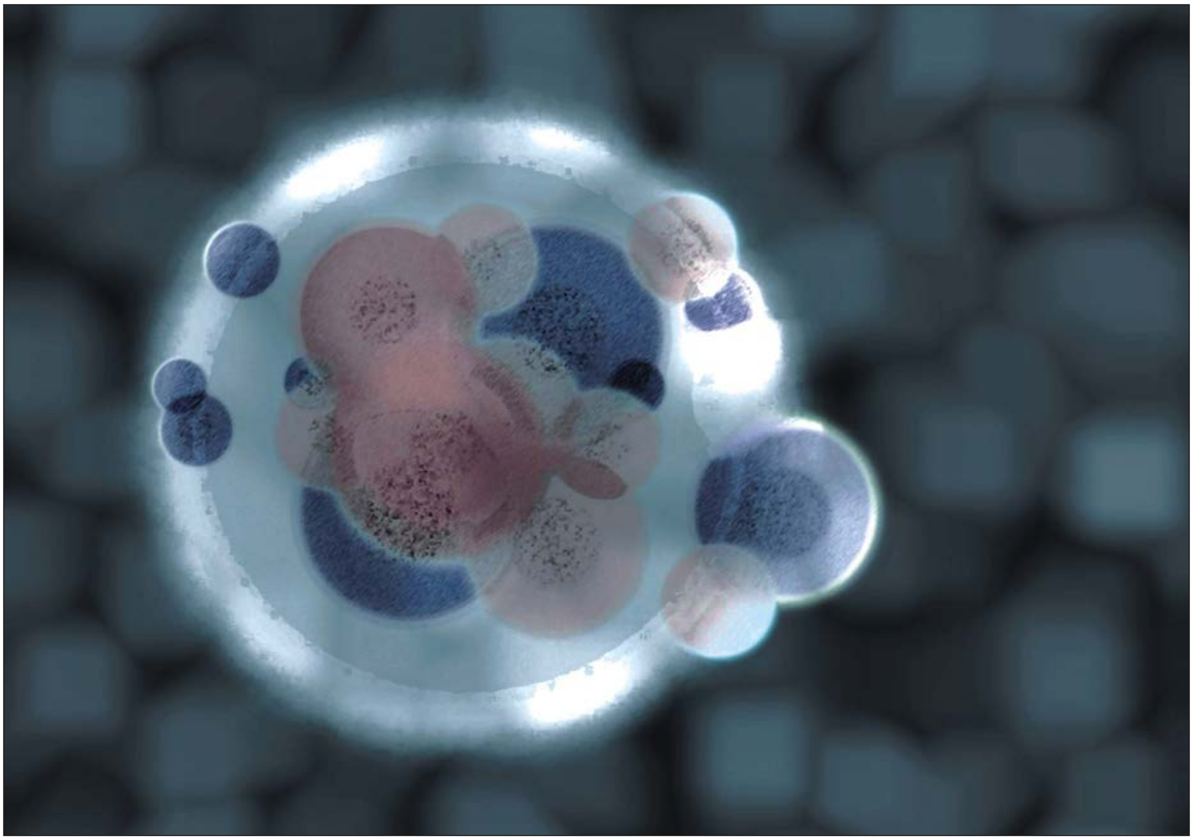

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Activities of Thiosulphate and 3-Mercaptopyruvate-cyanide-sulphurtransferases in Poultry Birds and the Fruit Bat

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Abstract: The activities of the sulphurtransferases in different tissues of some poultry birds and the fruit bats that, presumably, feed on cyanogenic plants and/or their products have been measured. Thiosulphate sulphurtransferase (rhodanese, EC 2.8.1.1) and 3-mercaptopyruvate sulphurtransferase (EC 2.8.1.2) were assayed in the crude extracts of some tissues (liver, kidney, lung, gizzard, heart and brain) of four domestic birds (local chicken, poultry chicken, pigeon and duck) and the fruit bat by the determination of the thiocyanate released from the reaction of potassium cyanide with respective sulphur donor by the Sorbo's ferric-nitrate reagent. The results showed that rhodanese (RHD) and 3-mercaptopyruvate sulphurtransferase (3-MST) are present in all the tissues studied. In the chickens, duck and fruit bat, the liver had the highest level of rhodanese while in the pigeon; the activity was highest in the kidney. The brain or the gizzard contained the lowest activity of RHD in all the birds. On the other hand, 3-mercaptopyruvate sulphurtransferase activities in the local chicken and pigeon were found to be highest in the liver, followed by the kidney. In poultry chicken, duck and the fruit bat, the level of the 3-MST was found to be highest in the kidney, followed by the lung in poultry chicken and the liver in the duck. Moreover, determination of the cyanide content of some food materials in the locality, poultry rations and parts of plants found at the roosting site of a colony of fruit bats on the Obafemi Awolowo University (Nigeria) campus showed different levels ranging from 1.82±0.37 to 15.73±0.18 mg 100 g^{-1}.

Key words: Thiosulphate sulphurtransferases (rhodanese), 3-mercaptopyruvate sulphurtransferase, cyanide, detoxication, chicken, duck, pigeon, fruit bat

INTRODUCTION

Cyanide poisoning to man and other animals is an important toxicological and environmental issue. This is against the backdrop that most substances used by man and animal as food come from plants. It is well known that cyanides, as glycosides, are widely distributed in the plant kingdom. Over two thousand species of plant are known to contain approximately twenty-five cyanogenic glycosides. Cyanide toxicity to mammals is relatively common due to these large numbers of cyanogenic forage plants (Al-qarawi *et al*., 2001; Kingsbury, 1964; Montgomery, 1965; Montgomery, 1980; Vennesland *et al*., 1982; Wokes and Willimott, 1951). Cassava and sorghum are especially important staple foods containing cyanogenic glycosides (Vennesland *et al*., 1982; Conn, 1979; 1979; Nartley, 1980; Oke, 1979; 1980; Rosling, 1987). These include amygadalin, dhurin, linamarin and lotaustralin.

The word poultry in agriculture refers to domesticated birds kept for egg or meat production. These birds include chickens (domestic fowls), turkeys, guinea fowls, pigeons and geese. Local chicken here refers to fowls kept on free-range while poultry chicken refers to those kept under the semi-intensive or intensive management systems (Komolafe *et al*., 1981). Birds on free-range find their own foods, which consist of leaves, grass, seeds, fruits and by scratching the grounds for insects, sand grains and earthworms. However, backyard and local poultry farmers do feed their birds with grains (such as maize, rice, wheat or sorghum) and mashed cooked tubers (such as yam, potatoes, cocoyams, cassava and its products) usual in the morning. When birds are confined, their basal (energy) feeds include poultry mashes usually consisting of ground cereals to which portions of vegetable protein meals and animal proteins had been added (Komolafe *et al*., 1981).

The purpose of this study was to determine the activities of the sulphurtransferases in domestic birds and in the fruit eating bats in correlation with their inherent enzymatic mechanisms to effectively detoxify cyanide. These birds (whose diets are composed basically of

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grains, cereals and other types of plant products) are constantly being exposed to cyanogenic glycosides in their foods and/or feeds. Domestic birds feed mainly on cereals, grains and tubers, as well as feedstuffs, which were composed from them. The fruit bats on the other hand feed mainly on a number of both normal and wild fruits. Many of these plant food materials have been found to be cyanogenic (Montgomery, 1965; Vennesland *et al.*, 1982; Wokes and Willimott, 1951; Egekeza and Qehme, 1980; Ellenhorn and Barceloux, 1988). Death of birds and grazing animals through exposure to cyanide salts or ingestion of cyanogenic plants have been reported (Aminlari *et al.*, 1997; Keeler *et al.*, 1978; http://www. inchem.org/documents/jecfa/jecmono/v30je18.htm). The potential toxicity of a cyanogenic plant depends primarily on the potential that its consumption will produce a concentration of prussic acid, HCN, which may be released by enzyme hydrolysis, which is toxic to exposed animals or humans. Two enzymes mediate the hydolysis of cyanogenic glycosides: beta-glucosidase and hydroxynitrile lyase that are present within the plants or in bacteria in the gastrointestinal tract of animals. The hydrolysis is triggered by physical disruption (mastication, tramping, etc.) or stress (drought. frost etc.). It is speculated that for these animals to survive the adverse effect of cyanide toxicity, they must possess effective cyanide detoxication mechanisms which are basically combination of processes but chiefly by enzyme-based reactions catalyzed by the sulphurtransferases (Aminlari and Shahbazi, 1994; Nagahara *et al.*, 1999; Ways, 1984).

Sulphurtransferases (EC 2.8.1) are a group of enzymes widely distributed in plants, animals and bacteria that catalyze the transfer of a sulphane atom from a donor molecule to a thiophilic acceptor substrate *in vitro*. Members of this group include rhodanese or thiosulphate-cyanide sulphurtransferase (RHD, EC 2.8.1.1), thiosulphate-thiol transference (EC 2.8.1.3) and 3-mercaptopyruvate-cyanide sulphurtransferase (MST, EC 2.8.1.2). Interestingly, RHD and MST are evolutionarily related as they display clear sequence homology in which they share up to 66% identical residues (Burrows, 1981; Gossellin *et al.*, 1984; Solomonson, 1981). The classical reaction of the sulphurtransferases is the formation of thiocyanate from thiosulphate and cyanide via a double displacement reaction (Westley, 1973). Sulphurtransferases seem to be essential but their *in vivo* function is still unknown in organisms investigated so far. Nonetheless, the physiological role of cyanide detoxification i.e., biotransformation of cyanide (CN^-) to the less toxic thiocyanate (SCN^-) has been attributed to the catalytic action of the sulphurtransferases among their other important biological functions (Al-qarawi *et al.*, 2001; Nagahara *et al.*, 1999). Furthermore, another enzyme, beta-cyanoalanine synthetase (beta-CAS, EC 4.4.1.9) in a reaction coupled to another enzyme, beta cyanoalanine hydratase (EC 4.2.1.65) has been implicated in the detoxification of HCN in insects (Urbanska *et al.*, 2002). Apart from cyanide detoxification, other proposed functions of the sulphurtransferases include formation of prosthetic groups in iron-sulphur proteins, maintenance of the sulphane pool, selenium metabolism and thiamin biosynthesis (Bordo and Bork, 2002) and regulation of mitochondrial respiration rate (Ogata and Dai, 1988; Ogata and Volini, 1986). Moreover, other mechanisms of cyanide detoxification include the formation of cyanomethemoglobin (instead of binding to cytochrome oxidase), reaction with mercaptopyruvate to form cyanohydrin (Nagahara *et al.*, 1999; Ways, 1984), the combination with endogenous hydrocobalamin (Vitamin B_{12}) to form cynacobalamin and a mechanism involving the thyroid gland in which cyanide is incorporated into asparagine (Oke, 1973; Nestle and MacIntyre, 1973).

MATERIALS AND METHODS

The study was conducted between April 2004 and July 2005 at either of our laboratories in Ile-Ife or Ikenne as indicated by the source of the material below. Domestic birds (local chicken, poultry chicken, pigeon and duck) were purchased from the local market at Ikenne, Ogun State, Nigeria. The birds were killed by cervical dislocation and then dissected. Fruit bats were collected from the Botanical Garden, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. They were knocked at the back of the head to unconsciousness and dissected. The tissues were quickly excised, placed in an ice bath and rinsed thoroughly with cold saline solution (0.9% NaCl), blotted and stored at freezing temperatures or used immediately. Freshly harvested cassava tubers were obtained from a farmland while fruits and cassava products were obtained from the local market in Ikenne, Ogun State, Nigeria. Leaves, seeds and fruits of plants in and around the colony of the fruit bat, *Eidolon helvum*, were collected within the campus of the Obafemi Awolowo University. Poultry feedstuffs were as formulated by and collected from the Commercial Farms Limited of the same University.

All chemicals were of analytical grade and were either purchased from Sigma Chemical Company or BDH Chemical Limited.

Tissue enzyme extract was prepared for either RHD or 3-MST assays according to the methods of Agboola *et al*. (2004). Rhodanase activity was measured according to the method of Lee *et al*. (1995) as described by Agboola and Okonji (2004). 3-MST was assayed according to the method of Taniguchi and Kimura (1974) using 2-mercaptoethanol as the substrate as described by Agboola *et al*. (2004). The unit of activity was expressed as Rhodanese Unit (RU for RHD) and Mercaptopyruvate sulphurtransferase Unit (MU for MST). 1RU or 1MU was taken as the amount of enzyme, which under the conditions of the assay gave an optical density reading of 1.08 at 460 nm per minute (Lee *et al*., 1995). Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976). Cyanide concentration determination was carried out by the distillation method of Yeoh and Oh (1979) as slightly modified by the Federal Industrial Research Institute, Oshodi, Lagos, Nigeria (Solomon, 1998).

RESULTS AND DISCUSSION

The activities of the sulphurtransferases in different tissues of some poultry birds and the fruit bats that, presumably, feed on cyanogenic plants and/or their products were measured. A summary of the specific activities of RHD and 3-MST in these tissue extracts are shown in Table 1 and 2. RHD and 3-MST were present in all the tissues studied. In the chickens, duck and fruit bat, the liver had the highest level of activity of RHD while in the pigeon; its highest level of activity was in the kidney. The activity was lowest in the brain for all the tissues except for the duck where the activity was lowest in gizzard. 3-MST activity was found to be highest in the kidney for all the animals tested followed by the liver or lung. The cyanide concentration estimated from some local foodstuffs and fruits in our locality as well as in two poultry feedstuffs are shown in Table 3. Table 4 shows the cyanide content of plants found at the roosting site of fruit bats on our campus. Cassava and its products contained very high levels of cyanide. Also yam and potato, which are other staple diet of man and animals, as well as pineapple, were found to contain high levels of cyanide. Furthermore, most plant materials used by the fruit bat as found in their roosting site in Ile-Ife contained cyanide in low concentrations.

This study showed that there is variation in RHD as well as 3-MST distribution in the tissues of these animals. Generally, the pattern of distribution of rhodanese in different tissues of animals is species specific. The values of activities in the tissues obtained for RHD are in the same order of magnitude as obtained for the chickens and pigens by Al-qarawi *et al*. (2001) but higher RHD enyzme activity were found in the fruit bat than the poultry birds and in other mammals as previously reported (Al-qarawi *et al*., 2001). It is also apparent that although, RHD or 3-MST are detectable in many of the organs studied, the amounts are low compared with those of the liver and/or kidney. This is consistent with the role of the liver as the main organ of biotransformation in detoxification processes in general and the kidney, which helps to eliminate the metabolites in the urine. Reports in the literature have shown that the liver is the major source of RHD and is believed to be the major site of cyanide detoxification (Al-qarawi *et al*., 2001; Nartley, 1980; Aminlari and Gilanpour, 1991; Drawbaugh and Marrs,

Table 1: Specific activity of rhodanese in the cytosolic fractions of different tissues of some birds and fruit bat

Tissues	Poultry chicken	Local chicken	Pigeon	Duck	Fruit bat
Liver	0.94±0.10	0.39±0.03	0.39±0.05	0.53±0.06	11.70±0.99
Kidney	0.70±0.06	0.49±0.02	0.78±0.08	1.11±0.04	11.63±1.24
Lungs	0.36±0.06	0.40±0.19	0.69±0.04	0.50±0.05	2.09±1.12
Heart	0.58±0.10	0.58±0.14	0.60±0.02	0.64±0.10	2.19±0.27
Brain	0.65±0.08	0.41±0.02	0.56±0.04	0.78±0.13	1.92±0.30
Gizzard	0.15±0.11	0.29±0.04	0.27±0.05	0.402±0.08	8.38±0.10*
Spleen	ND	ND	ND	ND	3.16±0.12

The values are in Rhodanese Unit (RU) per milligram (mg) of protein. The values are means±SD for five determinations. The extraction and enzyme assay were as described in the text. ND means Not Determined. *intestine

Table 2: Specific activity of 3-mst in the cytosolic fractions of some bird tissues and fruit bat

Tissue sample	Local chicken	Poultry chicken	Pigeon	Duck	Fruit bat
Liver	0.21±0.04	0.13±0.02	0.19±0.02	0.09±0.01	0.25±0.01
Kidney	0.24±0.02	0.18±0.05	0.34±0.09	0.28±0.02	0.31±0.01
Lung	0.12±0.01	0.17±0.02	0.22±0.02	0.27±0.02	0.09±0.01
Gizzard	0.09±0.01	0.27±0.04	0.14±0.01	0.45±0.01	0.14± 0.01*
Heart	0.09±0.01	0.03±0.01	0.15± 0.04	0.10±0.02	0.16± 0.01
Brain	0.08±0.02	0.10±0.01	0.16±0.02	0.06±0.00	0.08±0.02
spleen	ND	ND	ND	ND	0.17±0.02

The values are in 3-MST Units (MU) per milligram of protein. The values are means±SD for five determinations. 3-MST was assayed using 2-mercaptoethanol as substrate instead of 3-mercaptopyruvate, the true substrate. Therefore, values may be slightly underestimated. ND (Not Determined). *intestine

1987). However, high levels of RHD activity in other tissues have also been reported and as a result there is the suggestion that RHD preformed primarily other biological functions and that cyanide detoxification is just a secondary role. For instance, Himmich and Saunders (1948) had earlier on reported that the highest activity of RHD in dogs was in the adrenal gland which is consistent with the role of the enzyme in the synthesis of iron-sulphur centers of adrenal ferredoxin (Taniguchi and Kimura, 1974). In the present study, it is noteworthy to find that, the highest activity of RHD in pigeon was found in the kidney in agreement with the work of Al-qarawi *et al.* (2001) whereas according to Aminlari and Shahbazi (1974), the chicken proventicus contained higher activity of RHD rather than the liver. The proventicus or glandular stomach can be compared to the rumen in ruminants in that it secretes gastric juice and hydrochloric acid into the gizzard (Komolafe *et al.*, 1981) and therefore might be the site for the hydrolysis of cyanogenic glycosides to release the toxic HCN. It is not surprising to find that 3-MST activity is highest in the gizzard of both poultry chickens and ducks because the poultry chicken is the species most exposed to cyanide through commercial feedstuffs while the duck is locally kept exclusively on the free-range system when viewed in relation to the function of the gizzard in bird's digestion.

Species differences in reaction to cyanogenic glycosides due to a difference of detoxifying ability due to anatomical structure have been observed. For example, ruminants such as cattle and sheep are supposed to be more susceptible to the acute toxic effects because of their larger flora of microorganisms and considerable quantities of enzyme emulsin which hydrolyses the glycosides (Oke, 1979). Also, hamsters have been reported to be more susceptible than rats to acute toxic effects of orally administered amygdalin and prunasin (Gomez *et al.*, 1988).

Low concentration of cyanide can be highly toxic to man and animals. The yield of cyanide from common foods and feeds sources range from 0-910 mg 100 g^{-1} (Conn, 1979; 1979; http://www.inchem.org/documents/jecfa/jecmono/v30je18.htm). Lethal dose of HCN in mg per kg body weight (bw) were reported for mouse, 3.7; dog, 4.0; cat, 2.0 and for cattle and sheep 2.0 (Conn, 1979). Burrows (1981) reported an LD_{50} for oral KCN of 3.8 mg kg^{-1} body weight in sheep. The acute oral lethal dose of HCN for human beings is reported to be 0.5-3.5 mg kg^{-1} bw corresponding to 1.0-7.0 mg kg^{-1} bw of KCN with clinical signs which include headache, dizziness, mental confusion, stupor, cyanosis with twitching and convulsions, followed by terminal coma (Conn, 1979; Gossellin *et al.*, 1984). The acute toxicity studies of cyanogenic glycosides or cyanogenic plant tissues gave an LD_{50} of 0.1 mmole amygdalin kg^{-1} bw for mouse when injected intraperitonially and 0.02-0.13 mmol kg^{-1} amygdalin kg^{-1} bw for man (Solomonson, 1981) when ingested and 20,000 mg linamarin kg^{-1} bw and 450 mg linamarin kg^{-1} bw for rat when administered intravenously and orally, respectively (Oke, 1979). When chicken were fed high-cyanide-containing Cassava Root Meal (CRM) supplying 3000 mg of total cyanide kg^{-1} for 63 days and Cassava Foliage Meal (CFM) supplying 156 mg total cyanide kg^{-1} for 56 days, most of it in the form of cyanogenic glycosides, no changes in the haematological parameters due to cassava were seen. Addition of up to 30% CRM failed to neither adversely affect broiler survival, performance nor feed efficiency, but the inclusion of CFM in the experimental diets increased mortality, decreased weight gain and decreased feed efficiency (http://www. inchem.org/documents/jecfa/jecmono/v30je18.htm). In both experiments, increased quantities of dietary cassava cyanate were associated with increased blood serum thiocyanate concentrations. Histopathological examination of thyroid, liver and kidney revealed no appreciable alterations due to the cassava feeding. The results showed that broiler were tolerant of relatively high levels of dietary cyanogenic glycosides (Gomez *et al.*, 1988), through the enzymatic conversion to thiocyanate and not through any other mechanism, in particular, the one involving the thyroid gland (Oke, 1973). However, aflatoxin contamination might have contributed to the high mortality rate associated with CFM diets (http://www. inchem.org/documents/jecfa/jecmono/v30je18.htm). The results of this work also showed that commercial broiler ration contained a high level of cyanide (Table 3).

The toxic effects of cyanide have been attributed to the inhibition of cytochrome oxidase, the terminal oxidase of the mitochondrial respiratory pathway and other metallo- and carbonyl- groups containing proteins (Nagahara *et al.*, 1999). Cyanide acts almost instantly and could cause death within a few seconds to minutes depending on the route of intoxication. These routes include inhalation, ingestion or dermal or percutaneous absorption. *In vitro* antidotes to acute cyanide intoxication that have been tested in animals include the Sulphane Sulphur Donors (SSDs) (Baskin *et al.*, 1999), l- or D-cysteine, cystine (Huang *et al.*, 1998; Wrobel *et al.*, 1997), 2(D-glucopentahydroxypentyl)-thiazolidine-4-carboxylate (Wrobel *et al.*, 1997) and some alpha-keto acids (Porter and Baskin, 1996).

The method of quantization of cyanide releasable from cyanogenic glycosides employed here is considered

Table 3: Cyanide concentrations in some local plant food materials and poultry feeds

Plant	Cyanide content mg 100 g^{-1} of sample
Cassava (*Manihot esculenta*) tuber	15.73±0.08
Cassava Garri (product)	4.42±0.06
Cassava flour(product)	8.06±0.05
Potato (*Solanum tuberosum)*	7.80±0.04
Yam (*Discorea esculenta*) tuber	7.84±0.08
Ground (*Arachis hypogea*) seed	5.46±0.04
Banana (*Musa* sp.) fruit	1.82±0.07
Almond (*Prunus amygdalus*) fruit	5.20±0.07
Guava (*Psidium guavaja*)	
(a) Seed	4.94±0.05
(b) Mesocarp/Endocarp	5.20±0.07
Grape (*Citrus paradisi*) fruit	3.64±0.02
Orange (*Citrus sineusis*)	1.82±0.07
Pineapple (*Aranas comosus*) fruit	5.72±0.03
Layer ration*	1.94±0.02
Broiler finisher*	4.81±0.06

The values obtained are means±SD of three determinations.
*Poultry feedstuffs

Table 4: Cyanide concentrations of some plants in fruit bat roosting site

Sample	Cyanide content mg 100 g^{-1} of sample
Fiscus sp. (Leaf)	1.59±0.01
Astonia boovei (Leaf)	1.91±0.02
Voacengia Africana	
(a) Leaf	2.10±0.01
(b) Mesocarp	0.26±0.00
Celtis zenkevi (Leaf)	4.51±0.02
Paw paw (*Carica papaya)*	
(a) Seeds	7.83±0.01
(b) Mesocarp	0.74±0.01
(c) Endocarp	0.43±0.01
Kola milleni	
(a) Seeds	1.37±0.01
(b) Pud	0.16±0.00
Mango (*Magnifera indica*)	
(a) Endocarp	0.54±0.01
(b) Mesocarp	0.25±0.01
Tetraplatra tetraptera (Fruit)	0.82±0.01
Walnut (*Juglems neotropica)*	3.18±0.01
Funtunia elastica	1.36±0.01
Cassia siamia	1.86± 0.01
Delonix sp.	2.35±0.01
Spondiao mumbin	2.69±0.02
Termindia catapa	
(a) Fruit	4.46±0.01
(b) Leaf	3.12±0.01

Values are means of duplicate analysis±SD

to be reliable and sensitive and can be applied for virtually all food materials unlike a number of specific tests that were designed to evaluate cyanide content in cassava and its products (Rao and Hahn, 1984; Yeoh and Egan, 1977). The cyanogenic glycoside content of a foodstuff is usually expressed in terms of the amount of cyanide released by acid hydrolysis. The concentration varies widely in different natural orders, varieties and species and in different parts of the same plant and also depends on age, cultivation and climatic condition (Wokes and Willimott, 1951). Wide fluctuation in cyanide content in the different cassava cultivars in Malaysia for example has been attributed to genetic makeup and also due to environmental conditions such as mineral nutrition, fertilizer applications, shading of plants and water stress (Yeoh and Oh, 1979). The toxicity of the levels of cyanide in these plants when ingested is not the focus of this present study. We have demonstrated again that food plants found in our region, which are used in both human and animal nutrition, contain certain amount of cyanogens and also that most fruits around us are cyanogenic. It is also important to note that domestic birds, whichever the system of keeping them, have their feeds composed from some of these plant materials. The question remains as to the significance of these small amounts of cyanide in them as only many kilograms will produce lethal effect. A possible role of these sulphurtranferases as they participate in the proposed synergistic mechanism of cyanide detoxification to douse the lethal effect of cyanide is assumed. A rough guide for the toxicity of cyanide in food plants is as follows: amount less than 5 mg HCN 100 g^{-1} is considered innocuous, 5-10 mg HCN 100 g^{-1} is moderately poisonous and more than 10 mg 100 g^{-1} is considered dangerously poisonous for human consumption (Bolhuis, 1954; De Bruijn, 1973).

Moreover, frugivorous bats are known to forage on virtually all available plant materials during roosting in the day and the nocturnal night food search (Montgomery, 1965; Wokes and Willimott, 1951). It feeds on a wide variety of ripe tropical fruit such as bananas (Musaceae), pawpaw (Caricaceae), muvule (Moraceae), figs (Moraceae), mangoes (Anacardiaceae), guavas (Myrtaceae), giant passion fruit (Passifloraceae), loquat (Rosaceae), soursop custard apple (Anoaceae) and many kinds of citrus fruits as well as on blossoms (Halstead and Segun, 1975; Mutere, 1967; Okon, 1974). It also feeds on wild fruits, which sources are not known but the bats encounter in their night foraging over long distances (Mutere, 1967). Wherever the bats are found they are highly gregarious with population of about a quarter of a million, thereby destroying economic trees on which they roost (Halstead and Segun, 1975; Mutere, 1967). An attempt to use cyanide spray to control fruit bats in East Africa had been reported to be ineffective (Constantine, 1970). The aforesaid as well as the high level of the sulphurtransferases found in the fruit bat than in other mammals and birds further gave credence to the notion of the presence of a powerful enzymatic mechanism for the detoxication of cyanide in this animal. This might have evolved due to the exposure of fruit bats, through the ages to high levels of cyanogenic glycosides in their diet. It is apparent from Table 3 and 4 that many normal and wild plant materials the fruit bats feed upon are cyanogenic. This is apart from the wide varieties of plants that they are exposed to during the night foraging. From the environmental point of view, fruit bats are pests and the study of the enzymology of the cyanide detoxifying enzymes, effective and environmental friendly biological control strategy (ies) might be feasible in the longterm. As a result of this preliminary work, fruit bat liver rhodanese has been purified and characterized in our laboratory (Agboola and Okonji, 2004).

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