Food Chemistry **20** (1986) 1–9

Fate of Ingested Linamarin in Malnourished Rats

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(Received: 6 August, 1985)

ABSTRACT

Pure linamarin at a dose level of 30 g per 100 g body weight was administered in food to a group of Wistar rats maintained on vitamin B_2-deficient, sufficient and excess diets for 5 weeks and to another group of kwashiorkor rats. Free and total cyanide, intact linamarin and thiocyanate levels were estimated in urine and faeces obtained at 0-, 24-, 48- and 72-h periods and in blood samples obtained in the seventy-second hour after the drug had been administered. There was no detectable cyanide or intact linamarin in the faecal samples. Vitamin B_2-sufficient and excess groups of rats excreted higher total and free cyanide than the respective vitamin B_2-deficient groups. Most of the linamarin was degraded after the first 24 h. The rate of breakdown of the glucoside within the first 24 h was slowest for the zero and half normal vitamin B_2 status, respectively, as evidenced by its appearance in large quantities in the urine. The kwashiorkor rats, on the other hand, excreted less thiocyanate than the controls. In addition, their control group excreted most of the thiocyanate (SCN^-) in the first 24 h whilst the kwashiorkor rats excreted theirs in the first 48 h. Dietary protein deficiency prolongs the time of metabolism and hence increases the toxicity of cyanogenic glycoside in the body. It is also suggested that excessive exposure of malnourished humans to cyanide could be a contributory factor in the rampant cases of tropical ataxic neuropathy (TAN).

Food Chemistry 0308-8146/86/$03·50

INTRODUCTION

The principal cyanogenic glucoside in cassava is linamarin. On ingestion of cassava, the intact linamarin is hydrolysed to hydrocyanic acid which, in the presence of a sulphur donor, is detoxified by the enzyme, rhodanese, to the less toxic thiocyanate (SCN^-) and excreted in urine (Oke, 1969). Also, intact linamarin is excreted in urine by rats which have received only a sublethal dose of the drug (Barrett *et al.*, 1977). Of the B complex group of vitamins, vitamin B_{12a} (hydroxocobalamin) is known to play a part in cyanide metabolism (Wokes & Pickard, 1955; Smith, 1961; Osuntokun, 1969). Dietary deficiency of vitamin B_{12} (cyanocobalamin) leads to increased thiocyanate excretion. Other B complex group vitamins, which act mainly as co-enzymes in cell metabolism, have been indirectly associated with the metabolism of cyanide. Osuntokun *et al.* (1970) suggested that deficiency of riboflavin may be a contributory factor in the disease tropical ataxic neuropathy in areas of high cassava consumption. Monekosso & Annan (1964) demonstrated the therapeutic effect of the B complex group of vitamins on ataxic neuropathy patients.

In southern Nigeria, cassava is the main staple and provides nearly 70% of the total daily caloric needs. Kwashiorkor and other protein energy malnutrition syndromes present major public health problems, particularly in vulnerable groups. Umoh & Bassir (1977) reported that most of the commonly eaten diets in the southern parts of the country contain low levels of the B vitamins and protein. It is therefore necessary to determine the extent to which kwashiorkor and dietary levels of vitamin B_2 would alter the metabolism of linamarin.

MATERIALS AND METHODS

Selection of rats

Sixty albino rats of the Wistar strain, weaned at 23–24 days of age, were obtained from the disease-free stock of the Preclinical Animal House, University of Ibadan, Ibadan, Nigeria, and reared on a commercial stock diet (Pfizer Livestock Feeds, Ikeja, Nigeria) until they were 30–31 days old and weighed between 50 and 60 g. These rats were then weighed and allocated, on the basis of weight and litter origin, to six groups of ten rats each. The rats were housed individually in cages and fed for 5 weeks on the

TABLE 1
Composition of the Experimental Diets
(Percentage dry weight basis)[a]

Dietary Mix	*Percentage in diet*
Soyabean powder	44·48
Garri (fried cassava flour)	44·48
Salt mix	4·74
L-Lysine	0·20
L-Methionine	0·10
Vitamin mix[b]	1·00
Corn oil	5·00

[a] Bassir & Loebel (1968).
[b] Cuthbertson (1957) but with varying amounts of riboflavin.

TABLE 2
(a) Kwashiorkorigenic Diet
(Boyd & Carsky, 1969)

Component	%
Protein	3·47
Carbohydrate	81·53
Corn oil	8·00
Salt mixture	4·00
All vitamin supplement	3·00

(b) Control Diet
(Bassir & Loebel, 1968)

Component	%
Soyabean powder	44·48
Garri (fried cassava flour)	44·48
Salt mixture	4·74
L-Lysine hydrochloride	0·20
L-Methionine	0·10
Vitamin mixture	1·00
Corn oil	5·00

basal diet (Table 1) containing varying amounts of riboflavin. Diet A had no riboflavin. Diets B, C and D contained, respectively, half normal, normal and twice normal the rats' daily riboflavin requirements.

Induction of kwashiorkor in rats

Rats in group E were placed on the kwashiorkorigenic diet (Table 2) of Boyd & Carsky (1969), for 5 weeks while its control, group F, was on the protein-rich diet of Bassir & Loebel (1968). Food and water were supplied to all the rats *ad libitum*.

The onset of the kwashiorkor syndrome was marked by the symptoms observed in the model animals as compared with the controls, that is: (a) a drastic reduction in body weight gain; (b) flaking off of hair; (c) reduced packed cell volume; (d) a drastic reduction of white blood cells; (e) lowered rectal temperatures and (f) a dramatic recovery of the affected animals within a few days of being placed on the control diet.

Administration of the Linamarin

At the end of the period of feeding on the experimental diets, the rats were transferred to metabolic cages. Urine and faeces were collected for each rat during the first 24 h and labelled the zero hour samples. Pure synthetic linamarin (2-hydroxyisobutyronitrile-β-D-glucoside), melting point 145–147 °C, $[\alpha]_D + 29{\cdot}5$, was purchased from Calbiochem, La Jolla, California, USA. It has identical physical characteristics to those of the naturally occurring compound. The linamarin, dissolved in distilled water, was administered in food to the rats so that each rat received a dose of 30 mg per 100 g of body weight (Barrett *et al.*, 1977). Care was taken to ensure that the whole of the 30 mg of the drug was ingested by the rat. This was achieved by adding the solution to about 5 g of the rats' food. After this was completely eaten more food was offered.

Collection of faeces, urine and blood samples

The urine and faecal samples were collected 24, 48 and 72 h after administration of the drug. The animals were then sacrificed and blood samples collected for analysis. The samples were stored in dark labelled bottles and kept in a refrigerator until required for analysis.

Determination of total, free and unmetabolised linamarin (bound cyanide) in faecal urine and blood samples

The enzymatic assay method of Cooke (1968) was employed in this assay. In this method, 0·2 ml aliquots of urine or blood plasma for bound cyanide (unmetabolized linamarin), or 0·4 ml for free cyanide, were neutralized and incubated for 15 min at 37 °C with 0·1 ml of appropriate crude linamarase preparation for bound cyanide prior to spectrophotometric analysis. For determination of the free cyanide, the colour was developed directly without any enzyme hydrolysis.

For faecal cyanide determination, weighed samples of the faeces were solubilized in 2M orthophosphoric acid, filtered and the filtrate neutralized. Spectrophotometric readings were taken after 90 min at 620 nm.

Determination of thiocyanate in urine samples

The thiocyanate levels in the urine samples from kwashiorkor and control rats were determined by the method of Bowler (1944).

RESULTS AND DISCUSSION

The results obtained in this study are summarised in Tables 3–6. No linamarin was detected in the rats' faeces or blood. Failure to find linamarin in the faeces of the rats must mean that, within the limits of the analytical method, the ingested linamarin was either completely absorbed intact or partially hydrolyzed and the products absorbed or lost in the faeces (Barrett *et al.*, 1977). Failure to find linamarin in the blood, on the other hand, even though it appears in the urine, may reflect a quite diluted level of linamarin in the blood, concentrated by the kidney, and possibly a temporary binding of linamarin with the blood proteins in a form not measurable by the analytical method used.

Table 3 shows the pattern of excretion of the total and free cyanide, as well as the unmetabolized glucoside, after 24 h of drug administration. Riboflavin-sufficient and excess groups excreted higher total cyanide and free cyanide than the vitamin B_2-deficient groups. The vitamin B_2-excess group (group D) equally excreted more unmetabolized glucoside than any of the other groups, including the control (group C). After 48 h

TABLE 3
Total Cyanide, Unmetabolized Linamarin and Free Cyanide Urinary Excretion in 24 h by Rats fed on Various Levels of Dietary Riboflavin (mg/24 h urine sample)[a]

Group[b]	*Total cyanide*	*Unmetabolized linamarin*	*Free cyanide*
A	0·333 ± 0·050	0·195 ± 0·040	0·138 ± 0·013
B	0·545 ± 0·080	0·232 ± 0·011	0·313 ± 0·021
C	0·643 ± 0·032	0·203 ± 0·022	0·440 ± 0·041
D	0·853 ± 0·043	0·290 ± 0·010	0·568 ± 0·041

[a] Mean ± SE for ten rats per group.
[b] A, B, C and D, respective groups on zero, half normal, normal and twice normal daily vitamin B_2 requirements.

(Table 4), the pattern of clearance of the total and free cyanide had not changed significantly. However, the unmetabolized linamarin was almost completely eliminated during this period by the control group (group C) and group A (with no vitamin B_2 supplementation at all) with values at 0·004 mg and 0·045 mg/24 h, respectively.

Some of the vitamins of the B complex have been implicated in cyanide metabolism. A major vitamin in this group is riboflavin (vitamin B_2). Cyanide interferes with the cytochrome oxidase system in the cell, thereby

TABLE 4
Total Cyanide, Unmetabolized Linamarin and Free Cyanide Urinary Excretion in 48 h by Rats Fed on Various Levels of Dietary Riboflavin (mg/24 h urine sample)[a]

Group[b]	*Total cyanide*	*Unmetabolized linamarin*	*Free cyanide*
A	0·163 ± 0·010	0·045 ± 0·010	0·035 ± 0·000
B	0·378 ± 0·031	0·100 ± 0·020	0·278 ± 0·040
C	0·350 ± 0·030	0·004 ± 0·000	0·346 ± 0·020
D	0·558 ± 0·010	0·150 ± 0·000	0·408 ± 0·090

[a] Mean + SE for ten rats per group.
[b] A, B, C and D, respective groups on zero, half normal, normal and twice normal daily vitamin B_2 requirements.

TABLE 5

Total Cyanide, Unmetabolized Linamarin, Free Cyanide and Thiocyanate Urinary Excretion in 24 h by Kwashiorkor and Well Fed Rats (mg/100 ml urine samples)[a]

Group[b]	*Total cyanide*	*Unmetabolized linamarin*	*Free Cyanide*	*Thiocyanate*
KWM	0·530 ± 0·152	0·184 ± 0·109[c]	0·346 ± 0·066[c]	0·594 ± 0·065[c]
CM	8·817 ± 2·393	6·952 ± 1·298	1·87 ± 0·202	3·63 ± 0·065
KWF	0·816 ± 0·181[c]	0·110 ± 0·038[c]	0·706 ± 0·270[c]	1·13 ± 0·250[c]
CF	14·3 + 3·517	11·3 + 1·992	3·00 + 0·467	6·00 + 0·933

[a] Mean ± SE for ten rats per group.
[b] KWM, KWF, kwashiorkor male and female rats; CM, CF, control male and female rats.
[c] Differences between KWM, KWF and CM, CF are significant at $p < 0·05$.

rendering oxygen unavailable to the tissues, resulting in death. However, in the absence of critical concentration of cyanide, flavin nucleotides (from vitamin B_2) could be utilized to complete the oxidation, though with much reduced efficiency on account of the low potentials of the resulting hydrogen peroxide. This may result in an increased requirement for riboflavin. Deficiency of riboflavin impairs the function of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) systems. Higher free and total cyanide in the urine of rats with sufficient and excess

TABLE 6

Total Cyanide, Unmetabolized Linamarin, Free Cyanide and Thiocyanate Urinary Excretion in 48 h by Kwashiorkor and Well Fed Rats (mg/100 ml urine samples)[a]

Group[b]	*Total cyanide*	*Unmetabolized linamarin*	*Free cyanide*	*Thiocyanate*
KWM	5·78 ± 2·160[c]	4·76 ± 1·414[c]	1·02 ± 0·066[c]	1·92 ± 0·720[c]
CM	0·489 ± 0·099	0·095 ± 0·012	0·403 ± 0·045	0·733 ± 0·029
KWF	9·30 ± 2·400[c]	7·69 ± 1·113[c]	1·61 ± 0·427[c]	3·54 ± 0·800[c]
CF	0·893 ± 0·341	0·146 ± 0·032	0·747 ± 0·195	1·43 ± 0·567

[a] Mean ± SE for ten rats per group.
[b] KWM, KWF, kwashiorkor male and female rats; CM, CF, control male and female rats.
[c] Differences are significant at $p < 0·05$.

riboflavin in their diet (groups C and D) indicates a possible therapeutic effect of this vitamin on cassava-induced neuropathy. Monekosso & Annan (1964) demonstrated the therapeutic effect of the B complex vitamins (comprising 100 g of thiamine, 4 mg of riboflavin, 4 mg of pyridoxine and 40 mg of nicotinamide) on ataxic neuropathy patients who recovered from the disease. Osuntokun *et al.* (1970) suggested that the deficiency of riboflavin may be a contributory factor in the disease ataxic neuropathy. These workers also suggested that riboflavin deficiency, which frequently—if not invariably—accompanies dietary protein deficiency, would have the same depressant effect on cell oxidation as the cyanide inhibition of cytochrome oxidase.

Tables 5 and 6 show the pattern of excretion of the glucoside and its metabolites in 24 and 48 h in well fed control and kwashiorkor male and female rats. The results indicate that, in the first 24 h, the control rats excreted significantly higher total and free cyanide, unmetabolized linamarin and thiocyanate than the corresponding kwashiorkor rats. The female rats in both cases excreted higher quantities than their corresponding males. After 48 h of administration of the drug, its elimination—and that of its metabolites—showed that the clearance rate of each was significantly higher in the kwashiorkor rats than in the controls. The control rats excreted their detoxification products in the first 24 h while the kwashiorkor rats excreted theirs in 48 h of drug administration. In kwashiorkor, fatty liver is one of the reported features (McDonald *et al.*, 1963; Theron & Liebenberg, 1963; Lewis *et al.*, 1964; Olowookere, 1980). Liver is the main site of cyanide metabolism. The ability of the kwashiorkor rats to metabolise cyanide to thiocyanate, despite the state of the liver, supports the observation of Stanstead *et al.* (1965) that the liver function remains unaltered despite structural changes occurring in the liver in PEM. However, the chronic shortage of protein which they underwent from their weaning must have been responsible for prolonging the time of the metabolism and eventual clearance of the thiocyanate in the kwashiorkor rats. The higher figures in Tables 5 and 6 for female rats than their corresponding males might indicate some hormonal influence in the clearance rate of the ingested drug. This aspect was not, however, investigated further. As a result of the prolonged metabolism of the free cyanide to thiocyanate, we suggest that dietary protein deficiency increases the toxicity of the cyanogenic glucoside (linamarin) in the body and is a contributory factor in the ataxic neuropathy syndrome rampant in areas of Nigeria where a considerable amount of cassava is consumed.

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