
S100/10	2.44	0.9	0.92	6.5
DWW/1	0	0.7	0.92	0
DWW/3	0	0.5	0.92	0

[a]Expressed as μmoles/100 mg fresh weight, first expanded leaf.

TABLE 4. Incorporation of ^{14}C-isobutyraldoxime into linamarin in white clover.

Plant	HCN content[a]	Fresh weight (g)	U-^{14}C isobutyraldoxime administered (μci)	Converted to linamarin (%)
S100/1	2.60	1.0	0.90	2.4
S100/1	2.60	0.7	0.90	2.5
S100/10	2.44	0.9	0.90	2.0
S100/10	2.44	0.6	0.90	2.6
DWW/1	0	0.6	0.90	0
DWW/1	0	1.0	0.90	0
DWW/3	0	0.7	0.90	0
DWW/3	0	0.7	0.90	0

[a]Expressed as μmoles/100 mg fresh weight, first expanded leaf.

TABLE 5. Incorporation of ^{14}C-valine into linamarin in white clover shoots treated with isobutyraldoxime.

Plant	HCN content[a]	Fresh weight (g)	Isobutyraldoxime administered ($\mu moles$)	U-^{14}C valine administered (μci)	Converted to linamarin (%)	Trapped as isobutyraldoxime (%)
S100/1	2.60	1.5	43.2	3.68	2.9	0.3
S100/10	2.44	0.6	21.6	1.84	3.5	0.7
DWW/1	0	1.3	43.2	3.68	0	0
DWW/3	0	0.7	21.6	1.84	0	0

[a]Expressed as μmoles/100 mg fresh weight, first expanded leaf.

contained neither cyanoglucoside nor the hydrolytic enzyme. It can be seen that the conversion of ^{14}C valine into linamarin has only occurred in those plants normally capable of synthesising cyanoglucosides. Table 4 shows the results of feeding U-^{14}C isobutyraldoxime to the same plants. Again, the incorporation of radioactivity into linamarin has only occurred in those plants (S100/1 and S100/10) normally capable of linamarin synthesis. This means that at least one step after the production of isobutyraldoxime is missing in plants DWW/3 and DWW/1.

To test for the presence of step-one function (isobutyraldoxime synthesis) in cyanoglucoside negative plants, ^{14}C-labelled valine was fed to the same four plants in the presence of excess cold isobutyraldoxime. The results of this experiment are shown in Table 5, where it can be seen that in

the cyanoglucoside-positive plants (S100/1 and S100/10) about 0.5% of the radioactivity fed as valine has been "trapped" by the cold isobutyraldoxime thus demonstrating the function of step one in these plants. In the cyanoglucoside-negative plants (DWW/1 and DWW/3), no measurable radioactivity was found in the isobutyraldoxime fraction. This result means that white clover plants unable to synthesise linamarin lack at least two steps in the biosynthetic pathway shown in Fig. 1.

This conclusion means either that the single gene nature of the *Ac* locus must be questioned or that this locus may not represent a gene specifying structural information for protein synthesis. Detailed studies of both the genes controlling cyanogenesis in white clover have therefore tended to suggest that they represent control elements rather than structural genes.

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