THE PERFORMANCE OF ANIMALS ON LINAMARASE - PROCESSED CASSAVA AND ASPECTS OF CYANOGENIC GLUCOSIDE METABOLISM

BY

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D E D I C A T I O N

This work is dedicated to the LORD JESUS CHRIST, OGUNDU's FAMILY and the SAINTS, UNIVERSAL.

THESIS APPROVAL

This thesis, by Emmanuel Chinedum Ogundu meets the regulations governing the Degree of Doctor of Philosophy of Ahmadu Bello University and is approved for its contribution to scientific knowledge and literacy presentation.

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ABSTRACT

The β-D-glucosidase produced by fungal isolates namely; Aspergillus sydowi, A. terreus, A. carneus and Fusarium equiseti failed to hydrolyze crude linamarin from cassava tuber but hydrolyzed p-nitrophenyl β-D-glucoside, while linamarase also a β-D-glucosidase from cassava cortex hydrolyzed both substrates and was therefore used for further studies. The bacterial isolates namely; Bacillus licheniformis, B. subtilis, B. polymyta, B. circulans and B. coagulans failed to produce β-D-glucosidase.

Total cyanide hydrolysis in cassava tuber was achieved with 3 units of cassava linamarase in 10 minutes.

Fermentation of grated cassava for 8 days with addition of exogenous linamarase produced the highest percentage detoxification of 98.77. Sundrying cassava chips for 3 days and fermenting grated cassava for 8 days without addition of exogenous linamarase produced 93.77% detoxification each. Grated cassava fermented for 5 days with and without addition of exogenous linamarase showed cyanide reduction of 91.38% and 92.54%, respectively.

Rats placed on enzyme - processed cassava-based diet showed better performance in terms of feed efficiency and daily weight gain than those placed on sundried cassava-based diet with and without palm oil supplementation but lower than those placed on maize-based diet. The differences were however not statistically significant.

The thiocyanate levels in serum, urine and gastrointestinal tract were higher in rats fed sundried cassava-based diet without palm oil supplementation than those fed the same cassava-based diet

with palm oil supplementation. The least levels were found in rats fed enzyme-processed cassava-based diet. Free and bound cyanide (linamarin) were not detected in all treatment groups.

The thiocyanate distribution in gastro intestinal tract showed that the stomach has the highest level (77-88%) followed by large intestine (5-12%) and small intestine (5-11%).

Cassava-based diet when compared to a maize-based diet did not affect the quantity, percentage crude protein and dry matter content of the cecotrope excreted by rabbits during the day and night periods. However, the percentage fat (ether extract) was higher in cecotropes of rabbits fed cassava-based diet than those on maize-based diet.

Calculated vitamin B-levels of reingested cecotrope were sufficient to meet the rabbit's dietary requirements.

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CHAPTER 1.

INTRODUCTION

The tropical root crop cassava (Manihot esculenta Crantz) provides a major source of calories for about 500 million people in the tropics and its use as an animal feed component is increasing (Cock, 1985) . Cassava contains the cyanogenic glucosides, linamarin, [2- (β-D-glucopyranosyloxy) isobutyronitrile] and lotaustralin, (methyl - linamarin) or [2 - (β-D-glucopyranosyloxy) methyl butyronitrile], which are hydrolyzed to hydrogen cyanide by endogeneous linamarase (EC 3.2.1.21, linamarin β-D-glucoside glucohydrolase) on tissue damage (Conn, 1969). Its growing use for human food and animal feeds, however, could be hampered by the presence of these toxic cyanogens in its tubers (Ikediobi et al., 1980).

Acute cyanide toxicity in human usually terminates in the death of the victim while chronic toxicity has been associated with goitre (Ekpechi, 1967; Delange et al., 1970) and tropical ataxic neuropathy (Osuntokun, 1973).

Over the last two decades, there has been growing interest in cassava cultivation and its use in animal nutrition. Research carried out in different parts of the world indicates that cassava can be used as cereal substitute in animal rations (Reveron and Rodrigues, 1982). Experiments have however shown that different animals can tolerate different levels of cassava root meal in relation to a cereal-based reference diets without any deleterious effect. (Montilla, 1977, Omole, 1977, Ngoka et al. 1983, Khajarern et al. 1977, Devendra 1977, Omole and

Eshiet 1976, Fomunyan et al. 1983). However these experiments have shown that after such levels, there could be depressive effects on animal performance such as average daily gains, feed efficiency, digestible energy intake, dry matter digestibility and egg hatchability.

A wide variety of traditional food preparation techniques are used for processing cassava in attempt to reduce the cyanogen content either by loss of liberated hydrogen cyanide (HCN) through volatilization or by solution in water (Coursey, 1973). But traditional processing of cassava is unlikely to remove all the cyanide, (Cooke and Maduagwu, 1978).

Cyanide in intact cassava exists largely as the aliphatic glucosides, linamarin and lotaustralin, but only the free cyanide is amenable to direct assay. The greatest problem in obtaining reliable and reproducable data lies with the difficulty in achieving quantitative autolysis of the cyanogens in cassava extracts and quantitative recovery of the released HCN (Zitnak, 1973). In extracts from fresh cassava tubers, autolysis is aided tremendously by the action of endogenous linamarase on the cynogenic glucoside to release free HCN. However in extracts from processed cassava, this is not possible since all enzymatic activity of the cassava parenchymatous tissue would have been destroyed during processing. Therefore quantitative hydrolysis can only be assured by the addition of exogenous linamarase of known activity (Ikediobi et al., 1980).

Recently Ikediobi and Onyike (1982a, 1982b) have succeeded in accelerating the rate of cassava pulp detoxification by the introduction of exogenous linamarase during fermentation of grated cassava for gari production, and established the precise role of this enzyme in cassava tissue during the fermentation process. These interesting developments have since created the need to have linamarase in sufficient quantities as to be able to satisfy the potential demand that would exist for this enzyme in research and commerce.

Several fungi are known to produce amyloglucosidases (Wiserman, 1979). Some locally isolated fungi have been implicated in linamarase production (Ikediobi and Onyike 1982a, Ikediobi and Ogundu, 1985).

The experience of workers in areas of industrial biochemistry and microbiology has shown that it is always cheaper, faster and more efficient to produce large quantities of enzymes from micro-organisms than from any other source whenever there is an opportunity to do so (Wang et al., 1979b; Reese and Maguire, 1969). The reasons are

a) Enzyme production by microbial fermentation is economical on a large scale because of short fermentation cycles and use of inexpensive media
b) the screening procedures are simple and thousands of cultures can be examined in a reasonably short time.
c) different species produce some what different enzymes catalyzing the same reaction, allowing flexibility with respect to desired operating conditions in the reactor, and

d) Enzyme levels may be increased by environmental and genetic manipulations with ease (Wang et al., 1979b).

Therefore suitable processing techniques and exogenous addition of linamarase during cassava fermentation could reduce the cyanide content of cassava to levels that would remove the present limitations of cassava utilization as food and feed component, (Ikediobi and Onyike, 1982a, b; Nambisan and Sundaresan, 1985).

Furthermore, addition of palm oil to cassava-based diets has been shown by some workers to result in better utilization of cassava root meal by animals (Hutagalung and Chang 1977; Hew, 1975).

Fomunyan et al. (1983) have suggested that palm oil might be preventing absorption of linamarin during digestion and effect its subsequent excretion.

In rabbit nutrition, coprophagy ensures maximum utilization of crude protein and fibre in high-forage and low-forage diets (Robinson et al., 1985). Coprophagy in conjuction with fermentation in the large intestine of rabbit supplies necessary amounts of most B-vitamins, possibly provides insurance against an essential amino acid deficiency and permits further digestion of fibre and other nutrients by second passage through digestive tract (Thompson and Wordern, 1956). This in turn contributes to the productive efficiency of the rabbit and accounts for its ability to thrive on poor quality diets.

This work is therefore undertaken with the following objectives in view:

1) Development of Laboratory-scale cassava processing using exogeneous linamrase
2) Feeding of animals with cassava fermented in the presence or absence of added linamarase so as to determine:
   i. if the incorporation of linamarase during fermentation improves its biological value
   ii. if the reduction of cyanide levels in cassava products as a result of linamarase fermentation improves digestible energy intake.
   iii. to what extent thiocyanate levels in plasma, urine and gastro-intestinal tract are affected by feeding linamarase-processed cassava.
3) Studying the effect of palm oil on cassava utilization and linamarin hydrolysis in the gastro intestinal tract.
4) Determining the effect of cassava-based diet on the quantity and quality of cecotropes produced by rabbits.

CHAPTER 2

LITERATURE REVIEW

2.1 ORIGIN OF CASSAVA

Several specific names are applied to cassava. The name Manihot utilissima is commonly used and also Manihot dulcis, Manihot aipi and Manihot palmata. These names differentiate between cultivars with different contents of cyanogenetic glucoside in the roots, but this distinction is taxonomically invalid and Crantz's epithet Manihot esculenta is generally accepted as the correct one (Cliferri, 1938, 1942).

Cassava (Manihot esculenta Crantz) belong to the family of euphobiaceace. It is herbaceous or semi-shrubby parennial whose botanical characteristics show tremendous variation within species (Magoon, 1967).

Native of tropical America, cassava was brought into cultivation probably 4,000 years ago (Coursey and Haynes, 1970); it was later taken to Africa, countries bordering the Indian Ocean, and finally to the Pacific. In Africa, the Portuguese took it to their stations around the mouth of the Congo River and to the Island in the Bight of Benin during the half of 16th century, and it was probably introduced to the countries bordering the Indian Ocean, Ceylon, Malaya and Indonesia during the 18th century (Jones, 1959).

2.1.1 Cassava Production

Although production statistics for cassava are notoriously unreliable, the best available evidence indicate

that, on a tonnage basis, annual world production is exceeded by that of six other crops, wheat, rice, maize, barley, potatoes and sugar beets (Nestle, 1973, FAO 1971).

Cassava is the most important root crop of the tropics and its production is much greater than all others put together (Coursey 1984, FAO 1982a). It is essentially a tropical crop, although some cultivars can withstand light frost. Cassava is produced in more than 80 countries, but two thirds of world production take place in only five of them (Brazil, Indonesia, Zaire, Nigeria and India) and 90% of global production comes from 19 countries (Nestle 1973; FAO 1982a).

Cassava is generally grown as a subsistence crop. It is particularly valued because of its drought tolerance, its ability to grow in poor soils, and its relative resistance to weeds and insect pests. These characteristics, plus the fact that it can be left in the ground without harvesting for a lengthy period of time, mean that it is a very useful crop that can be used as a secuirty against famine (Nestle, 1973). Furthermore, it is not season-bound and can therefore, be planted and harvested at any period of the year.

Cassava also possesses certain characteristics which make it of particular interest to the biologist and to the economist concerned with resource development in tropical areas. This is due to the fact that cassava productivity in terms of calories per unit land area per unit of time appears to be significantly higher than that of other staple crops (Varies et al., 1967).

Coursey and Haynes (1970) indicated that cassava can produce 250 x 10^3 cal/ha per day compared to 176 x 10^3 for rice, 110 x 10^3 for wheat, 200 x 10^3 for maize, and 114 x10^3 for sorghum. They pointed out that the grain crops have been subjected to considerable research to improve their genetic potential, whereas cassava offers considerable scope for genetic improvement. They also indicate that root crops have higher biological efficiency as food producers.

FOA estimates show that in the 1979-1981 period, world production of cassava was about 120 million tons annually. Although Africa has slightly more than 50% the world's land area devoted to cassava cultivation, it accounts for only 37% of the world's cassava production.

These estimates show that yield has been nearly constant at 6.4t/ha in recent years, but between 1969-1971 and 1980 production climbed from 39.4 million tons to 46.5 million tons; Zaire, Nigeria, and Tanzania being the major African cassava producers. Average yields in these countries range from 9t/ha in Nigeria to less than 5t/ha in Tanzania (Cock 1985, FAO 1981).

2.1.2 Cassava utilization

The most important use of cassava is as human food (Nestle, 1973). Consumption figures (Nestle 1973, 1974) from 14 countries in which cassava is an important dietary staple show that, for example in Southern Nigeria 25-50% of the dietary calories come from cassava (Nicol, 1952) as opposed to

a National figure of 15% (Nestle 1974). Bailey's (1961) surveys in Java showed that 63.5% of calorie intake came from cassava as opposed to a National figure of 15% (Nestle 1974) while Normanha (1970) reported that in Brazil in 1962-1963, cassava consumption was 124kg/person per annum at the National level representing an urban intake of 42kg/person and 200kg/person in rural areas.

A very large proportion of the total crop is consumed in the production areas where traditionally it is processed into dry products and long-distance trade exists in these areas (Coursey 1978a; Lancaster et al., 1982).

Cassava is largely used as experimental feed in Latin America, and in general, satisfactory results have been obtained although problems, particularly of acceptability, still exist (Nestle and Graham, 1977).

Apart from its use as direct food and feed, cassava is used to produce industrial starch. The low-amylose content of cassava starch, 17% as opposed to 22% in potatoe starch and 27% in corn starch, makes it posses unusual viscousity characteristics. The large percentage of branch-chained amylopectins in cassava starch gives it great dimensional strength, which makes it in particular demand for sizing paper or fibers, to give them greater tensile strength (Ayres 1972; Nestel 1973).

Cassava starch also provides good parent material from which to hydrolyze dextrins for formulating adhesives..

Such adhesives appear to have a greater flexibility and less brittleness at low humidities than dextrins derived from cereal starches. Cassava starches also possess specific characteristics that are in demand in the food industry (Evans and Wurzburg 1967; Nestel 1973).

Cassava can be hydrolyzed to produce glucose and cassava-based glucose industries are in operation in some countries (Nestel 1974, Coursey 1984).

Considerable attention has been given to cassava as a source of ethanol. Brazil in particular produces alcohol for power purposes from cassava though more from sugar cane (Coursey 1984).

Cassava stalk have also been used to make particle board (Flaws and Palmer, 1968) on an experimental scale.

2.1.3 Nutritive Value

Fresh unprocessed cassava is very rich in ascorbic acid and calcium and appears to have nutritionally significant amounts of thiamine, riboflavin, niacin and iron (Jones 1959; Oke, 1968; Jennings, 1970). The protein content is very small lacks the sulphur-containing amino-acids, cystine and methionine and possibly lysine and threonine (CIAT 1972). Cassava roots have 30 to 40 percent dry matter (which is of higher proportion than most other roots and tubers) of which more than 80% is starch. (CIAT 1972, Cock 1985). Crude protein (i.e. N x 6.25) ranges from less than 1 percent up to 7.5 percent. Crude fiber and fat are very low. Ash is about 4 percent. The quality of starch shows low variability, so that all cassava starch is potentially of the same quality (CIAT, 1972).

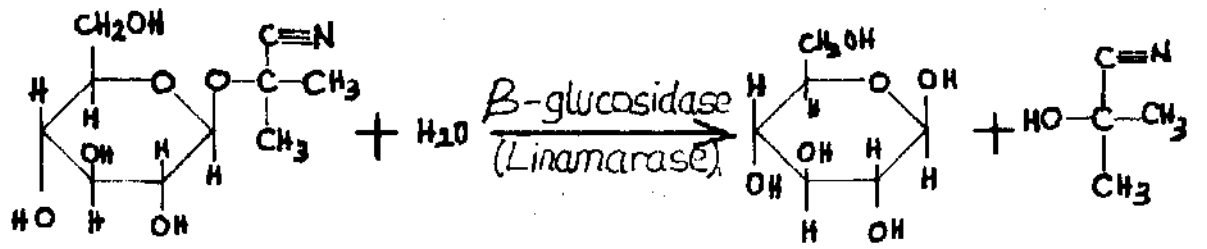
Unprocessed cassava roots also have high amounts of toxic cyanogens which exist largely as the aliphatic glucosides, linamarin and lotaustralin (Butler and Butler, 1960; Butler and Conn, 1964; Butler, 1965).

Processed and unprocessed cassava tubers are rich in carbohydrates and so are a rich source of calories. Processed cassava, especially that which has undergone the process of fermentation has protein content of 1-5 percent (Jones, 1959; Oke, 1968). Values from different sources vary, depending largely on the method of analysis. There is evidence that processing increases the protein content of gari, a popular cassava product (Strasser et al., 1970).

Proteins, vitamin and mineral contents of cassava are reduced to insignificant amounts by processing (Oke, 1968; Coursey, 1973). The amount that remains in the final products depends on the kind of treatment it has undergone. As food for human consumption and animal feed, cassava products are a good source of calories (Coursey 1973). It is generally eaten for its caloric rather than for nutritional purposes. Vegetable, meat and fish stew or soups are commonly eaten along with it (Oke, 1968). There have been several attempts to fortify cassava products with protein and/or vitamins from fungi or soya beans, but none has been quite satisfactory (Reade and Gregory, 1975; Joseph, 1973).

2.2 CASSAVA TOXICITY

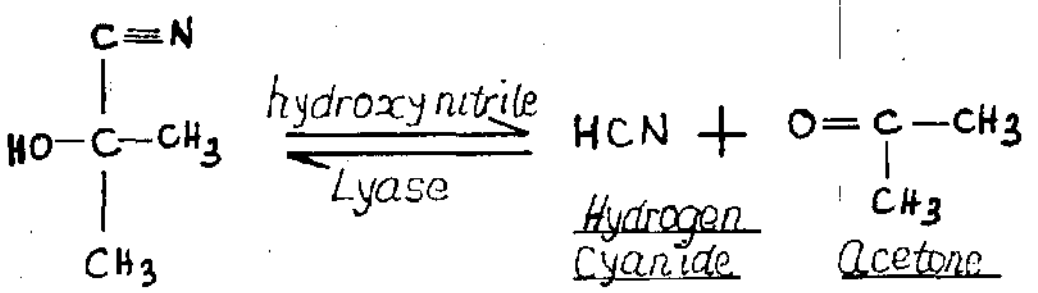
The fact that cassava(Manihot esculenta Crantz) can be toxic must have been known to the Amerindians since the earliest days of its domentication, if not before (Coursey 1973). The first reference to cassava toxicity in the Western literature appears to be in the writings of Clusius (1605). The association of the toxicity with the presence of hydrocyanic acid (HCN) was first made by Henry and Boutron-Charland (1836), while the identification of the occurence of the HCN in the form of a cyanogenic glucoside, originally named manihotoxin is attributed to Peckolt (1886, quoted by Cerighelli 1955). This compound, the principal cyanogen of cassava, was subsequently (Dunstan et al., 1906) shown to be identical with the better-known glucosides, Phaseolunatin and linamarin of Phaseolus and Linum, respectively. The presence of HCN in "Sweet" and "bitter" cassava was established by Francis (1878) and investigated further by Connaby (1900), Collens (1915) and Turnock (1937)who concluded that HCN was the only poisonous substance in cassava. Linamarin is structurally 2- (β-D-glucopyranosyl-oxy) isobutyronitrile; under enzymatic or acidic hydrolysis it liberates free HCN, together with acetone and glucose; fig. 1.1 (Coursey 1973). In step (a) the β-glucosidic bond linking β - (D) - glucose (II) to 2-hydroxy isobutyronitrile (acetone cyanohydrin) (III) is hydrolyzed by the endogenous β-glucosidase (linamarase)



(a) Linamarin — I

β-glucose — II

2-hydroxyisobutyronitrile — III



(b) 2-hydroxyisobutyronitrile — III

IV

FIG. 1.1 ENZYMATIC HYROLYSIS OF LINAMARIN.

to form those two compounds. In step (b), the hydroxynitrile is cleaved into acetone (IV) and HCN (Conn, 1973). While this process can and does readily occur non-enzymatically, enzymes catalyzing this type of reaction (hydroxynitrile-lyase) are known and have been studied in sorghum vulgare (Seely et al., 1966). The presence of such an enzyme in higher plants such as cassava that contain linamarin and lotaustralin may therefore be predicted (Conn, 1973).

Earlier work on the occurence of linamarin in cassava has been reviewed (Jones, 1959; Wood, 1965a; Johnson and Raymond, 1965; Oke, 1968). Other studies (Butler, 1965; Nartey, 1968, Bissett et al. 1969; Bruijn, 1971) have shown that a small proportion, between 2 and 8%, of the total cyanogenic glucoside present in cassava tubers consists of a methyllinamarin, believed to be identical with lotaustralin. This substance is hydrolysed under conditions similar to those of linamarin hydrolysis to yield HCN, methyl ethyl ketone, and glucose (fig. 1.2). The toxicity of cassava thus appears to arise from the presence of cyanogenic glycosides which may hydrolyze readily to free HCN (Coursey, 1973).

2.2.1 Distribution of Cyanogenic Compounds in Cassava Tuber.

The cyanogenic glycosides are distributed throughout the cassava plant, but the concentration varies greatly between varieties, and also with climatic, and cultural

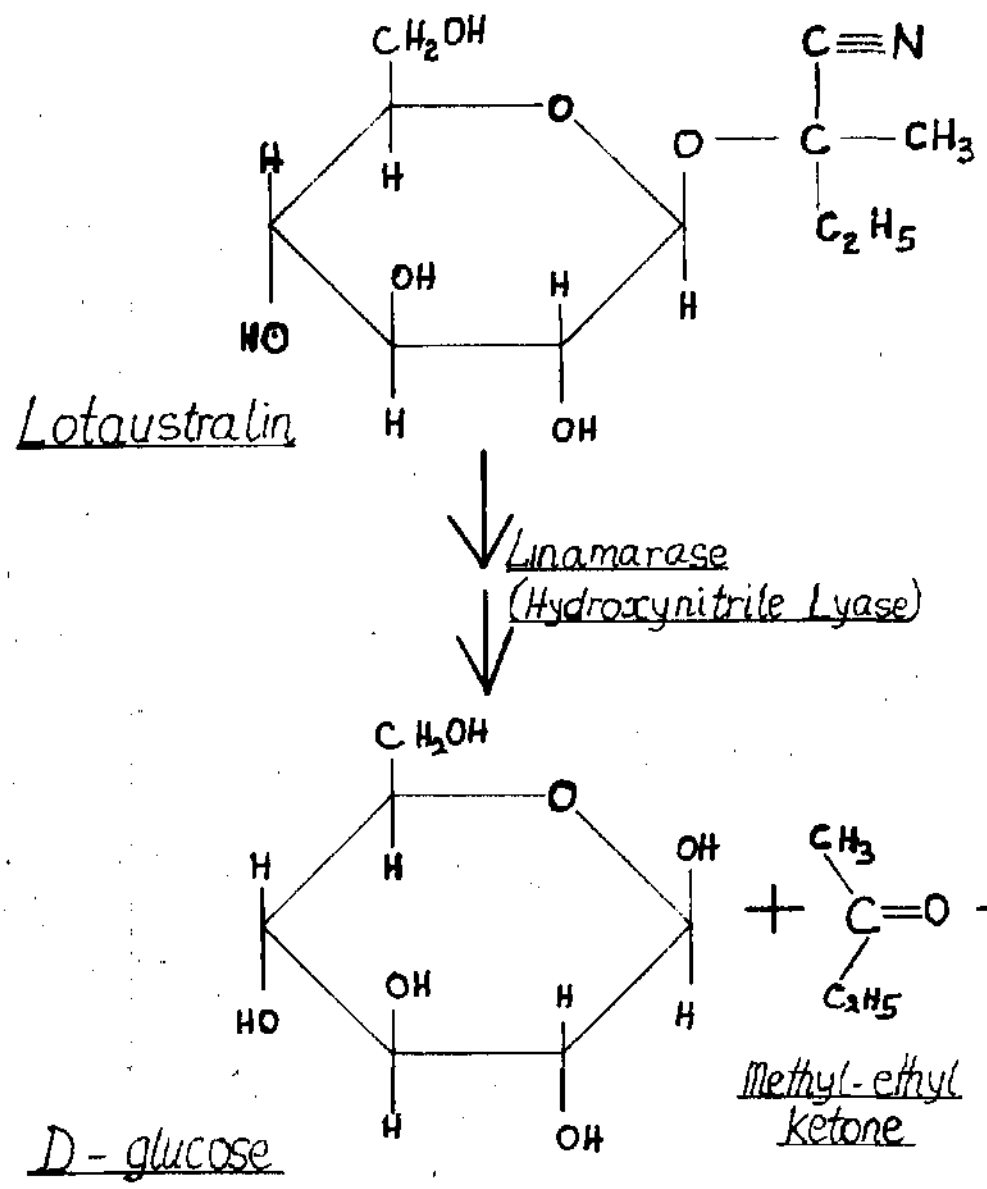


FIG 1.2. ENZYMATIC HYDROLYSIS OF LOTAUSTRALIN.

conditions. The normal range of cyanogen content is from 15 to 400ppm, calculated as mg HCN/kg fresh weight but occassional samples containing as low as 10mg/kg or over 2000mg (Rogers, 1963) have been reported. Most commonly, cyanogen content falls between 30 and 150mg/kg (Coursey, 1973).

Cassava is often described as "bitter" or "sweet" according to the amount of cyanide present, but these are at best only approximate terms, and to attempt to associate cyanide levels with particular botanical taxa is quite incorrect (Bolhuis, 1954, CIAT 1972). No exact correlation between sweetness or bitterness of taste can be made (Pereira and Pinto, 1962). In general, bitter cassava has a high cyanide content while sweet cassava tends to have lower values but there is a great deal of overlapping between classes (Sinha and Nair, 1968). In most varieties, under most cultural conditions, the concentration of cyanogenic glycoside is very substantially higher in the "peel" fraction of the tuber than in the "flesh", the ratio being usually 5 or 10 to 1.

The cyanogenic glycosides of cassava are accompanied in the plant tissue by a hydrolytic enzyme, linamarase (also known as linase) similar but not identical to emulsin (Wood, 1965b , Wood, 1966). In active, healthy tissue of the growing plant, enzyme and substrate do not come in contact, but contact occurs when the tissue is

mechanically damaged, or when loss of physiological integrity occurs as a result of post-harvest deterioration of the tubers, or of wilting of the leaves; hydrolysis then take place, liberating HCN (Coursey, 1973).

2.2.2 Nature of the Toxicity

Toxicity in cassava and its products is associated primarily with the free HCN in the material that is formed when the cyanogenic glycoside have been hydrolysed. Although the toxicity of the glycosides present in cassava may be in doubt, HCN itself is one of the most potent poisons known; large doses can cause acute poisoning, usually resulting in death, while the habitual ingestion of smaller quantities, even so small as to produce no immediate symptoms can result in chronic effects (Coursey, 1973).

The division of cassava into "bitter" and "sweet" types relates to the likelihood of acute symptoms occuring after consumption of the product if special care is not taken to detoxify it. The classification already given is based on the statement of Boorsma (1905) that 50-60mg HCN is a lethal dose for an adult male weighing 50kg (Bolhius, 1954).

Acute poisoning as a result of eating cassava by man or domestic animals occurs now and again but is by no means an everyday affair. Chronic effects are probably potentially a much more serious factor limiting the use of cassava in human or animal nutrition (Coursey, 1973).

2.3 Biosynthesis of Cyanogenic Glucoside in Cassava Plant (Manihot spp.)

A common biochemical feature of the cassava plant is that it synthesizes and accumulates cyanogenic materials in its vegetative tissues especially the edible leaves and tubers (Nartey, 1973). These materials, on hydrolysis, give rise to moderate to lethal concentrations of hydrocyanic acid or hydrogen cyanide (HCN), a powerful specific and nonspecific inhibitor of several essential enzyme-catalysed systems in respiration (Dixon and Webb, 1965).

Genetically, Manihot spp. appears to be cyanophoric with some dominant cyanogenesis - controlling gene being present in successive cultivars and phenotypes. Thus all cassava varieties probably contain all the enzymes which catalyze the biosynthesis of cyanogenic materials and their degradation (Nartey, 1973). Variations in the concentrations of cyanogenic glucosides in cassava tissues and cultivars may, therefore, determine enzyme activities, substrate concentrations, and availability, as well as rates of transport, storage, and degradation in specific tissues and cultivars (Nartey, 1973).

Linamarin, 2 (β-D-glucopyranosyloxy) isobutyronitrile earlier, was known to be the only cyanogenic glucoside in cassava and it was isolated and characterized by Dunstan et al. (1906). Butler (1965) showed that most plants containing linamarin also contained a higher homologue

of this glucoside, methyl-linamarin or lotaustralin, 2 (β-D-glucopyranosyloxy)2-methylbutyronitrile, a glucoside of 2-hydroxy-2-methlbutyronitrile.

Linamarin and lotaustralin have been shown by Nartey (1968) to constitute the cyanogenic materials of cassava (Manihot utilissima and Manihot esculenta) in the proportion of 93% to 7%. The occurence of lotaustralin with linamarin in cassava tissues was later confirmed by Bisset et al. (1969).

2.3.1 Biosynthesis of Linamarin and Lotaustralin

In different laboratories (Conn, 1969, 1973; Nartey, 1968) it has been demonstrated that the aglycone moieties of cyanogenic glycosides may be synthesised from structurally related amino acids. This motivated studies on the effectiveness of valine and isoleucine as precursors of the aglycone moities of linamarin and lotaustralin, respectively (Nartey, 1973). Results (Nartey, 1968) show that both 'sweet' and 'bitter' cassava seeds contained only trace amounts of valine and isoleucine, but during germination, large amounts of these amino acids accumulated through the degradative action of proteolytic enzymes on seed storage proteins. Concurrently, the concentrations of linamarin and lotaustralin increased sharply. When uniformly labelled L-valine- ^{14}C and L-isoleucine - ^{14}C were administered

to cassava seedlings during the period of active cyanogen synthesis (Nartey, 1969), large amount of radioactivity were subsequently found incorporated in the aglycone moieties of linamarin and lotaustralin, respectively. From these it is clear that linamarin and lotaustralin are synthesised from valine and isoleucine, respectively, by the cassava plant (Nartey, 1973). The proposed biosynthetic pathway is shown in fig. 1.3. The pathway incorporates data from Conn and Butler (1969), Nartey (1968, 1969) and Tapper et al. (1972).

Their experiments suggest that the biosynthetic pathway involves an initial decarboxylation of the amino acid, followed by a four electron oxidation of α- carbon to a nitrile function in which the α- amino nitrogen is retained. Subsequently, a two electron oxidation of the β - carbon leads to its hydroxylation followed by a glycosylation to yield linamarin since most plants containing linamarin also synthesize lotaustralin, with the probable exception of Hevea brasiliensis (Butler, 1965). It may be assumed that the pathway for biosynthesis of lotaustralin proceeds from L-isoleucine via hydroxy-β-methylbutyronitrile followed by glycosylation to form the cyanogenic compound.

2.4 CYANIDE DETOXIFICATION IN THE BODY

There are essentially five ways in which the minute amounts of cyanide formed in the human body during metabolism or minute amounts taken in food are detoxified. When HCN

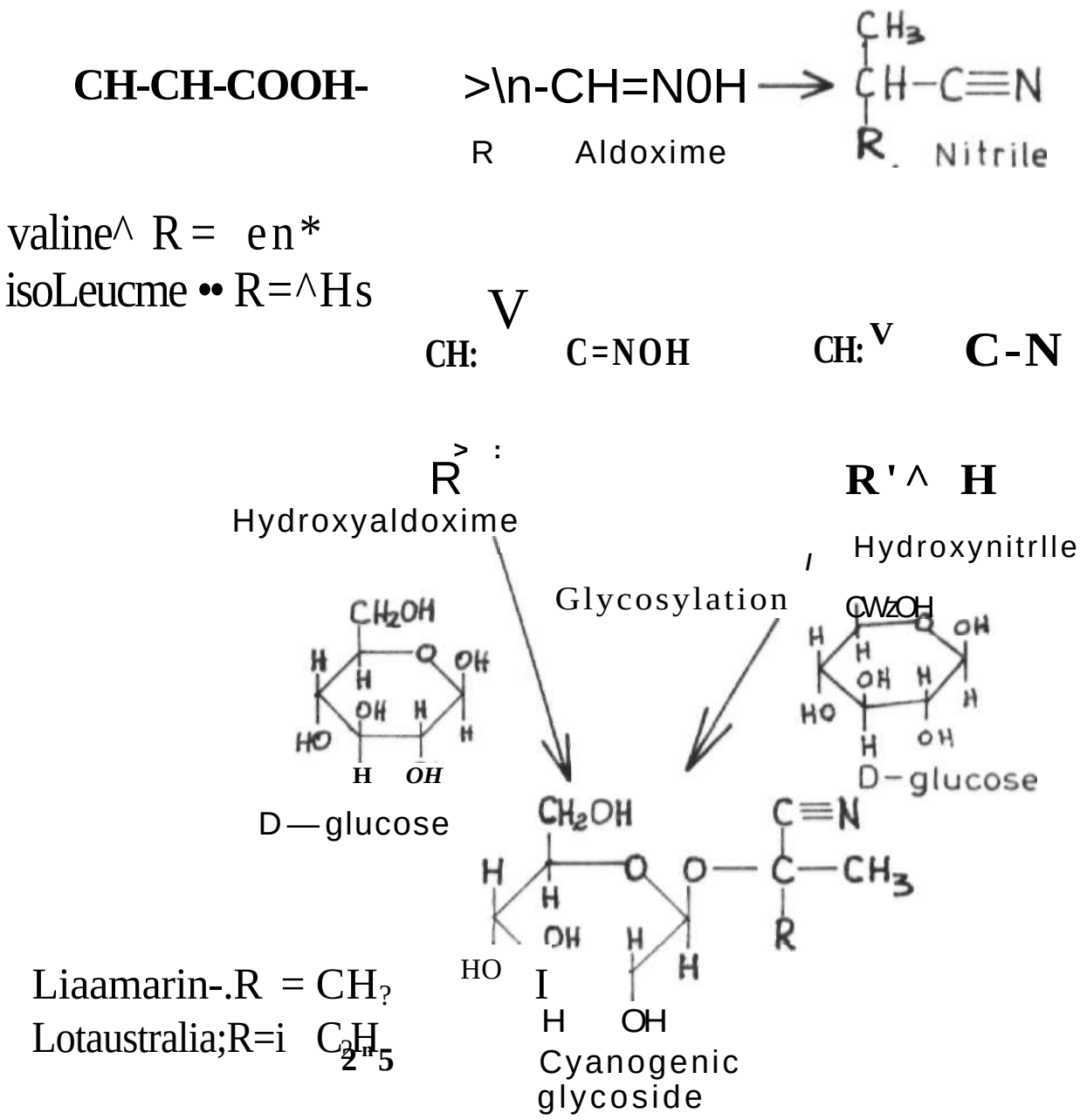


FIG-1-5: The biosynthesis of Linamarin and lotaustratin in cassava. Pathway incorporates data from Conn *and* Butter, 1969; Narfey, 1968 and J969; and Tapper et. at. 1972.

is converted to thiocyanic acid, there is a 200-fold reduction in toxicity (Oke, 1973). Most of the detoxifications due to conversion of HCN to thiocyanic acid are due to the action of rhodanese (Lang, 1933a, b, Saunders and Himwich, 1950). The enzyme rhodanese, catalyses the reaction in which cyanide accepts sulphur from inorganic and organic sulphur donors with the resultant formation of thiocyanate (Foos, 1950, Sorbo, 1951a, b 1953, Himwich and Sanders; 1948).

Lang (1894) showed that injections of cyanide or aliphatic cyanides into rabbit increased the thiocyanate excretion and this has been confirmed by Heymanns and Mesoin (1896). Pascheles (1894) showed by in-vitro studies that when liver or muscle tissue from a dog was digested with NaCN, thiocyanate was produced, the liver tissue being more active than the muscle. This enzyme represents the chief site of detoxification and occurs in all parts of the body with the largest concentration in the liver (Lang 1933a, b)

$$CN^- + (S_2O_3)^{2-} \xrightarrow{\text{Rhodanese}} SCN^- + SO_3^{2-}.$$

The amount of rhodanese in the liver varies with different animals so that detoxification may be expected to take place at different rates. Himwich and Saunders (1948) found the following levels for the livers of animals.

0.78 - 1.46mg/g for dog

10.08 - 15.16mg/g for rhesus monkey

7.98 - 18.92mg/g for rabbit

14.24 - 28.38mg/g for rat

This probably explains the results obtained by Makerji and Smith (1943) that nearly all the cyanide ingested by rabbits was recovered in the form of thiocyanate in the urine in 24-48h whereas in dog less than 25% was recovered in 7 days. On the other hand, the activity of the enzyme in parts of the brain and the central nevrous system seemed to be the same for the different species of animal.

Cyanocobalamin (Vit B_{12}) occurs in the liver to some extent as hydroxocobalamin (Vitamin B_{12a}) which is capable of reacting with cyanide to give cyanocobalamin, providing another independent pathway for cyanide detoxification (Mushet et al, 1952).

3-mercaptopyruvate, arising from cysteine by transamination or deamination can provide sulphur as rapidly as thiosulphate for cyanide detoxification (Meister, 1953, Wood and Fiedler, 1953). This is made possible by the transfer of the sulphur in 3-mercaptopyruvate to sulphite resulting in the formation of thiosulphate and pyruvic acid (Fig. 1.4). The thiosulphate formed then provide the sulphur for the conversion of cyanide to thiocyanate by the rhodanese enzyme (thiosulphate sulphur transferase). The sulphite formed from the rhodanese

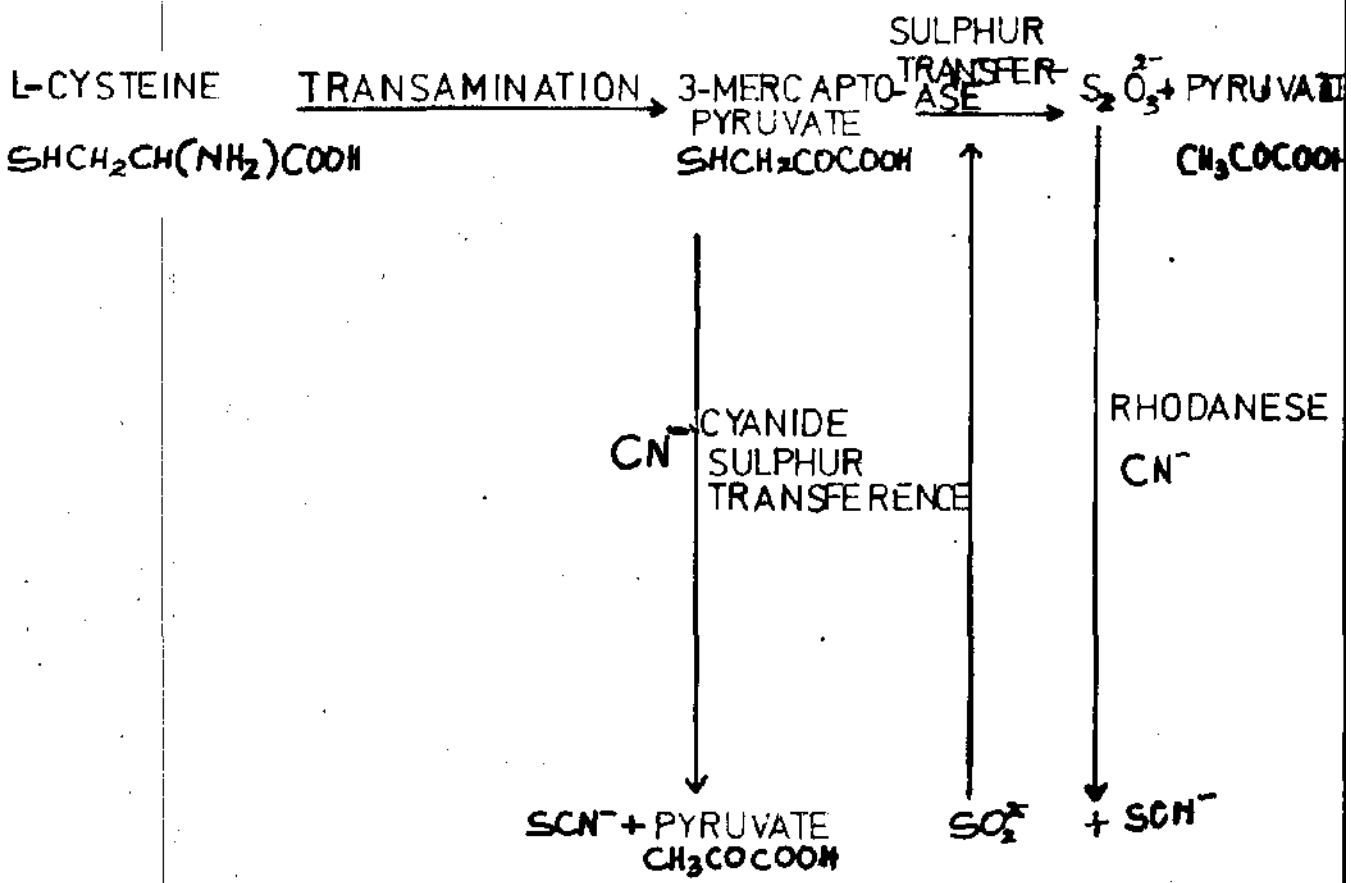


FIG 1·4 THE CYCLE FORMED BY THE REACTION OF CYANIDE WITH 3-MERCAPTOPYRUVATE (OKE 1973)

reaction is again ready for use by the sulphur transferase. A cycle is then formed as shown in Fig. 1.4 (Oke 1973). Cyanide can also react with 3-mercaptopyruvate to yield thiocyanate and pyruvic acid (Fiedler and Wood, 1956). This reaction is catalyzed by the enzyme cyanide sulphur transferase (Fig. 1.4).

Cyanide can react with cystine to split the disulphide linkage to form cysteine and β - thiocyanoalanine which tautomerises to 2-aminothiazoline-4-carboxylic acid or its equivalent, 2-imino-4-thiazolidine carboxylic acid. β-thiocyanoalanine could be a source of thiocyanate in the body since it undergoes oxidative deamination to produce thiocyanopyruvic acid which decomposes readily to yield thiocyanate (Schubert et al, 1951) (Fig. 1.5).

The thyroid glands show some detoxificating effect (Hunt, 1905 - 1906). This author showed that in the presence of powdered sheep thyroid, the lethal dose of acetonitrile for mice was 1.1mg/g whereas it was 0.32mg/g for those not fed the powdered sheep thyroid indicating that probably thyroid has a detoxificating effect. Later Baumann et al, (1933) found that rabbits injected with acetonitrile excreted 3-5% which was increased on feeding desiccated thyroid. Since thyreidectomy had little effect on the conversion of benzyl cyanide to thiocyanate, they were of the opinion that the mechanism of detoxification by thyroid depends on the demethylation of acetonitrile.

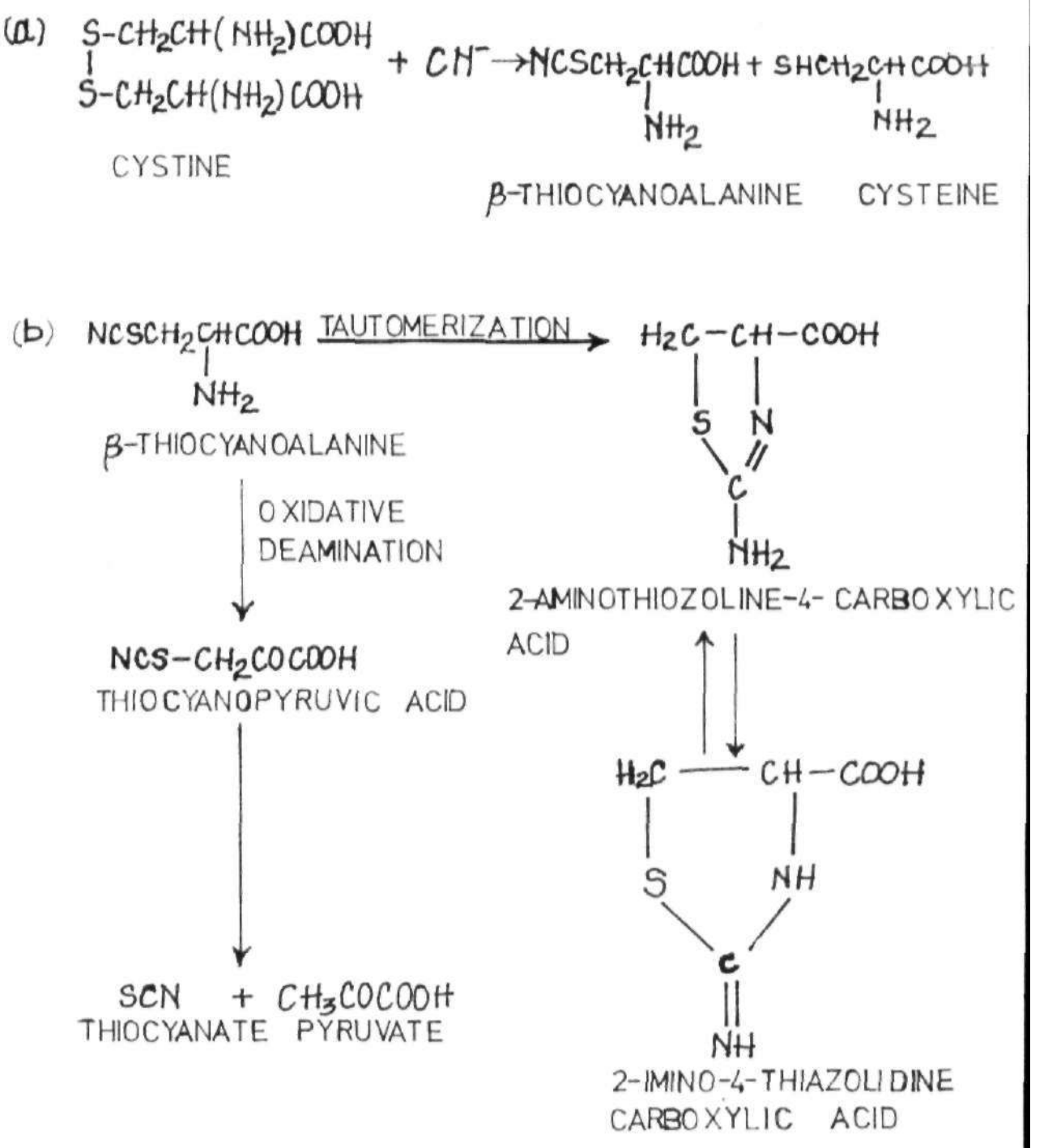


FIG. 1-5 THE REACTION OF CYANIDE WITH CYSTINE

Another pathway for cyanide detoxification is provided by methemoglobin which form complexes with cyanide. The resulting cyanomethemoglobin is itself non toxic and can be slowly metabolized. Since the body contains considerable amount of hemoglobin, therapy for cyanide-poisoned individuals consists in accelerating methemoglobin formation, e.g. by amyl nitrile administration (White et al. 1978).

2.5 CASSAVA CYANIDE AND KWASHIORKOR DISEASE

The utilization of cassava root tuber and products as a major staple food presents a variety of health problems (Nartey, 1973). Because of the low protein content, cassava food products have been implicated in the high incidence of kwashiorkor, the common protein deficiency syndrome of the developing countries (Jones, 1959). The high moisture content and the high ratio of carbohydrates to nitrogen make cassava tubers an excellent substrate for microbial growth and production of high levels of toxic metabolites. Nartey (1968) has shown that Aspergillus flavus thrives on cassava meal substrate and produce relatively high levels of aflatoxins. These heat-stable carcinogenic metabolites inhibit protein synthesis and cause liver damage and hepatoma in animals (Butler and Barnes, 1963).

The chronic uptake of cyanide in small doses, which as single doses do not cause clinical symptoms, is assumed to be decisive for the pathogenesis of a few diseases (Baumeister et al., 1975). An inhibition of

some routes of cyanide metabolism are probably responsible in some cases (Schievelbein et al., 1969; Baumeister, 1972).

In Nigerians, a syndrome referred to as tropical ataxic neuropathy comprising lesions of the skin, mucous membranes, optic and auditory nerves, spinal cord and peripheral nerves has been associated with cyanide intoxication due to high cassava diets. (Money, 1959; Montgomery et al., 1964; Osuntokun, 1968). The disease affects males and females equally and all age groups but occurs rarely in children under 10 years. The peak incidence is in the 40-50 years - old group (Osuntokun, 1973). The cyanide and thiocyanate contents of various foodstuffs commonly eaten by Nigerians have been estimated (Osuntokun, 1973). The result shows that the cyanide content of gari, eba, and purupuru, which are derivatives of cassava, is higher than that of other food items.

Death following cassava meals are occasionally reported, but intragastric release of cyanide occurs (Osuntokun and Aladetoyinbo, 1970). A diminution or a complete absence of the sulphur-containing amino acids cysteine and methionine in plasma is also observed in this disease. It seems that the detoxification of cyanide via formation of 2-imino-4-thiazolidine carboxylic acid is prevented because of the lack of cysteine (Osuntokun, 1968). Foodstuffs containing high concentrations of thiocyanate, such as milk, and vegetables of the Brassica family including cabbage, are only rarely consumed by Nigerians who suffer from tropical ataxic neuropathy (Osuntokun, 1973).

The essential neurological components of the disease are myelopathy, bilateral optic atrophy, bilateral perceptive deafness, and polyneuropathy. In about 35% of the patients stomatoglossitis is present. Motor neuron disease, Parkinsons disease, cerebellar degeneration, psychosis and dementia have also been found in association with the disease (Asun, 1967; Ocunt•kun, 1968). These findings support earlier suggestions by Moore (1934a, b, 1937); Clark (1935, 1936 and Oluwole (1935) that an important factor in the etiology of tropical ataxic neuropathy in Nigerians is chronic cyanide intoxication from cassava diet.

An endemic goitre survey (Ekpechi, 1964) in high-cassava consuming areas in the Eastern parts of Nigeria has disclosed a marked variation in incidence of goitre from village to village. Studies of the possible goitrogenic action of dry, unfermented cassava on rats (Ekpechi, 1973) have shown that cassava has an adverse action on the function of the thyroid gland. This work showed that the striking degree of thyroidal iodine depletion caused in 7 days is unlikely to have been caused by its low iodine content.

Epidemiological and metabolic studies concerning the etiology of endemic goitre on Idjwi (Kivu Lake, Republic of Zaire) carried out by Delange et al., (1968) led to the conclusion that iodine deficiency was not the single casual factor. The work of Delange et al., (1973) have shown that the absorption of cassava in men induces an inhibition of the penetration of iodine into the thyroid as expressed by a considerable drop in radioiodine thyroid uptake and a rise

in urinary excretion of stable and labelled iodine. Inhibition of iodine uptake by the thyroid is also obtained in rats fed with cassava and this effect is accompanied by a striking rise in the plasma thiocyanate concentration and urinary thiocyanate excretion (Delange et al., 1973).

2.6 CASSAVA AS ANIMAL FEED

One of the main obstacles to efficient animal production in the tropics is the lack of excess agricultural products that can be used as raw materials for animal rations. Cassava products (roots and foliage) appear to be one of the best possibilities for overcoming the chronic deficit in tropical agricultural production (Montilla 1977). Cassava (Manihot esculenta) has been widely used as a feedstuff and provides a major source of energy for livestock in Asia, Africa, Europe and South America (Nestel, 1975). However, numerous research findings and field reports have indicated that its extensive use in poultry and swine feeds has encountered some nutritional problems and diseases, including: its low protein, mineral, and vitamin content (Huntagalung, et al., 1973; Huntagalung, 1977); variation in HCN content resulting in acute and chronic toxicity, i.e. ataxic neuropathy; suspected goitrogenic substances causing iodine deficiency (goitre); reduction in availability of certain mineral elements resulting in zinc parakeratosis in pigs; low palatability due to dry texture, high ash, and crude fibre content causing poor digestibility and diarrhea, enzyme-inhibiting factors causing poor absoprtion of vitamins and minerals,

poor performance and lack of skin and egg yolk pigmentation; and contamination by pathogenic microorganisms causing aflatoxicosis (Huntagalung and Tan 1976). Nevertheless, these problems are not confined only to feeding of cassava but they have been encountered in feeding other feedstuffs, such as rice bran, soybean, and cottonseed meal (Huntagalung, 1977).

Within the last decade, efforts have been made by various workers to overcome these problems and to improve the nutritive value of cassava by investigating the effects of supplementation of nutritive and non nutritive feed additives to cassava-based diets. These additives include : source and level of energy and protein, synthetic amino acids, minerals, vitamins, antibiotics and antifungals, pigments, flavouring agents, hormones and enzymes (Huntagalung, 1977).

2.6.1. Cassava in Poultry Nutrition

The use of cassava root meal as an energy substitute for maize and other cereal grains in poultry feed is becoming increasingly acceptable based on research findings (Ngoka et al, 1983).

Much of the research carried out during the last 20 years shows that cassava root flour can be substituted for cereal in broiler rations at levels of up to 30 percent. When the diets are prepared in the form of pellets it appears possible to use cassava root meal and cassava foliage meal at levels up to 50 and 20 percent, respectively (Montilla, 1977).

Yoshida et al. (1966) found that young birds grew well when fed with 10% cassava root, but that higher levels resulted in delayed growth. Vogt (1966) affirmed that only after the fourth week can broiler consume cassava root flour at levels higher than 10%.

Olson et al. (1969b) found that peeled cassava root flour could be incorporated into diets for chicks at levels of up to 30% without affecting weight increase if the feed was balanced for energy and protein.

Vogt and Stute (1964) had already observed that better results were obtained when dehydrated cassava root flour was pelleted, and they suggested that the feed intake could be negatively influenced by the excessively fine nature of the flour. Chou and Muller (1972) substituted pelleted cassava root flour for corn flour at levels up to 50%, and found that this substitution was possible provided that the diets were duly balanced with regard to other nutrients.

Rendon et al. (1969) studied the incorporation of 0, 15, 30, and 45% cassava root flour in diets for broilers, and reported a decrease in weight gain and feed efficiency for all levels studied, however, with the 15% level the differences were lower. The authors confirmed the existence in cassava flour of a factor that reduced feed intake in birds receiving the highest levels.

Armas and Chicco (1973) replaced corn with cassava flour in broiler diets containing corn flour, soybean flour, sesame flour, meat meal, and fish meal. The cassava flour

was incorporated at levels of 18, 36 and 54% in the diets (which were isocaloric and isoproteinaceous), and they found no significant differences with respect to weight increase and feed efficiency, although with the diet 54% cassava flour the weight increase was reduced by 8.1%. The fact that the diets contained 8 or 16% animal protein, or were supplemented with 0.3% methionine and 0.3% lysine, did not affect the results. According to Sebastia et al. (1973), feed consumption was not affected in broilers by the substitution of up to 50% of the sorghum by cassava root flour. The best diet in terms of weight gain was that in which 30% of the sorghum was substituted by cassava root flour. Higher levels of substitution gave rise to a marked deterioration in weight increase and feed efficiency.

Montilla et al. (1975) partially (30% of the diet) and totally (37% of the ration)replaced corn flour with sun dried "sour" cassava root flour (785ppm HCN in fresh cassava and 50ppm in the flour). In both cases 11% of the corn had been replaced by 9% sugar cane molasses and 2% animal fat to eliminate the powdery characteristics of the rations. These rations, given to 1-day-old Ventress X White Rock chicks, resulted in very similar weight gains at the 4th week. In the case of diets containing 0 and 30% "sour" cassava flour; at the 37% level the weight gain dropped by 9.0% (highly significant). The same authors compared based rations (starters and finishers) with rations

containing 30% "sweet" cassava root flour (0ppm HCN) or 30% "sour" cassava root flour (50ppm HCN). The "sour" cassava behaved in a slightly inferior manner with regard to weight increase and feed efficiency, probably because of its HCN content (50ppm). It would appear that even accepting the affirmations by Tejada and Brambila (1969) and Jalaludin and Yin (1972) that birds (Gallus) are exceptionally resistant to HCN intoxication, it is necessary to obtain by adequate processing a product that does not contain HCN, or which contains it in a very low proportion (Montilla 1977).

Temperton and Dudley (1941) used cassava meal as a substitute for maize and ground oats. They observed that laying birds could tolerate up to 40% cassava meal in a dry mash without the ration being unpalatable or causing constipation.

In more recent studies, higher levels of cassava meal have been used in the diet of layers. Pillai et al. (1968) replaced 50% of ragi flour (Eleusine corocana) by cassava spent pulp, a fibrous waste of starch extraction, in the diet of laying hens. They observed a significant 12% increase in egg laying even though the two diets were isoproteic and isocaloric, but were unable to explain the cause of improvement resulting from the inclusion of tapioca in the diet. Similarly, Enriquez and Rous (1972) fed cassava roots as far as up to 50% of the ration to white Leghorn pullets

and observed no adverse effects on egg production and efficiency of feed conversion. Hamid and Jalaludin (1972) replaced maize with cassava root meal in the diets for laying birds at levels of 0, 20, 40, 60 and 76%. A non significant increase in feed consumption and egg production was reported up to the 60% level of substitution. Supplementation of the 76% cassava meal with 0.15% methionine gave no significant improvement in performance. Supplementation with 0.3% methionine, however, gave a significant increase in egg production. They concluded from this study that cassava could be successfully used to replace maize up to a level of 60% in the diets of laying birds, but that above that, there may be difficulty in maintaining the protein content of the ration.

Jalaludin and Leong (1973) in two separate experiment observed that low dietary levels of cassava meal (5, 10%) had no deleterious effects on egg production and efficiency of feed conversion, but 50 or 60% levels of cassava root meal tended to decrease production and feed efficiency. They further observed that the depressing effect of high dietary levels of cassava meal could be overcome by supplementing with fish meal.

Ngoka et al. (1983) fed Yafic hens and Rhode Island Red cocks cassava root meal in maize-based rations at four dietary levels, substituting for maize at 0, 50, 75 and 100% levels. Their results show that the eggs from bird fed the highest level of cassava showed significantly ($P<0.05$) higher infertility

and lower hatchability than did the others. There were no significant differences in motility, and shell intergrity while hatching time was not prolonged for any of the treatments.

2.6.2 Cassava in swine Nutrition

Cassava roots have been used for feeding swine since the early 1900s from which time attempts made by several workers to evaluate their nutritive value and physiological effects on pigs have been continuously reported (Khajarern et al. 1977). The modern view of cassava in swine nutrition originated with the report of Oyenuga and Opeke in 1957 (Nestel 1974).

A comprehensive work has been carried out by Maner (1972, 1973) in which up to 60% cassava has been used in the ration. However, at this level, gain and efficiency declined, although these could be improved by methionine supplementation which compensated for the low methionine level of the cassava and also provided a sulphur donor for the detoxification of HCN. Hew and Hutagalung(1973) found that feed intake in swine decreased as the cassava level of the ration was increased. Performance improved significantly following the addition of 0.2% methionine to a 50% cassava diet but did not improve with higher methionine levels. The further addition of palm oil (which also enabled the pigs to better utilize higher methionine levels) and glucose (which may have reduced HCN levels by forming glyconohydrin and improving palatability) improved both daily gain and food conversion.

Mullider et al. (1972) demonstrated that pigs on cassava-soybean meal diets containing lower crude protein than control diets grew as fast and as efficiently as control pigs. Khajarern et al. (1977) showed that cassava root products of moderate quality can be successfully incorporated into growing-finishing pigs diets at as high levels as 50% for 17-35kg pigs and 60-70% for those of heavier weight. However, it should be noted that the diet must be carefully formulated to balance it for vitamins, minerals, and limiting amino acids. Also special care must be taken as to the form of the diet, especially when the cassava is of a lower quality (higher CF and ash thus lower digestibility). Diets must be fed in form promoting optimum feed intake to ensure an adequate supply of digestible nutrients. These workers showed that as light, fluffy and dusty high cassava content of the diet increased from 0 to 65%, pigs grew at progressively slower rates and required more feed per kilogram gain. Where the equivalence of the daily consumption of pelletized cassava-based diets to the control diets eliminated the poorer growth performance.

Khajarern et al. (1977) suggests that subclinical cyanide toxicity may occur with low levels of methionine, and as indicated by Maner and Gomez (1973), could be detected by increased urinary thiocyanate excretion. Several workers have demonstrated that pigs fed a cassava-based diet grew at a slower rate and less efficiently than those in cereal control

group (. Velloso et al. 1967; How and Hutagalung 1976, Kitpanit 1975). However, increasing evidence suggest that cassava-based diets, once properly prepared and fed in the proper form, could be totally substituted for cereals in swine rations without detrimental effects on either growth performance or carcass quality (Khajarern et al. 1977 ; Tewe, 1982).

2.6.3 Cassava in Ruminant Nutrition

Both balance and feeding trials indicate that about 20-30% dietary levels of cassava chips are most efficiently utilized. Higher levels, while supplying increased energy are less well utilized due to reduced digestibility, probably because of reduced amylolytic activity in the intestinal tract posterior to the rumen (Devendra 1977).

Cassava feeding generally increases milk yield (Cardoso et al. 1968; Olaloku et al., 1971). In dairy heifers, Pineda and Rubior (1972) reported that cassava feeding increased total live weight gain and reduced both age at first breeding and the average number of services per conception. Assis (1962) in his study showed that cassava significantly increased milk production by 19.5% compared with 7.0% for sweet potatoes. The response to cassava is probably a result of the relatively higher supply of ME from cassava (11.9-14.6 MJ/kg) compared with sweet potatoes (11.0-13.4 MJ/kg) for cattle.

Several reports suggest increased live weight gains of beef cattle fed cassava. Johnson et al., (1968) fed four groups of Angoni X Boran steers for 150 days with one of four diets based on : bran; cassava; corn and cob; or a commercial concentrate mixture. The calves fed on the commercial concentrate and cassava-based diets gained weight significantly ($P<0.01$) faster than those fed bran or corn and cob-based diets, and were similar to calves that were suckled by the dams and weaned at 7 months of age. Better performance due to cassava has also been reported by Montilla (1971) in young bulls fed with a diet containing 40% cassava meal, which gave a much better performance than maize meal. In Africa, supplementing poor quality hay with cassava significantly increased the dry matter intake of Zebu cattle (Karue et al., 1973).

The effect of feeding increasing levels of cassava meal (30, 40, 60 and 80%) in the concentrate fraction (25% of total dry matter intake and 75% from Napier grass) to Kedah-Kelantan heifers was studied (Devendra 1977). Significant differences were found ($P<0.05$) between the 30 and 80% levels for both of the daily live weight gains and the efficiency of feed conversion. The best growth rates and efficiencies of feed conversion were recorded with the 30% level of inclusion. For all treatments, correlations of mean life weight with heart girth, height at withers, body length, and hip width were highly significant ($P<0.01$). Devendra and Lee Kok Choo (1976b) also reported improved carcass characteristics due to cassava feeding. Cassava has also been used to replace 50 or 100% of

the barley in diets for growing calves in India, and was found to improve growth performance (Mudgal and Sampath 1978).

The addition of grated cassava to Napier grass prior to ensiling significantly increased dry matter intake, apparent digestibility of dry matter ($P<0.01$) and N balance ($P<0.05$) in castrated sheep in Brazil (Pereira et al., 1974). In Venezuela, growth and metabolism studies have been conducted on West African wolless wethers (Chicco et al. 1971) involving cassava and molasses in the utilization of urea. Cassava addition significantly increased body weight gain and bacterial protein ($P<0.01$).

Balanced trial experiments with sheep (Devendra 1977) have been reported. The results show that the digestibility of dry matter in the rice straw-based diets increased significantly ($P<0.05$) with the inclusion of dietary cassava but decreased significantly with increasing level of cassava inclusion ($P<0.05$). The highest digestibility of dry matter (74.4%) was noted for the 20% level of cassava inclusion. With 80% cassava in the diet, the digestibility of dry matter decreased to 79.9% compared with 93.2% for the 20% level of inclusion. A similar trend was also noted for organic matter, crude protein, ash, ether extract, nitrogen free extract, and energy . CP digestibilities was not significantly different between the 40, 60 and 80% levels of cassava inclusion, but these treatments were significantly ($P<0.05$) lower than the 20% level of inclusion. A parallel finding was also noted for N retention.

When the diets with 20, 40, 60 and 80% cassava were supplemented with methionine, no significant differences were noted in the parameters measured except for crude fibre digestibility which showed significant difference ($P<0.05$) between the 80% level and 40 and 60% levels of cassava inclusion. The highest crude fibre digestibility was noted for the 80% level of cassava inclusion supplemented with 0.6% methionine.

Very little is known about HCN toxicity in ruminants, and present evidence (Devendra 1977) suggests that feeding high levels (20-80%) of cassava chips and chopped roots does not produce any ill effects. This may perhaps be due to the fact that sweet varieties were fed, because feeding raw fresh leaves of bitter varieties as a forage (with 180-240 mg/kg fresh weight) has produced acute toxicity and death. In a feeding trial involving Kambing katjang weaner kids in Serdang, up to 40% mortality was noted within 2 weeks of starting the trial, and a deletcrious effect was produced on live weight gain (Devendra 1977; Tewe *et al* 1982).

Nevertheless, white clover containing cyanogenic glucosides equivlaent to 129mg of cyanide per 100g dry matter produced no deleterious effects in dairy cows, but plasma thiocyanate increased producing evidence for a metabolic effect (Butler *et al.* 1957). Several species of *Cynodon* with HCN in them have not been reported to have any serious effects in ruminants (Henderson Research Station 1970). On the other hand, high levels of cyanogens (180ppm HCN)

and low levels of iodine (±0.05 p.p.m I) have been reported to cause thyroid dysfunction (Hemmington et al. 1971) in sheep grazing Cynodon plectostachys. Worker (1957) noted that blood thiocyanate levels were constant throughout the season in Jersey cows grazing white clover pastures, and that urinary clearance of thiocyanate could be greater than in sheep. They therefore suggested that the cow probably has an alternative detoxification mechanism.

2.6.4 Cassava-Based Rations for Rabbits

The work of Omole and Babiet (1976) on feeding cassava diets to rabbits shows that the rate of gain decreased with increasing levels of cassava in the ration, although this decrease was not significant until the ration contained up to 45% cassava root meal. Preliminary results from this work showed that the addition of 0.2% methionine to the control and 45% cassava diets resulted in very slight improvement (0.22 and 0.48%, respectively) in live weight gain over the unsupplemented diets. When the 15 and 30% cassava diets were supplemented, however, there was 5.9 and 3.5% increase in weight gain over the unsupplemented rations. Addition of methionine to the ration did not improve the efficiency of feed utilization significantly; neither did it affect the quantity of urinary thiocyanate excreted by the animals.

Fomunyan et al. (1983) has also shown that cassava levels of up to 45% supported good growth in rabbits. Rabbits fed cassava and corn-based diets showed similarity in reproductive growth, and carcass traits at the 45% level of cassava inclusion. There was also similarity in thyroid weights of the rabbits on both diets suggesting that the levels of thiocyanate were below the threshold causing goitre (Oke, 1973). The similarity for reproductive, growth, and carcass traits of the rabbits at 45% level of cassava inclusion suggest that the cyanide did not affect the rabbits or that it was rapidly detoxified by rhodanese of the liver (Fomunyan et al , 1983). Rabbit liver contains 8-9mg rhodanese/g. The value is surpassed only by the rhodanese content of rat liver which is 14-28mg/g (Himwich and Saunders 1948, Oke, 1973 and Fomunyan et al.1983).

2.7.1 CASSAVA PROCESSING TECHNOLOGY AND DETOXIFICATION

A wide variety of techniques have been devised in various parts of the world to detoxify the more poisonous varieties of cassava. Most traditional food preparation techniques appear to be designed to bring together enzymes and substrate (bound cyanide, linamarin) by cell rupture, followed by elimination of the liberated HCN by either volatilization or solution in water. In some processes, the initial hydrolysis of the linamarin is assisted by the use of fermentation processes (Coursey, 1973).

The traditional methods include drying, soaking, boiling , and fermenting whole or fragmented roots; all of these processes reduce the total cyanide content of the material (Coursey, 1973).

In Nigeria, sun drying of cassava (root, slices, chips, flour) is one of the popular processing methods. The materials are spread out, usually on mats, concrete floors, or disused portions of main roads, and left in the sun to dry gradually with occasional turning. The amount of cyanide that is lost from the materials depends on such factors as shape and size of fragments (Thani and Lohani, 1978) and relative humidity, air temperature and wind velocity (Rao and Cock, 1973).

The HCN level (milligrams HCN per kilogram) of samples of a number of African cassava-based food products have been given by Oke (1968); Fresh cassava 380, Gari 19, Fufu 25, Lafun 10 and Kpokpogari 11. Wood (1965b) gives a value of 25mg HCN/kg in fresh guri, falling to 2mg HCN/kg after storage for 1 month. During the extensive investigation conducted in Nigeria in connection with the mechanised production of gari (Akinrele et al. 1962), a value of 30mg HCN/kg was regarded as acceptable in the product. In Brazil, where cassava flours are extensively used in the baking industry, Paula and Bengel (1939) stated that crude flours made from bitter cassava contain 10-200mg HCN/kg, but the bitter grade of table flour are free from HCN : Nemoto (1940) gives 27-37mg HCN/kg as the normal range, but grated

manioc flour may contain as much as 125mg HCN/kg.

Both reports state that these residual quantities of HCN are destroyed in the baking process when the flours are incorporated in bread. However, in studies on the manufacture of rotis (a roasted product) with cassava flour containing 57-118mg HCN/kg, Joachim and Panditteseker (1944) found losses ranging from 47 to 80% during cooking, which would indicate high degree of retention.

The changes in concentration of free cyanide (non-glycosidic) and bound cyanide (cyanogenic glucoside) in fresh cassava chips during dehydration, boiling or soaking in water have been studied (Cooke and Maduagwu 1978). Their findings show that all of these processes rapidly removed free cyanide from the chips, but only 8-12% of the total cyanide is present as free cyanide. Air-drying in oven showed that 2% of the bound cyanide was removed by drying at 46.5°C; smaller losses were recorded at the higher temperatures. Greater losses occured on sun drying, presumably because of the longer drying time at moisture contents and temperatures at which endogenous linamarase is active. The corresponding losses of free cyanide was 82.5% (Cooke and Maduagwu, 1978).

On soaking (leaching) and boiling of fresh cassava chips, the same authors found that the free cyanide was rapidly removed in boiling water (over 90% removed within 15min); the bound cyanide was removed at a slower rate but

a thorough cooking for 25 minutes removed over 50% of the bound cyanide.

During sun drying of cassav slices, Maduagwu and Adewale (1980) reported a progressive loss of CN with time, most of it within the first 8h, during which time 46-58% free and 69-74% bound cyanide disappeared from the "bitter" variety and about 74% of bound cyanide disappeared from the "sweet" cultivar. These losses, they suggested could be due to optimal hydrolytic activity of the β-glucosidase, linamarase.

Nambisan and Sundaresan (1985) on evaluating the efficiency of different processes in reducing the cyanogenic glucoside content of cassava found that the maximum retention of cyanogenic glucoside(80%)was observed in baked, fried, and steamed tubers. Cyanogenic glucoside retention in sun-dried chips varied from 30-60%, the retention being governed by the chip thickness. In the case of cassava boiled in water smaller chip size and sufficient water was found to be the ideal condition for maximum cyanogenic glucoside removal (25-75% retention) depending on the chip size. These authors found that the most effective method for cyanogenic glucoside removal was by crushing fresh tuber and subsequent sun-drying, whereby 95% cyanogenic glucoside was eliminated.

The extent of loss of hydrocyanic acid during fermentation of cassava tubers for 3-4 days (in the traditional method) and 8 days have been studied (El Tinay et al., 1984). Loss of cyanide from whole tubers was 80-87% after 8 days and was only 51-53% after 4 days. When water is added to the crushed pulp the reduction in cyanide was 83-91% with marked decrease in bound cyanide in the 1st day of fermentation. This result suggests that autohydrolysis is enhanced by addition of water to the crushed pulp (El Tinay et al. 1984). The high cyanide content of the water necessitates dewatering of the pulp before consumption (El Tinay et al., 1984; Cooke and Maduagwu 1978). Maduagwu and Oben (1981) used a screw press for dewatering fermented cassava and reported a 63.8% reduction in cyanide content as a result of the processing step.

The variable and inconsistent literature data for cyanide losses during processing is probably because of problems associated with assay methodologies (Zitnak, 1973; Cooke, 1978). Important process parameters such as cassava piece size, dehydration rates, temperatures, etc. were often not specified. Also the different forms of cyanide respond differently to processing (Cooke and Maduagwu 1978) and have different toxicities (Montgomery, 1969).

It thus appears that cassava food products, as commonly prepared by many of the various detoxification methods, can still contain appreciable traces of HCN

(Coursey, 1973, Cooke and Meduagwu, 1978, Maduagwu and Oben, 1981).

2.7.2 CASSAVA FERMENTATION

The fermentation of cassava is very important during the preparation of "gari" a popular food among the low income group in the southern regions of Nigeria and which is also eaten extensively along the coast of West Africa (Akinrele, 1964). Its significance as already reported (Collard and Levi, 1959) is that it brings about the detoxification of cassava by the liberation of hydrocyanic acid at low pH, through the combined activities of Corynebacterium manihot and Geotricum candida, and the development of the characteristics flavour of gari. This fermentation which normally takes 3-4 days during village processing can be shortened to 24 hours by seeding a fresh pulp with 4-day old cassava juice (Akinrele, 1964). It is confirmed (Akinrele, 1964) that the fermentation of cassava proceeds in two stages during which the mash is generally sterilised against adventitious microbial growth. During the first phase, cassava bacteria Corynebacterium manihot attack the starch with the production of lactic and formic acids, a reaction accompanied by the evolution of heat. When the pH of the medium has fallen to about 4.25, a mould, Geotricum candida, begins to proliferate rapidly bringing

about further acidification and the production of the characteristic aroma of gari.

Earlier studies (Collard and Levi 1959; Callard 1963 and Akinrele, 1964) of cassava fermentation have attributed detoxification to the spontaneous hydrolysis of the constituent cyanogenic glycosides, principally linamarin, by the organic acids produced during starch degradation by the fermentative action of Corynebacterium manihot at low pH. More recent work, (Wood 1966, and Okafor 1977) has demonstrated the unusual stability of linamarin in dilute acids even at 100°C thus making it unlikely that detoxification is due solely to chemical hydrolysis by the weak acids of microbial fermentation. Ikediobi and Onyike (1982a) have shown that detoxification is due mainly to the action of linamarase on the cyanogenic glucosides of fermenting cassava. It is also observed that some of the formic acid is broken down by a hydrogenase system to form carbondioxide and probably hydrogen. All these gases tend to render the medium anaerobic (Akinrele, 1964). Fermentation seems to proceed best at a temperature of about 35°C. Frequent mixing of the mash and exposure to light appear to accelerate fermentation particularly during the second stage. A continous system is possible and in fact, gives a better fermentation than does the batch process.

Exposure to air or oxygen and contact with iron should be reduced to a minimum to avoid discolouration (Akinrele, 1964).

2.7.3 USE OF LINAMARASE IN CASSAVA DETOXIFICATION DURING FERMENTATION.

The use of exogenous linamarase to assist cassava detoxification during fermentation has been reported (Wood, 1966; Bruijn, 1971; Ikediobi and Onyike 1982a). Fermenting grated cassava was detoxified by 87.5% when exogenous linamarase was added to fermenting mash as opposed to 50% without any enzyme addition. This is an indication of the enzymatic basis of the detoxification process (Ikediobi and Onyike, 1982a). The work of these authors have shown that higher fold detoxification is achieved after 24 hours of fermentation in the presence of added linamarase than after 5 days of fermentation without enzyme addition and that very little additional detoxification is achieved by adding more enzyme to the fermenting mash beyound the first 24 hours, these observations are explained by the development of a pH favourable for linamarase activity within the first 24 hours of fermentation and accounts for the rather high cyanide seen within this period (Ikediobi and Onyike, 1982a; Tewe, 1975).

2.8 THE FUNGAL CELL AND ENZYME PRODUCTION

2.8 .1 THE FUNGAL CELL

The fungi comprise a large, heterogeneous, and ubiquitous group of heterotrophic organisms living as symbiots (Hickman, 1965). They are characterized by a distinctive, filamentous, multinucleate vegetative structure

known as the myceliums. It consists of a branching system of walled tubes, the hyphae, which contain protoplasm and continually extend by apical growth and lateral branching (Hickman, 1965). Food material is absorbed over the whole surface of the mycelium, representing a highly effective system for exploiting the resources of the substratum (Hickman, 1965). After a period of growth, reproduction occurs with the formation of spores uni - or multicellular bodies that become detached from the parent to give rise to new individuals (Ingold, 1953).

Sussman et al., (1957) have drawn attention to the cell surface in fungi, pointing out that it is not an inert region, but rather a region of manifold activities. Such activity is manifested in the surface localization of certain enzymes. It is not known precisely to what extent surface enzymes are physically integrated into the cell wall, but such a locus would apparently be appropriate for enzyme involved in the synthesis of wall components and extracellular polysaccharides (Aronson, 1965). In addition, the cell wall may function as a reservoir for various ions (Harold, 1962).

2.8.2 Media for fungal growth

Fungi are dependent upon the medium or substrate, for all the elements and compounds they require or utilize except molecular oxygen and, possibly, a little carbon dioxide, which are obtained from the atmosphere. From these elements and compounds they synthesize their cellular

constitutents and obtain the energy necessary for their life processes. Some compounds, such as vitamins and some amino acids enter the cells without modification. High molecular weight compounds, including cellulose, starch and proteins, are hydrolyzed before they can be utilized (Lilly, 1965).

The animal composition of a medium must include all the elements that are essential which must be present in compounds that can be utilized (Lilly, 1965). The carbon-containing compounds reported to be utilized by fungi for growth in synthetic media include a wide variety of amino acids, organic acids, sugars, sugar derivatives, alcoholic and more complex organic compounds (Perlman, 1965). Strain specificity in utilization of carbon is common. Glucose is utilized by more fungi than any other sugar and is nearly a universal carbon source (Perlman, 1965). The measurement of conversion of carbon sources to mycelium has shown that in general 2-3g glucose is required to produce 1g dry mycelium (Lilly, 1965; Perlman, 1965).

Fungi usually utilize simple inorganic nitrogen compounds and ammonium salts or nitrates as suitable sources of nitrogen although with nitrate there may be a lag in growth before the necessary enzymes concerned in nitrate reduction are produced (Nicholas, 1965).

Other chemical elements necessary for fungal growth include sulphur, potassium, magnesium and phosphorus. Sulphur can be utilized as sulphate sulphur by others

(Lilly, 1965). Phosphorus can be derived from any soluble non-toxic phosphate such as potassium dihydrogen phosphate or dipotassium hydrogen phosphate. Potassium is supplied as a phosphate and its other sources include potassium chloride, potassium sulphate, and potassium nitrate. Magnesium is supplied from various soluble non-toxic magnesium salts as magnesium sulphate heptahydrate (Lilly, 1965).

For routine use, it is convenient to prepare a microelement solution of such strength that 1ml/litre will supply the concentration desired. The following salts have been satisfactorily used (Lilly, 1965); iron, $Fe(NO_3)_3$. $9H_2,0$; Zinc, $ZnSO_4$. $7H_2O$; magnesium, $MnSO_4$. H_2O; Copper $CuSO_4$. $5H_2O$; molybdenum, $NaMo\ O_4$. $2H_2O$; calcium $Cacl_2$. $6H_2O$. The stock solution should be acidified to prevent precipitation of iron.

As a basic requirement, water used should not contain toxic materials. For exacting work, deionized distilled water, or distilled water from glass or quartz stills may be required. Water from metal stills may be very impure (Nicholas, 1952).

Vitamins and other organic growth factors such as biotin, thiamine, pyridoxine and inositol are required for fungal growth (Fries, 1965).

Since the nutritional requirements of fungi differ, it follows that no one medium, or substrate, will be suitable for

all fungi (Lilly, 1965).

2.8.3 Fungal enzyme production

Most of the fungal enzymes are located internal to the cell membrane (cryptic) but some species have a number of enzymes external to the membrane in or on the wall; still others secrete enzymes into the medium. The same enzyme can be present in all the three locations at the same time (Gottlieb, 1978). For a spore to utilize a water-insoluble substrate such as starch, cellulose, protein or fat, the free soluble form of the enzyme secreted into the medium has the greatest advantage in that the enzyme can act at a distance from the cell (Gottlieb, 1978). In the absence of secretion of an enzyme into the medium, the cell wall-bound form is helpful to the spore especially if the spore is in direct contact with the insoluble substrate. Enzymes which exist in the bound or free form often have hydrolytic functions enabling them to degrade the insoluble substrate to simple molecules, such as starch to glucose. The simple sugar can then permeate the spore and be metabolized by it (Gottlieb, 1978).

Enzymes in the basic metabolic pathways are constitutive and are present in the cell whether or not relevant substrates are available to them. A number of other enzymes, however, are not detected in spores unless the substrates are present to increase their synthesis - the adaptive enzymes (Gottlieb, 1978).

About six fungal isolates namely, Aspergillus sydowi, Aspergillus ochraceus, Aspergillus carneus, Aspergillus terreus, Fusarium equiseti and Penicillium steeki have been shown to produce linamarase in sorghum extract medium (Ikediobi and Onyike 1982a, Ikediobi and Ogundu, 1985). Ikediobi and Ogundu (1985) showed that at least 70% of the total enzyme activity was extracellular. Wiseman (1979) had earlier shown that these fungi among others were capable of amyloglucosidase production.

2.8.4 Factors important to fungal enzyme production

Strain Selection:

In devising a system for enzyme production, it is important to begin with the most active fungal strain available. In the absence of a known enzyme-producing strains, a simple screening programme must be devised in which cultures from nature or from culture collections are examined for enzyme activity (Wang et al. 1979b).

Improving Production

Once a good strain is obtained, growth parameters must be optimized to maximise fungal growth and enzyme production. Hence, temperature, pH, and oxygen transfer are important. Also nutrition of the microorganism, especially with respect to source of carbon, nitrogen, phosphorus, sulphur and mineral salts are important (Wang et al. 1979b). Often, especially with extracellular enzymes, the addition of surfactants is important (Faith et al., 1971). Non-ionic

surfactants are generally preferred over ionic and cationic agents which are usually toxic to the microorganism (Faith et al., 1971).

Growth cycle Peak

If the fungal growth is conducted as a batch culture, the stage of the growth cycle at which enzyme content in the growth medium is highest must be ascertained (Wang et al. 1979b). In many cases enzymes rapidly disappear after reaching their peak activity (Thurston, 1972). On the other hand, a chemostat can be quite useful for enzyme production since the formation of many enzymes is controlled by growth rate, which can be kept constant in continuous culture (Wang et al. 1979b). The use of both continuous cluture and continuous flow of isolation of β-galactosidase, and intracellular enzyme has been described in the literature (Gray et al. 1972).

In addition to the factors outlined, it is of extreme importance to exploit the genetic regulation of enzyme biosynthesis (Wang et al., 1979b).

2.8.5 Fungal enzyme induction

Enzyme induction is defined as a relative increase in the rate of synthesis of a specific enzyme, resulting from exposure to a chemical substance - the inducer (Wang et al. 1979a). Inducible enzymes are necessary when the organism finds itself limited to, for example, a polysaccharide, an oligosaccharide or an amino acid as its sole source of carbon and energy. Induction ensures that energy and amino acids are not wasted in making

unnecessary enzymes but that when needed, the enzymes can be formed rapidly (Wang et al. 1979a).

The structural genes coding for the production of many enzymes are normally inactive in absence of the enzyme's substrate. That is, enzyme production is normally repressed. However, when a substrate is added, the structural gene is turned on and the enzyme is produced. Such a process is termed "induction" or derepression and the enzyme is considered "inducible" Wang et al. (1979b). Many catabolic enzymes fall into the inducible category. Typical inducers are substrates, and often most potent inducers are analogues of the substrates which are poor or inactive enzyme substrates (Wang et al. 1979a,b). These are called gratuitous inducers. Products, rather than substrates are sometimes the inducers of enzymes, (Wang et al. 1979a, b). Enzyme induction sometimes occurs upon addition of a coenzyme to the growth medium. Thus thiamine increase production of pyruvate decarboxylase (Witt and Neufang, 1970), and pyridoxine enhances formation of tyrosine phenollyase (Yamada et al. 1972).

Surfactants such as Triton X-100 and Tween 80 at concentrations of 1 and 5 percent have been found to cause 3- and 4 - fold induction of linamarase production respectively. Tween-80 caused about 3-fold induction in A. Sydowl at a concentration of 0.5 percent and about 2-fold induction in F. equiseti at a concentration of 0.2 percent (Ikediobi et al. 1985). Reese and Maguire (1969) had also used Tween-80 to stimulate enzyme

production in microorganisms at concentrations between 0.05 and 0.2 percent. They observed inductions of between 2 - and 13 folds in most of the micro-organisms.

2.9 THE BACTERIAL CELL AND ENZYME PRODUCTION

The bacteria form a large group of unicellular forms which in their manner of life bear a general resemblance to the fungi. They are usually clearly distinguished from the fungi, but as throughout the whole biological system, boarderline forms exist (Galloway and Burgess, 1952a). Bacterial structure shows little differentiation and its main function is the apparently simple one of dividing by binary fission. However the bacterial cell has a complicated structure built up by the different enzymes which manufacture its components and its proper function depends not only on the kinds of enzymes it can produce but on the structural organization within which they can operate (Wilson and Miles, 1964a).

Certain physical conditions must be satisfied in order that nutrients can enter the bacterial cell. Of these, most is known of the effects of temperature and hydrogen ion concentration. Bacteria have widely different optimum growth temperatures, from a little above freezing point to at most 70°C. The great majority of bacteria are mesophilic, with optimum temperatures in the region of 25°C and 37°C. The hydrogen ion concentration range for growth extends from pH 1.0 or less for the chemosynthetic

organisms to pH 11.0 for certain bacteria living in alkaline soils. The optimum for most bacteria is slightly to the alkaline side of neutrality (Bisset 1952). The existence of bacterial cell walls show that food can only be absorbed in solution, and no solid food can be utilized unless it is first converted into soluble form. This is achieved by the production of exogenous enzymes which liquifies the substrate outside the cell wall before its absorption (Galloway and Burgess 1952b).

It is probably characteristic of all bacteria that, at a given moment their enzymic apparatus has only a small fraction of the activity of which it is potentially capable. The capacity to increase or decrease the activity of individual enzymes or sets of enzymes in a particular biosynthetic pathway in response to environmental changes has great survival value for the organism (Wilson and Miles 1964b). Concentration of some enzyme may be increased when its substrate is present, but there are enzymes whose concentrations are almost unaffected by presence of their substrates (Wilson and Miles, 1964b).

Ejiofor and Okafor (1983) have reported the ability of some bacteria and yeasts such as Alcaligenes faecalus, Leuconostoc mesenteroides, Saccharomyces cerevisiae and Rhodotorula mimuta to produce linamarase in fermenting cassava pulp.

2.10 COPROPHAGY IN RABBITS

Rabbits along with a variety of animals from the rodent family practice coprophagy on a regular basis. Rabbits were at one time classified as rodents but are now part of a separate order, the Lagomorphs (Cheeke, et al., 1982). A unique feature of the rabbit is its ability to expel its caecal contents at intervals as cecotropes and consume them directly from the anus (Robinson et al. 1985). The phenomenon of consuming one's own faeces is referred to as coprophagy. It is usually practiced by domestic rabbits as young as three weeks of age and by wild rabbits while they are in their burrow during the day time (Watson, 1954).

In contrast to other monogastric animals such as swine, rats and poultry, rabbits digest the protein in forages quite efficiently, inspite of the fact that fiber digestibility in the rabbit is very low (Maynard et al., 1979; Slade and Hintz, 1969). The ability of the rabbit to selectively digest the protein fraction suggests that some characteristic of its digestive tract accomplishes this more effectively than is the case with swine (Robinson et al., 1985). In the hindgut of the rabbit, selective excretion of larger fiber particles occurs with reverse peristalsis moving small particles and fluids into the caecum where bacterial fermentation takes place. The caecal contents are expelled at intervals as caecotropes and consumed directly from the anus by the rabbit (Robinson et al., 1985).

The soft faeces of the rabbit is known to contain more vitamin B_{12} than the hard faeces, and these animals probably satisfy their vitamin B_{12} requirements by eating soft faeces (Kulwich et al. 1953).

It has been indicated (Robinson et al. 1985) that in a highly digestible concentrate diet, normal digestive efficiency and normal protein digestion in the rabbit are dependent on hindgut activity while in swine and other monogastric animals, digestion of high concentrate diets in the small intestine is very efficient. This shows that, in rabbits the digestion of grains and high quality protein supplements in the small intestine is incomplete, and further digestion and coprophagy are essential for full utilization of dietary nutrients.

2.11 Effect of prevention of Coprophagy

The importance of coprophagy is shown by the fact that if it is prevented, the growth rate of the rabbits drops by 12 to 25% (Barnes et al. 1963) and they show symptoms even of vitamin B deficiency (Kulwich et al. 1953). Other workers (Proto, 1965; Stephens, 1977 ; Fraga and De Blas 1977) have shown that the prevention of coprophagy exerts a significantly unfavourable effects on the apparent digestibility of protein. Robinson et al. (1985) have also shown that when coprophagy is prevented, there is reduction in dry matter digestibility, in the digestibility of acid detergent fibre (ADF) in the high concentrate and grass diets,

and in the digestibility of acid detergent fibre-nitrogen (ADF-N) in grass; though no reduction in the digestibility of alfalfa ADF.

2.12 THE EFFECT OF PALM OIL ON CASSAVA-BASED DIETS

Growth depression of animals fed cassava-based diets were often corrected by the addition of fats and oils (Hutagalung 1972; Hew and Hutagalung 1972; Lee and Hutagalung 1972). Fats and oils have been used extensively as source of energy for feeding livestock. Studies in pigs and poultry on the use of palm oil and stearin as an energy source have shown favourable responses in rate of gain and feed efficiency (Hutagalung 1972; Ng and Hutagalung 1974; Hutagalung and Chey 1975). Sulphur and fats enable better utilization of cassava flour by animals. Fats, especially, have been noted to influence feed intake regardless of the density of the feed (Carew et al. 1963). In terms of energy and digestibility, palm oil has been found to be superior to fats of animal origin (Hutagalung 1977).

Various sources and levels of fats and oils have been added to cassava-based diets. Supplementation of 5% palm oil to 50% cassava- 0.2 percent methionine diet improved the performance of pigs (Hew and Hutagalung, 1972; Ross and Enriques 1967). Hutagalung and Chang (1977b) studied the effects of palm oil, stearin and lard, addition to a cassava-based diet on the performance and carcass quality of

growing and finishing pigs. Supplementation of either palm oil or stearin within range of 5-10% to a 30% cassava-based diet significantly improved the rate of gain and feed efficiency of pigs compared to those on the basal and lard cassava-based diets. Carcass quality evaluation showed that palm oil or stearin addition at 5% to cassava-based diets produced better carcass quality compared to those on basal and lard supplemented diets.

Devendra and Hew (1977) fed pigs varying levels of palm oil (0, 5, 10, 20, 25, 30%) in diets containing cassava-root meal ranging from 10 to 21%. They found no apparent differences in rates of gain and feed efficiency due to levels of palm oil addition, carcass characteristics were not affected by either palm oil or cassava, even at the 30% palm oil level. However, they suggested that the optimum level of palm oil inclusion in the cassava-based diets of pigs was 5%.

Hew (1975) stated that an 8% palm oil supplementation of a cassava-based diet enabled faster grwoth by animals and beyond this level a plateau was reached.

The improved performance of animals fed palm oil supplemented cassava diets may be attributed to the increased energy intake (Hew 1975). Formuyam et al. (1980) have shown that palm oil does slow down the decomposition of acetone cyanohydrin, an intermediate product of cassava linamarin. They also suggested that palm oil could prevent the absorption of linamarin and effects its subsequent excretion.

CHAPTER 3

MATERIALS AND METHODS

3.1 Fungal and Bacterial isolates

Pure fungal and bacterial cultures used were obtained from the Departments of Crop Protection, Institute for Agricultural Research (IAR) and Microbiology respectively, Ahmadu Bello University, Zaria. The fungi and their identification numbers are as follows:

Aspergillus	sydowi	SA 1107
Aspergillus	ochraceus	SA 1322
Aspergillus	carneus	SA 1326
Aspergillus	terreus	SA 562
Fusarium	equiseti	SA 1109

The bacterial isolates and their strain codes are as follows

Bacillus	Licheniformis	B 5.1
Bacillus	Subtilis	G.3
Bacillus	polymyxa	Wa 42
Bacillus	circulans	Wa 11_3
Bacillus	coagulans	ST 21

3.2 Media Materials

Potatoes, sorghum and cassava used for preparing culture and growth media were purchased from local market in Zaria.

3.3 Diets

The following ingreidnets were used in the diet of experimental animals; cassava ("sweet" variety), maize, soybean meal, bone meal, vitamin/mineral

premix and methionine. The last two ingredients were obtained from Roche Nig, Ltd.

3.4 Animals

The male dutch-breed rabbits and male white albino rats (Normatensive strain) used were obtained from Departments of Animal Science and Biochemistry respectively, Ahmadu Bello University, Zaria.

3.5 Equipment

The following equipment were used; orbital thermostated incubator/shaker for growing the fungi and bacteria; spectronic 20, refrigirated high speed and swing out centrifuges, aeration apparatus, 'Econo-Columns' used for ion-exchange chromatography, metabolic cages for rats and rabbits, Kjeldahl apparatus, soxhlet extractor, drying oven and dialysis tubes.

3.6 Chemicals

P-nitrophenyl B-D-glucoside (PNP-glucoside) used for enzyme assay was obtained from Sigma Chemicals Company, St. Louis, M.O. U.S.A. Dextrose and agar powder for preparing culture media, Bovine Serum Albumin fraction V for preparing protein standard curve, and ammonium sulphate for precipitating proteins were products of Hopkin and Williams Co., Chadwell Heath Essex England.

Potassium cyanide, potassium thiocyanate, anhydrous picric acid, pyridine, boric acid, sodium borate, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate , acetic acid were purchased from British Drug House (BDH) Chemical Co., Poole, England.

Barbituric acid and sodium perchlorate are products of Merck, Schuchardt and Merck, Darmstadt, Germany, respectively while the sodium hypochlorite used is product of Phillip and Harris Ltd. Berlingham, England. Anion exchange resin; Amberlyst A.21 mesh size 20-30 used for thiocyanate estimation is from Serva Feinbiochemica, Heidelberg.

Silicon antiform used is a product of Fluka AG Chemische Febrik CH-9470 Buchs, while the sodium hydroxide and hydrochloric acid were products of May and Baker Ltd. Dagenham, England. All other routine chemicals and solvents used were of analytical grade.

3.7 Preparation of potato-Dextrose-Agar-Streptomycin (PDAS) medium

Peeled potatoes were cut into small pieces and boiled for one hour in distilled water (200g potatoes per litre of water). The extract was filtered through glass wool. Dextrose and agar (20g each per litre of filtrate) were added to the filtrate. The volume was made up to the required volume and boiled till the agar dissolved. Streptomycin sulphate was added (0.05g streptomycin sulphate per litre of solution), 10ml of the resulting solution was poured into Universal MacCartney bottles with a 10ml dispensing syringe. They were tightly capped and sterilized at 121°C in an autoclave for 20 minutes under a pressure of 15psi. The bottles were allowed to cool to room temperature before use.

3.8 Culturing of the Fungi and Bacteria isolates

The pure fungi and bacteria isolates were transferred into the bottles of PDAS medium in an innoculating hood in a sterilized room. The innoculating needles used for the transfer were constantly sterilized with smokeless flame of a spirit lamp placed inside the innoculating hood. The bottles were left half-cocked and stored in an incubator for 4-7 days to ensure full growth of the fungi and 2-4 days for bacteria.

3.9 Preparation of sorghum - extract growth medium for fungal growth

The chemical composition of sorghum in different concentrations include starch, protein, fibre, pentosans, ash, dextrose, sucrose and minerals such as P, Mg, K, Si, Ca, Na, Cu, Mn, Fe, Mo, Zn (Savile and Thorpe 1951).

About 1.4kg of sorghum was boiled in 7 litres of distilled water (200g/litre) and left to cook for one hour. The cooked grain was upon cooling carefully filtered through a clean sheet of muslin cloth. The volume of the filtrate was made up to 7 litres. Two hundred millilitres of the flitrate was poured into each of the 250ml Erlenmeyer flasks and carefully plugged with cotton wads, the latter and their contents were autoclaved for 15 minutes at 121°C under a pressure of 15psi. The flasks were removed and allowed to cool before use for the fungal growth.

3.10 Fungal growth

The fungus fully grown in potatoe Dextrose Agar Streptomycin slant was innoculated into the flasks containing the autoclaved liquid growth media inside an innoculating hood in a sterilized innoculating room. The wads of cotton wool were gently and carefully plugged firm after innoculation. The flasks were transferred to a rotary shaker maintained at a speed of 100 revs/min. The fungus was allowed to grow for 18 days. Possible contaminations by bacteria was eliminated by incorporating streptomycin sulphate in the growth medium at the concentration of 2% (w/v).

3.11 Preparation of Mineral-salt-cassava medium (MSC) and bacterial growth

One litre of mineral-salt cassava medium contain KH_2PO_4 1g; $MgSO_4.7H_2O$ 0.2g; $CaCl_2$ 0.2g; NH_4NO_3 1.0g; cassava flour 22.0g; 200ml was dispersed into 250ml Erlenmeyer flasks. They were autoclaved at 121°C for 15 minutes under a pressure of 15psi. After cooling, a loopfull of bacteria was transferred into the flasks in duplicate and grown in orbital shaker. Ten milliliter was removed aseptically at three days intervals and assayed for linamarase.

3.12 Extraction of crude fungal linamarase

At the end of the growth period, the contents of the growth flasks were pooled and centrifuged at 5,000 x g for 15 minutes. The supernatant was saved for linamarase assay while the precipitated mycelia were discarded.

3.13 Ammonium sulphate precipitation

The weight of ammonium sulphate required to give 90 percent saturation of a given volume of enzyme extract was determined from a table (Ferdinand 1978). This amount was dissolved in the enzyme extract with continuous stirring till a preicipitate was observed. This was left in refrigirator at $4^{o}C$ for 2 days for the precipitate to settle down and come together. The supernatant was carefully pipetted out using pipette filler. The precipitate was dialyzed against water for 24 hours at room temperature to remove remaining ammonium sulphate. The extract obtained after dialysis was assayed for enzyme activity and subsequently used for cyanide assay.

3.14 Preparation of linamarase from cassava tubers

The thin brown outer skin of freshly harvested cassava tubers was carefully removed revealing the underlying cortex. The latter was removed and sliced into small pieces. Approximately 100g of the slices were homogenized in 300ml of prechilled 0.1M acetate buffer of pH 5.50. The homogenate was collected in a prechilled container and filtered by suction through a 1cm-layer of Kieselguhr. The filtrate was used to extract a second 100g batch of sliced cassava cortex as described above. The filtrate from the second extraction was similarly used to extract a third 100g batch. This final extract was kept overnight at $4^{o}C$.

To this extract were added 2.3 volumes of cold acetone and the

resulting solution swirled several times for 2 minutes within a 2-hour period. A pale-green precipitate was recovered upon decantation. This precipitate was subsequently extracted several times with 10ml portions of cold 0.1M acetate buffer of pH 5.5C. The extract as centrifuged at 500ox g for 5 minutes at room temperature and the resultant supernatant was dialyzed either at room temperature or in the cold room against 0,1M acetate buffer of pH 5.50 to remove any residual linamarin. The extract obtained after dialysis was assayed for activity and subsequently used for cyanide assays.

3.15 Assay of enzyme activity using p-nitrophenyl β-D-glucoside as the substrate

The assay method was essentially that described by Ikediobi et al. (1980). P-nitrophenyl β-D-glucopyranoside (PNP-glucoside), an artificial analogue of linamarin, was used as the substrate.

One millilitre of a solution containing 21mg of PNP-glucoside in 0.01M sodium phosphate buffer of pH 6.8 was incubated for one hour at room temperature ($25 \pm 2^{\circ}C$) with 0.5ml of appropriately diluted enzyme extract (200 times dilution). At the end of the incubation period, the reaction was stopped by the addition of 2ml of 0.2M borate buffer of pH 9.8. The reaction solution was then brought up to 4ml with distilled water and the absorbance of the resulting solution read at 400nm in spectronic 20 spectrophotometer.

One unit of linamarase activity was defined as that amount of enzymes which produced one micromole of P-nitrophenolate ion at 400nm/min under the described assay conditions.

3.16 Protein determination (Lowry et al. 1951)

The protein content of the crude extracts was determined by Folin-Lowry method using bovine serum albumin as standard. The reagents used were:

A. 50% Folin-Coicattaou reagent (1:1% H_2O)

B. 2% Sodium potassium tartarate

D. 1% $CuSO_4 \cdot 5H_2O$

D. 2% $Na_2 CO_3$ in 0.1N NaOH

E. Bovine serum albumin (BSA) stock solution - 200 µg/ml.

Standard solutions of BSA (concentration range 0-200 µg/ml) were added in different test tubes; 5ml of a mixture (50mls of reagent D plus 0.5ml each of B and C) were added to the test tubes and let to stand for 10 minutes at room temperature after mixing thoroughly. Then 0,5ml of the diluted Folin-Coicalteau reagent (immediately prepared) was added to each tube, shaken vigorously and allowed to stand for 30 minutes for colour development. Absorbance of the contents in each tube was read at 750nm against a blank which had no BSA. A calibration curve was prepared by plotting absorbance readings against protein (BSA) concentrations. (fig. 4.6). The protein content of enzyme extracts were determined in the same way and the corresponding protein concentrations were read from the calibration curve.

3.17 Construction of cyanide standard curve :

(Per Lundquist et al. 1985)

A standard stock solution of 0.1mM KCN containing 2.6 µg CN^-/ml was prepared. Varying concentrations ranging from 0.26 - 1.82 µg CN^- corresponding to 0.1ml - 0.7ml were measured into different stoppered test tubes. Each was made up to 4mls with 0.3M NaOH. 0.4ml of 2M acetic acid was added and vortex-mixed briefly. 0.1ml of 5mM sodium hypochlorite (NaclO) was added and mixed again. Within 1 minute, 0.5ml of barbituric acid-pyridine reagent (six grammes of barbituric acid dissolved in a mixture of 30ml of pyridine and 64ml of water, and 6.0ml of concentrated Hcl added in a fume chamber) was added and vortex mixed. The absorbance was determined at 580nm within 5 to 15 minutes latter. A blank determination was performed by substituting the cyanide concentrations with equal volumes of water.

3.18 Preparation of cassava and cassava product extracts for cyanide analysis

About 20g of fresh cassava parenchyma tissue is homogenized in 50ml of 0.1M Hcl for 3 minutes. The homogenate is filtered by vacuum and the resulting filtrate adjusted to pH 6.8 with base. This solution is then centrifuged at 5000 x g for 3 minutes. The resulting supernatant is then stored in the freezer until analyzed. Preparation from various cassava products are made in the same way as from the cassava tuber except that 150ml of 0.1M sodium phosphate buffer pH 6.8 is used in the extraction.

3.19 Fermentation of cassava, using exogenous linamarase

Freshly harvested cassava tubers were washed, peeled to remove the skin and cortex and the exposed parenchymatous tissue was grated. Four hundred and thirty seven millilitre of enzyme extract with enzyme activity of 2,753.1 units (6.3 units/ml) was added into 2kg of the grated mash. These were thoroughly mixed. Water was added to make the volume of liquid (enzyme extract plus water) to weight of cassava a ratio of 1:4 (500ml of liquid; 2,000g of cassava). Fermentation was allowed to proceed at room temperature in covered plastic tray. The same amount of cassava was fermented without enzyme but with water at the same ratio. The pH of the fermenting mash was monitored at regular intervals to ensure that fermentation was going on normally. To measure the pH, an aliquot of the fermenting cassava is removed, hand-squeezed in a cheese-cloth and the pH of the ex-pressed juice determined with a pH meter. Samples were taken after five days and sun dried while fermentation was terminated at the 8th day. After squeezing out the liquid using jute bags, the mash was sun dried for 2 days. These were stored in lynon bags till when used, both for cyanide analysis and animal feeding experiment.

3.20 Preparation of cassava flour

Freshly harvested cassava tubers (same source as that in section 3.19) were washed, peeled to remove the skin and cortex and the exposed parenchymatous tissue was chipped into small, flat sizes. These were sundried on a concrete floor for 3 sunny consecutive days. The chips were ground/milled into coarsed floor. This was stored in lynon bags till when used for cyanide analysis and animal feeding experiment.

3.21 Quantitative determination of free and total cyanide in cassava, cassava product, and Animal tissue extracts

About 0.1 - 0.5ml of cassava extract or cassava product extracts was incubated with 1.0ml of linamarase preparation containing 3· units of activity for 10-15 minutes at room temperature in a tall stoppered test tube. The volume of the incubation mixture is made up to 2ml with 0.2M sodium phosphate buffer of pH 6.8. At the end of the incubation period, the reaction was stopped by the addition of 2ml of 0.2M borate buffer of pH 9.8. 0.4ml of 2M acetic acid was added and vortex mixed briefly. 0.1ml of 5mM sodium hypochlorite was added and mixed again. Within 1 minute, 0.5ml of barbituric acid - pyridine reagent was added, mixed, and the absorbance determined at 580nm 5 to 15 minutes later.

The determination of free cyanide was performed as the above method except that the exogenous linamarase preparation

is not added to the aliquot of the extracts to be analysed for free cyanide. Instead 1ml of 0.2M sodium phosphate buffer is added.

The difference between the total and free cyanide gives the bound cyanide or unhydrolyzed cyanogenic glucosides.

3.22: FEEDING EXPERIMENT : 1 - Feeding of rats with processed, unprocessed and palm oil supplemented cassava - based diets

Twenty four male white albino rats (normatensive), 120-140gm initial weight were divided into four groups containing six animals each. Each animal was placed into a seperate metabolic cage. Experimental diets (Table 3.1) was fed to each group of rats for 21 days after one week adjustment period. Feed consumption was determined for each group of animals daily. The weight gain of each animal was determined weekly and the figures used to determine feed efficiency ratio (i.e. grams of feed consumed per gram of gain).

3.23: FEEDING EXPERIMENT II: Feeding of rabbits with cassava and maize-based diets

Eight male Dutch rabbits (1.3 - 2.05kg initial) weight) were fed on two different diets: Cassava-based diet and maized-based diet. The composition of the diets are given in Table 3.2. Four rabbits were placed on each of the diet in seperate cages for eight weeks. Both feed and water were given ad libitum. Cassava and maize were respectively the main source of energy while the crude protein content of the diets was 20%.

Table 3.1 : Percentage composition of diets for the rat-feeding experiment

Ingredient	Maize-based diet	Unprocessed cassava-based diet	Unprocessed cassava + palm oil based diet	Enzyme processed cassava-based diet
Maize	58.90	-	-	-
Unprocessed cassava	-	49.78	53.13	-
Enzyme processed cassava	-	-	-	51.13
Soybean meal	31.10	40.22	40.22	38.87
Palm oil	-	-	5.00	-
Sucrose	8.37	8.37	-	8.37
Vitamin/mineral Premix	1.00	1.00	1.00	1.00
Methionine	0.50	0.50	0.50	0.50
Salt (Nacl)	0.13	0.13	0.13	0.13
	100.00	100.00	100.00	100.00
Crude protein (%)	16.00	16.00	16.09	16.00
Gross Energy (Kcal/g)	3.83	3.60	3.87	3.60

Table 3.2 : The composition of cassava - and maize-based diets for rabbit experiment

Ingredients	Cassava-based diet %	Maize based diet %
Cassava meal	22.75	
Maize	-	26.25
Wheat offal	47.00	47.00
Molasses	3.00	3.00
Groundnut cake	23.50	20.00
Bone meal	3.00	3.00
Vitamin premix	0.25	0.25
Salt	0.50	0.50
CRUDE PROTEIN CONTENT = 20%		
Metabolizable Energy (Kcal/kg) =	2,797	2,681

3.24 Collection of urine, faeces, serum, gastro-intestinal tract (GIT) content and wall of the rats

To avoid fungal and bacterial contaminations urine and faeces were collected daily stored in the refrigirator and pooled together at the end of the week. The total volume of the urine and weight of the faeces for each week were recorded. They were stored in freezer till analyzed.

Whole blood was collected by slaughtering the rats at the end of the experiment in a prechilled container placed in ice water. This was centrifuged, at a speed of 5000 x g for 5 minutes. The serum (the supernatant) was collected and stored in a freezer till use.

The gastro-intestinal tract (GIT) was seperated into the stomach small intestine and large intestine. The content of each was collected by rinsing with small amount of 0.1M phosphate buffer, pH 6.8 and the total volume recorded. A known weight of the GIT wall was homogenized in the same buffer solution. The total volume was recorded and they were all stored in a freezer till used for analysis.

The weight of the kidney and liver of each animal was recorded immediately after slaughter.

3.25 Collection of soft and hard faeces of the rabbits

Collection of the faeces started after three weeks adjustment period, during which the rabbits were adapted to the cages and the diets. During each collection, the rabbits

were housed in the cages (Plate 3.1) which prevented them from consuming the soft faeces from the anus.

In the experiment, five day-periods and five night-periods collections were made. In the day-period collections, the animals were caged from 7.00am to 6.00pm. while for the night-period collections, the animals were caged from 6.00pm to 7.00am. At the end of each collection the faeces defecated by each rabbit was weighed and recorded separately.(Plate 3.2).

To ensure that the animals were not stressed, day and night period collections were made only once in a week on different days.

3.26 Estimation of bound and free cyanide in faeces, serum, GIT content and wall

The hard faeces was ground in a grinder while the wet faeces was thoroughly mixed in a mortar. The gastro-intertinal tract content was collected with rinsing using 0.1M phosphate buffer pH 6.6. A known weight of the GIT wall was homogenized in 0.1M phosphate buffer, pH 6.8 and stored.

Cyanide was measured by separating it from the samples, absorbing it into sodium hydroxide and estimating the cyanide in the alkaline extract. Seperation is achieved by a nitrogen aeration method (Boxer and Rickards 1952, Lundquist et al. 1985). The aeration vessel contained 1g of faecal sample or 1ml of sample in the case of serum, GIT content or GIT wall



Plate 3.1 : A rabbit in a cage to prevent coprophagy.

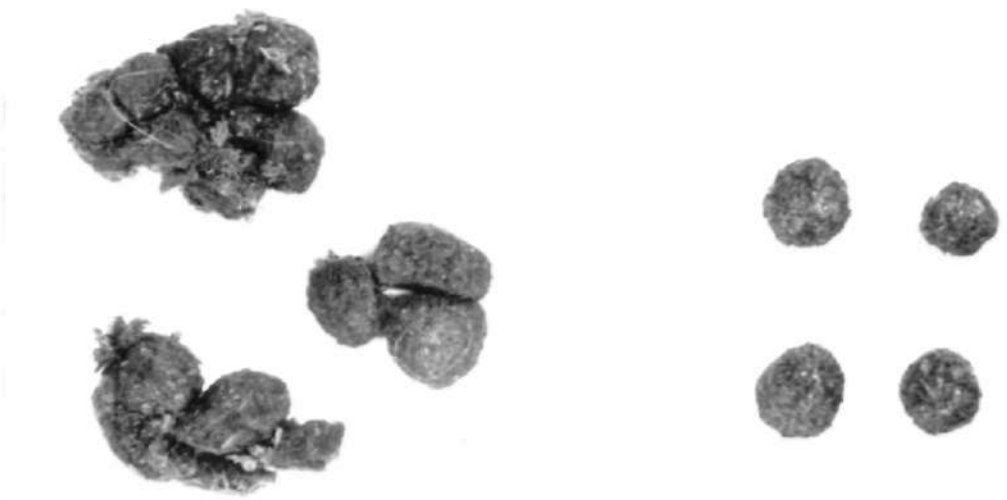


Plate 3.2 : Cluster of soft faeces or secotropes (left) and hard faeces (right) of rabbits.

or buffer (for blank determination). Ten milliliter of de-ionized water and five drops of antifoam agent were added. The sample was acidified by addition of 1.0ml of 0.5M H_2SO_4 and the liberated HCN was then transferred by aeration into 4.0ml of 0.1M NaOH for 45 min.

For total cyanide, the sample was incubated with 1ml of linamarase with 3 units of activity for 15 minutes before the addition of water, antifoam agent, H_2SO_4 and aeration.

After the aeration, the stoppers from the NaOH traps were removed with care to avoid the sucking back of any NaOH from the inlet tube into the trap. The cyanide was estimated by modified konig reaction (· Lundquit et al. 1979). 0.4ml of 2M acetic acid was added to the trap and vortex-mixed briefly; 0.1ml of sodium hypochlorite (Phillip and Harris Ltd. diluted 10 fold) was added and again mixed. Within 1min. 0.5ml of the barbituric acid - pyridine reagent was added, mixed and the absorbance determined at 560nm 5 to 15 min. later. The cyanide concentration of the sample was read with the cyanide standard curve.

3.27 Construction of thiocyanate standard cruve

A standard stock solution of 0.1M potassium thiocyanate was prepared. The working standard of 0.2M was prepared by diluting the standard stock solution 500 fold with distilled water to give 11.6 µg SCN/ml equivalent to 200 µM SCN. The working standard was again diluted 10 fold to give 1.16 µg SCN/ml. 0.1 - 0.7ml of this were placed in test tubes in

duplicate. The volume was made up to 4ml with 1M sodium perchlorate solution. This was acidified by 0.2ml of 0.5M acetic acid and vortex mixed. 0.1ml of sodium hypochlorite (Phillip and Harrison Ltd. diluted 2 fold) was added and mixed again. Within 1min 0.5ml of barbituric acid-pyridine reagent was added and the absorbance determined at 580nm 5 to 15min later against water blank.

3.28 Activation of the resin for ion-exchange column chromatography

Amberlyst A.21, 20-30 mesh size was washed with water and suspended in hydrochloric acid, 1M. The resin is filtered on a suction filter and washed with water until the pH of the washings is 4.5 or greater. The resin is then spread out on a glass disk and dried at 100°C for 12 hours (if it is not needed for immediate use). It is then suspended in water and left to stand for at least 15 min. The water is decanted off and the resin resuspended in an equal volume of sodium hydroxide, 1M. After 15 min. the resin is washed with water until the washings attain a neutral pH; it is then ready for use. If the pretreatment of the resin according to the description given above is omitted, blank values are higher.

3.29 Estimation of thiocyanate in serum, urine faeces, gastro-intestinal tract content and wall

The method of Lunquist et al. 1979 was used. Econo-columns 0.7cm i.d and 4cm long were used for the chromatographic step. The column was packed wet and 0.5ml of the sample diluted

with 5.0ml of 0.1M NaOH, was applied. The column was washed three times with 5ml portions of water, then the absorbed thiocyanate was eluted with 8.0ml of 1M sodium perenlorate ($NaClO_4$). 0.2ml of 0.5M acetic acid was added to 4.0ml of the eluate and mixed. 0.1ml of sodium hypochlorite (Phillip and Harris Ltd) diluted to 2 fold was added and mixed again within 1min. 0.5ml of barbituric acid pyridine reagent was added, mixed, and the absorbance was determined at 580nm, 5 to 15min later. Simultaneously a blank was determined where the sample was replaced with water. The absorbance of the blank should be 0.020. The thiocyanate concentration in the sample was read from the standard curve prepared with thiocyanate standards of known concentrations.

The technique of Lunquist et al. (1979) for the chromatographic separation of thiocyanate has previously been only applied to urine and plasma samples. For this reason, a recovery experiment was conducted to investigate if the thiocyanate present in the extracts from faeces or other animal tissues (which are sometimes highly coloured) could be quantitatively recovered.

A known amount of standard thiocyanate solution was added to homogenized faeces, GIT wall or contents from rabbit. Each was mixed thoroughly and centrifuged at 9000 x g for 5 minutes. The sediment was washed 3 times with 5ml portions of 0.1M NaOH and centrifuged as above.

The pooled supernatant of each tissue extract was applied to column and washed three times with 5ml portions of water. The adsorbed thiocyanate was eluted with sodium perchlorate and twelve 1ml fractions were collected in separate test tubes. Thiocyanate in each tube was estimated as already described.

CHAPTER 4

RESULTS

4.1 β-D-glucosidase production from fungal bacterial and cassava sources

Four fungal isolates, Aspergillus terreus, Aspergillus sydowi, Aspergillus carneus, Fusarium equseti and five bacterial isolates, Bacillus licheniformis, Bacillus subtilis, Bacillus polymyxa, Bacillus circulans and Bacillus coagulans were grown in sorghum extract liquid medium. β-D-glucosidase was tested for in the liquid growth medium after 18 days of growth. Extract was made from fresh cassava cortex and the same enzyme was tested for.

Results in tables 4.1 and 4.6 show that all the fungi isolates and cassava extract produced β-D-glucosidase while the bacterial isolates did not; Table 4.2 shows that the specific activity of the enzyme is highest in cassava extract followed by that from F. equseti, A. terreus, A. carneus and lastly A. sydowi.

4.2 Enzyme specificty using cassava linamarin and PNP glucosidase as substrates.

The ability of the β-D-glucosidase produced by the fungal isolates and that from cassava extract to hydrolyse cassava linamarin and PNP - glucoside was tested. Results in tables 4.3, 4.5 and 4.6 show that the enzyme from cassava was able to hydrolyse crude linamarin in cassava and PNP-glucoside while fungal enzyme was able to

Table 4.1 : The activity and protein content of concentrated extracellular β-D-glucosidase produced by fungi grown in sorghum extract liquid medium.

Source of Enzyme	Activity of Crude enzyme (units/ml)	Protein content (mg/ml) $X10^{-1}$
A. terreus	2.04	3.36
A. sydowi	0.67	6.88
A. carneus	0.44	0.84
F. equiseti	1.78	1.42
Cassava[a]	2.96[a]	0.72[a]

[a] Denotes activity and protein content of linamarase from cassava cortex.

Table 4.2 : Specific activity of crude concentrated extraceullular β-D-glucosidase from fungi grown in sorghum extract liquid medium.

Source of Enzyme	Specific activity (units/mg protein)
A. terreus	6.07
A. sydowi	0.97
A. carneus	5.24
F. equiseti	12.54
Cassava[a]	41.11[a]

a Denote specific activity of linamarase from cassava cortex.

Table 4.3 : Bound cyanide in fresh cassava tubers determined using enzyme from fungal and cassava sources

Source of enzyme	Enzyme activity (units/ml)	Bound cyanide (µg/gm tuber X10)
A. terreus	2.04	0.00
A. sydowi	0.67	0.00
A. carneus	0.44	0.00
F. equiseti	1.78	0.00
Cassava	2.96	4.77

Table 4.4 : Bound and total cyanide in fresh cassava tubers determined using different levels of cassava cortex linamarase.

Enzyme activity (units/ml)	Total cyanide (µg/gm tuber X10)	Bound cyanide (µg/gm tuber X10)
0.000	0.27*	0.00
0.592	2.91	2.64
1.480	4.73	4.46
2.960	4.95	4.68
5.920	4.45	4.18
14.800	3.68	3.41
29.600	3.68	3.41

*Free cyanide

Table 4.5 : Specificity of β-D-glucosidase from fungal and cassava sources using PNP-glucosidase and cassava linamarin as substrates

Enzyme source	PNP glucoside	Cassava linamarin
A. terreus	++	-
A. sydowi	+	-
A. carneus	+	-
F. equiseti	++	-
Cassava	++++	++++

Table 4.6 : β-D-glucosidase and linamarase production by fungal and bacterial isolates using PNP-glucoside and cassava linamarin as substrates.

Enzyme source	PNP glucoside	Cassava linamarin
A. terreus	++	-
A. sydowi	+	-
A. carneus	+	-
F. equiseti	++	-
B. licheniformis	-	-
B. subsilis	-	-
B. polymyxa	-	-
B. circulans	-	-
B. coagulans	-	-
Cassava[a]	++++	++++

[a]Denotes activity of enzyme from cassava cortex.

hydrolyse PNP-glucoside but not crude linamarin from cassava. Bacterial growth medium extract did not show any enzyme activity nor hydroltic ability on the two substrates. The enzyme activities shown on tables 4.1, 4.2, and 4.3 were determined using PNP glucoside as defined in section 3.15.

4.3 Cyanide determination and time of incubation for total cyanide hydrolysis in fresh cassava tubers using linamarase from cassava cortex.

Different levels of linamarase from cassava cortex were used to determine the enzyme concentration that will cause total cyanide hydrolysis and the time of incubation that will accomplish this. Results in table 4.4 and fig. 4.1 show that as the enzyme concentration increased, the hydrolysis of bound cyanide increased till a maximum of 3 units, above which there was a decline in cyanide hydrolysis.

The time of incubation in table 4.7 and fig. 4.2 show that hydrolysis of bound cyanide by linamarase increased with time till 10 minutes when it remained constant till the 30th minute. There was a decline after the 30th minute till the 40th minute and remained constant till the 50th minute.

4.4 The effect of processing on total cyanide content of cassava tubers.

Table 4.8 shows the cyanide content of processed and unprocessed cassava tubers used in the animal feeding experiments. The results show that the highest percentage cyanide detoxification of 98.11 was from grated

Table 4.7 : Incubation time for total hydrolysis of bound cyanide in fresh cassava extract.

Enzyme concentration : 2.96 units

Time (min)	Bound cyanide (µg/gm tuber)X10
5	3.82
10	4.68
20	4.68
30	4.68
40	4.41
50	4.41

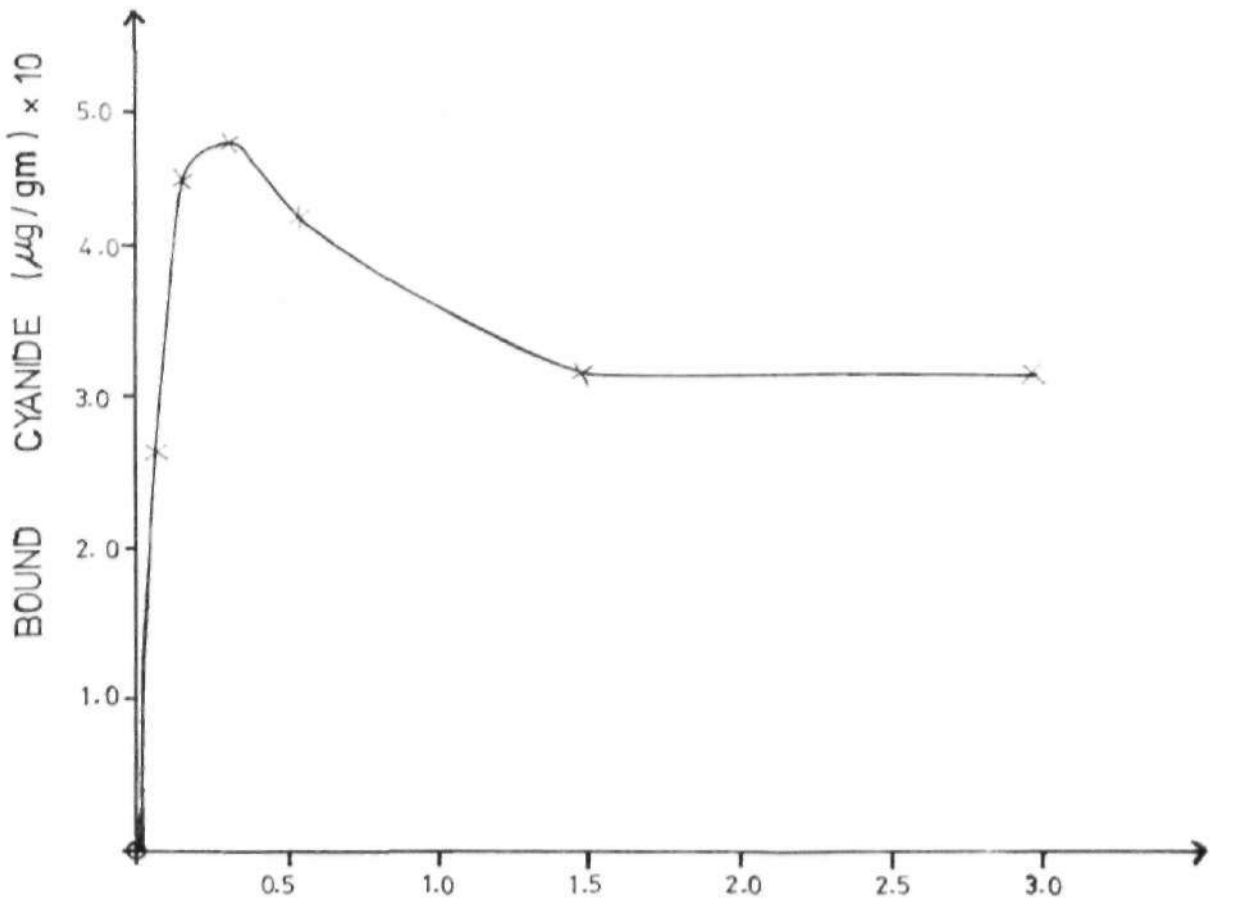


FIG 4.1 MAXIMUM ENZYME ACTIVITY FOR TOTAL HYDROLYSIS OF BOUND CYANIDE

Table 4.8 : Total cyanide content in processed and unprocessed cassava and the effect of processing on cyanide reduction.

Cassava sample	Total cyanide (µg/gm)	Percentage cyanide reduction
Fresh cassava (edible portion)	52.95	-
3 days sundried cassava chips	3.30	93.77
Linamarase treated grated cassava fermented for 5 days and then dried in the sun	4.30	91.88
Grated cassava fermented for 5 days and dried in the sun	3.95	92.54
Linamarase treated grated cassava fermented for 8 days and sundried	1.00	98.11
Grated cassava fermented for 8 days and sundried	3.30	93.77

cassava fermented for 8 days with the addition of exogenous linamarase followed by sundrying. This was followed by the 8-day fermented grated cassava-tuber without added exogenous enzyme which showed a 93.77% detoxification. This was the same value obtained from the 3 days sundried cassava chips (no fermentation). Grated cassava fermented for 5 days and sundried without exogenous enzyme has 92.54% cyanide detoxification while the 5 days fermentation with added exogenous linamrase followed by sundrying had 91.88% cyanide detoxification.

The pH profile of the fermenting pulp in table 4.9 showed a sharp decrease in pH from 6.1 to 4.3 within 48 hours of fermentation and a gradual increase to 4.8 and 5.0 in fermentation with and without exogenous enzyme respectively. Cassava chips were sundried on concrete floor for 3 days at a mean temperature range of 21 - 31°C.

4.5 Performance of rats fed various types of processed cassava-based diet with or without palm oil supplementation

Three cassava-based diets and a maize-based diet used as control were fed to male albino rats. Six rats were placed on each diet and each rat was kept in a seperate metabolic cage. Results in tables 4.10 and 4.11 show that rats placed on maize-based diet had better performance in terms of feed efficiency ratio and daily gain. This was closely followed by rats placed on

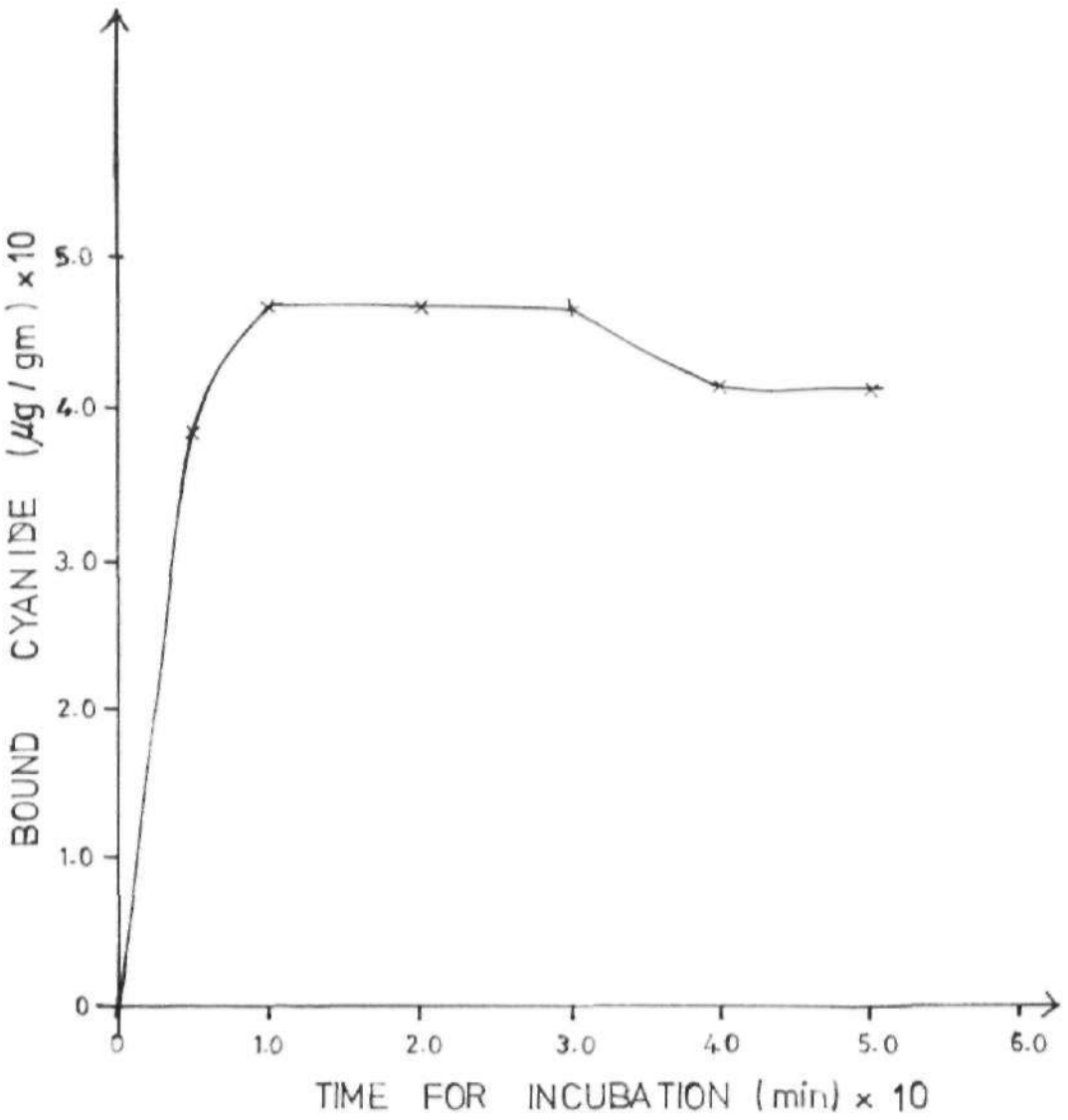


FIG 4.2 THE CURVE FOR TOTAL HYDROLYSIS OF BOUND CYANIDE

Table 4.9 : The pH profile of fermenting cassava pulp

Day	pH	
	Fermentation with enzyme	Fermentation without enzyme
0	6.1	6.1
2	4.3	4.3
4	4.4	4.4
6	4.6	4.7
8	4.8	5.0

Table 4.10 : Performance of Rats fed on Diets Containg Maize, Sundried, Palm oil Supplemented Sundried and Enzyme Processed Cassava.

Diet	Maize-based diet	Sundried cassava-based diet	Palm oil supplemented cassava-based diet	Enzyme processed cassava-based diet	
No. of animals	6	6	6	6	S.E
Average initial weight (g)	132.40	132.30	132.26	132.51	±0.06
Average final weight (g)	203.55	187.44	189.75	189.44	±3.71
Average weight gain (g)	71.15	55.12	56.93	57.49	±3.69
Average daily gain (g)	3.39	2.62	2.71	2.74	±0.18
Average feed intake (g)	285.85	248.90	243.02	235.72	±11.16
Average daily intake (g)	13.61	11.85	11.57	11.22	±0.54
Feed efficiency ratio	4.01[a]	4.52[a]	4.27[a]	4.10[a]	±0.11

Mean values with the same superscript show no significant difference.

Table 4.11 : Feed Efficiency Ratio of Rats Fed on Diets Containing Maize, Sundried, Palm oil Supplemented sundried and Enzyme Processed Cassava

Animal	Maize-based diet	Sundried cassava-based diet	Enzyme Processed sundried cassava-based diet	Palm oil supplemented sundried cassava-based diet
1	3.96	5.61	4.63	3.41
2	4.62	4.51	4.06	4.83
3	3.39	3.76	5.22	4.02
4	5.23	4.47	3.26	5.66
5	3.95	4.70	4.30	3.41
6	3.78	4.50	4.93	4.80
Mean±SE	4.14 ± 0.53^{a}	4.59 ± 0.30^{a}	4.36 ± 0.45^{a}	4.40 ± 0.35^{a}

Mean values with the same superscript show no significant difference.

enzyme processed cassava-based diet. The next was rats placed on palm-oil supplemented-cassava based diet while the least was rats placed on sundried cassava-based diet. The differences were however not statistically significant within any pair of treatment.

4.6 Thiocyanate and cyanide levels in the serum of rats fed various types of processed cassava-based diet with or without palm oil supplementation.

Results in table 4.12 show that the mean thiocyanate level in serum of rats fed on sundried cassava was the highest, followed by that in the serum of palm oil-supplemented cassava-based diet. The least thiocyanate levels were obtained from serum of rats placed on enzyme-processed cassava-based diet and maize-based diet which were almost the same value. The thiocyanate in serum of rats fed on sundried cassava based diet was significantly higher ($P<0.05$) than that in the serum of rats fed on the other three diets. The thiocyanate level in the serum of rats fed on palm oil-supplemented diet was significantly higher ($P<0.05$) than that in the serum of rats on enzyme processed cassava-based diet, and maize-based diet., whose values were not significantly different from one another. Free and bound cyanide were not detected in the serum of all groups.

Table 4.12 : Thiocyanate levels (µg/L) in serum of rats fed on diets containing maize, sundried, enzyme-processed and palm oil supplemented sundried cassava.

Animal	Maize-based diet	Sundried cassava-based diet	Palm-oil supplemented cassava-based diet	Enzyme-processed cassava-based diet
1	0.44	1.02	0.67	0.58
2	0.48	1.00	0.88	0.67
3	0.42	1.02	0.76	0.46
4	0.67	1.21	0.48	0.64
5	0.81	1.06	1.00	0.62
6	0.78	1.12	1.18	0.64
Mean±SE	0.6000±0.10[b]	1.072±0.04[a]	0.828±0.124[b]	0.602±0.04[b]

Means in the same row with different superscripts differ significantly P/0.05

4.7 Thiocyanate and cyanide levels in urine of rats fed various types of processed cassava diet with or without palm oil supplementation.

The mean urinary thiocyanate collected on weekly basis were summerized in table 4.13. The results show that the urine of rats fed on sundried cassava-based diet contained the highest level of thiocyanate. This was significantly higher ($P<0.05$) than the level in urine of rats on maize-based diet but not significantly higher than the levels in urine of rats on palm oil supplemented or enzyme processed cassava diets. Thiocyanate levels in urine of rats fed on sundried cassava diet supplemented with palm oil, was higher than those in urine of rats either on the enzyme processed cassava or maize diet, but the difference was statistically not significant. The urinary thiocyanate of rats on enzyme-processed cassava diet was higher than those of rats on maize-based diet but the difference was also not statistically significant. Free and bound cyanide were not detected in the urine of all groups.

4.8 Thiocyanate and cyanide levels in the gastro-intestinal contents and walls of rats fed various types of processed cassava-based diet with or without palm oil supplementation

The procedure used to assay thiocyanate in the faeces and parts of the gastrointestinal tract is that developed by Lundquist et al. (1979) for the assay

Table 4.13 : Thiocyanate levels (µg) in the urine of rats fed on diets containing maize, palm-oil supplemented sundried and enzyme processed cassava

Week	Maize-based diet	Sundried cassava-based diet	Palm oil supplemented cassava-based diet	Enzyme processed cassava-based diet
1	13.363 ± 4.10	22.82 ± 10.98	28.445 ± 6.17	14.020 ± 5.91
2	10.788 ± 3.45	33.26 ± 10.60	21.895 ± 4.38	19.935 ± 10.25
3	13.763 ± 6.25	15.31 ± 5.26	15.603 ± 5.91	20.49 ± 10.20
Mean±SE	12.638 ± 0.81 [b]	23.80 ± 4.51 [a]	21.98 ± 3.21 [a]	18.15 ± 1.80 [a]

Means in the same row with different superscripts differ significantly at P/0.05

of this substance in urine and serum. In this procedure, thiocyanate in serum or urine is purified by first absorbing it on an anion exchange resin and washing out impurities with distilled water and dilute Hcl solution before eluting with perchlorate. As this technique was only previously applied to urine and serum samples, it was necessary to check recovery when the method was applied to extracts from faeces and other tissues which were usually highly coloured. The result of the recovery experiment is shown in fig. 4.3. The thiocyanate was eluted in a volume of about 4ml with a mean recovery of 98.3%. This recovery is quite close to the mean value of 101% obtained by Lundquist et al. (1979) for serum samples.

The data in table 4.14 show that the total thiocyanate level in the gastrointestinal tract (stomach, small intestinal and large intestinal content and wall) of rats fed on sundried cassava-based diet was significantly higher ($P<0.05$) than that in rats fed on the other three diets. The thiocyanate level in the GIT of rats fed on sundried cassava diet supplemented with palm oil was significantly higher ($P<0.05$) than these in rats on maize-based diet but not significantly higher than that in rats on enzyme-processed cassava-based diet. The difference between the thiocyanate levels in rats on enzyme-processed cassava and maize-based diet were not statistically significant.

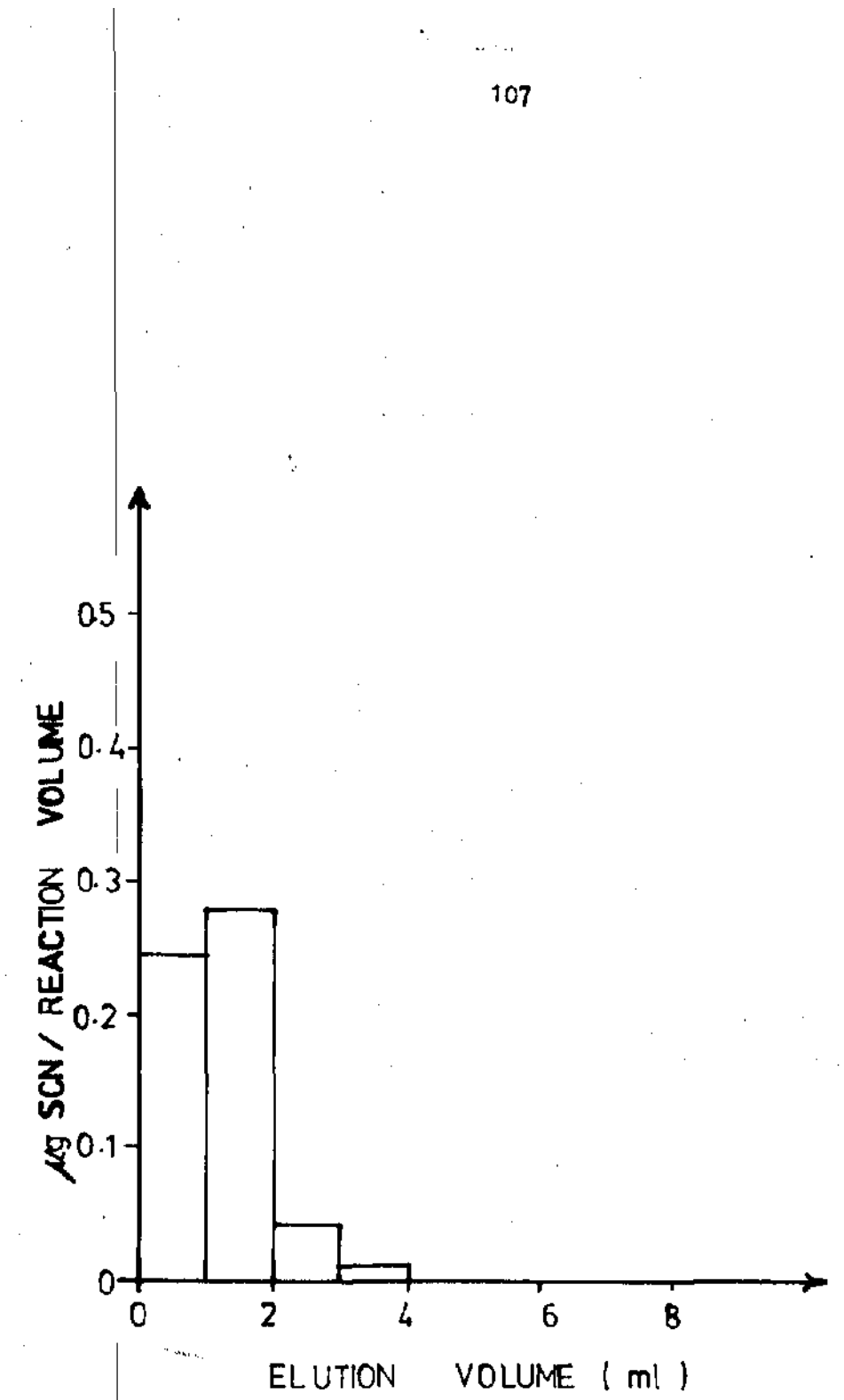


FIG. 4.3 ELUTION DIAGRAM OF THIOCYANATE IN FAECES

Table 4.14 : Thiocyanate levels (µg) in the gastrointestinal tract content and walls of rats fed on diets containing maize, sundried, enzyme processed and palm oil supplemented sundried cassava.

GIT Content and Wall	Maize-based diet *mean ± SE	Sundried cassava-based diet mean ± SE	Palm oil supplemented cassava-based diet mean ± SE	Enzyme-processed cassava-based diet mean ± SE
Stomach content	10.52 ± 2.95	15.19 ± 2.90	14.48 ± 1.69	9.79 ± 2.59
Stomach wall	5.19 ± 1.10	9.59 ± 2.27	5.84 ± 1.18	6.52 ± 1.24
Small intestinal content	1.100 ± 0.43	1.02 ± 0.36	1.18 ± 0.14	1.22 ± 0.55
Small intestinal wall	0.76 ± 0.47	0.72 ± 0.42	0.42 ± 0.15	0.99 ± 0.56
Large intestinal content	1.62 ± 0.42	2.02 ± 0.74	1.09 ± 0.49	1.98 ± 0.80
Large intestinal wall	0.52 ± 0.15	0.68 ± 0.25	0.14 ± 0.08	0.51 ± 0.15
Total SCN	19.61^{c}	29.22^{a}	23.15^{b}	21.01^{bc}

*Mean ±SE : Standard error of means

Means in the same row with different superscripts differ significantly at P/0.05

The thiocyanate levels (Tables 4.14 and 4.15) in the stomach content within each dietary group were significantly higher ($P<0.01$) than the levels in the stomach wall, small and large intestinal content and wall. The levels in stomach wall were significantly higher ($P<0.01$) than those in small and large intestinal content and wall. The thiocyanate levels in small intestinal content were higher than those in small intestinal wall but the difference was not statistically significant. The thiocyanate level in large intestinal content was higher than those in the small and large intestinal wall while the level in small intestinal content was slightly higher than that in large intestinal wall. These differences were however of no statistical significance.

The percentage of the total GIT thiocyanate present in the stomach was 80.11% for maize-based diet, 84.80% for sundried cassava-based diet, 87.78% for palm oil supplemented cassava-based diet and 77.63% for enzyme-processed cassava-based diet. The small intestinal content and wall contained 8.98%, 5.95%, 6.91% and 10.52% while large intestinal content and wall had 10.91%, 9.24%, 5.31% and 11.85% for the respective diets. Bound and free cyanide was however not detected in the gastro intestinal tract.

Table 4.15 : Percentage thiocyanate levels in Gastro intestinal tract content and walls of rats fed on diets containing maize, sundried, enzyme processed and palm oil supplemented sundried cassava.

GIT content and walls	Maize-based diet	Sundried cassava-based diet	Palm oil-supplemented cassava-based diet	Enzyme processed cassava-based diet
Stomach content	53.65	51.98	62.55	46.60
Stomach wall	26.47	32.82	25.23	31.03
Small intestinal content	5.10	3.49	5.10	5.81
Small intestinal wall	3.88	2.46	1.81	4.71
Large intestinal content	8.26	6.91	4.71	9.42
Large intestinal wall	2.65	2.33	0.60	2.43
Stomach content and wall	80.11	84.80	87.78	77.63
Small intestinal content and wall	8.98	5.95	6.91	10.52
Large intestinal content and wall	10.91	9.24	5 .31	11.85

4.9 The effect of cassava-based diet

on the quantity of cecotropes produced by rabbits during the day and night periods

4.9.1 Day-period collection

Results obtained from the five weeks faecal collection (once a week) were summarized in table 4.16. The mean weight of hard faeces excreted per rabbit per day-period was 38.60g for cassava-based diet and 38.81g for the maize-based group. The quantity of soft faeces collected during the day period showed that the mean soft faeces excreted per rabbit was higher for the maize-based group (11.32g) compared with the cassava-based group (9.59g). This difference was however not statistically significant.

Comparing the quantity of hard and soft faeces excreted during the day-period, the hard faeces were significantly higher ($P<0.01$) than the soft faeces in both dietary groups.

4.9.2 Night-period collection

Results obtainted for the faecal collections at night period (Table 4.17) show that all rabbits in the two dietary groups excreted both hard and soft faeces. The mean hard faeces excreted per rabbit per night period on maize-based diet (35.15g) was very close to that obtained from those on cassava-based diet (34.69g). The mean of the soft faeces collected during the night for maize-based diet (23.92g) was slightly lower than that from cassava-based diet (26.95g). These

Table 4.16 : The amount of hard and soft faeces of rabbits collected during the day (Day Collection)

Week	Animals fed on cassava-based diet		Animals fed on maize-based diet	
	Hard faeces (g) *Mean ± SE	Soft faeces (g) Mean ± SE	Hard faeces (g) Mean ± SE	Soft faeces (g) Mean ± SE
1	39.45 ± 5.53	9.46 ± 5.7	39.80 ± 8.33	12.18 ± 7.1
2	39.22 ± 3.94	7.81 ± 5.3	44.20 ± 7.26	13.88 ± 4.81
3	49.16 ± 7.27	5.68 ± 5.19	38.35 ± 3.74	9.95 ± 5.74
4	30.92 ± 4.52	11.71± 1.61	33.44 ± 4.75	10.65 ± 4.01
5	34.27 ± 2.04	13.29 ± 1.66	38.27 ± 6.11	9.93 ± 3.84
Mean±SE	38.60 ± 3.45 [a]	9.59 ± 1.51[b]	38.81 ± 1.93[a]	11.32 ± 0.85[b]

*Mean ± SE = Standard error of means for 4 animals per group. One collection per week.

Mean in the same row with different superscript differ significantly at P/0.05

Table 4.17 : The amount of hard and soft faeces of rabbits collected during the night (Night Collection)

Week	Animals fed on cassava-based diet		Animals fed on maize-based diets	
	Hard faeces (g) *Mean ± SE	Soft faeces (g) Mean ± SE	Hard faeces (g) Mean ± SE	Soft faeces (g) Mean ± SE
1	25.16 ± 1.46	18.86 ± 5.81	27.37 ± 8.19	25.43 ± 5.76
2	25.00 ± 10.91	27.70 ± 7.16	23.71 ± 8.52	33.57 ± 11.15
3	34.70 ± 9.04	45.47 ± 13.3	35.15 ± 7.61	20.03 ± 0.17
4	41.07 ± 2.67	20.51 ± 1.92	47.76 ± 3.09	20.82 ± 0.79
5	47.54 ± 2.98	22.19 ± 0.55	41.75 ± 3.01	19.76 ± 0.99
Mean±SE	34.69 ± 4.94[a]	26.95 ± 5.44[b]	35.15 ± 4.96[a]	23.92 ± 2.93[b]

*Mean ± SE = Standard error of means for 4 animals per group. One collection per week.

Means in the same row with different superscript differ significantly at P/0.01

differences were not statistically significant.

The quantity of hard faeces collected during the night period on each of the dietary group was significantly higher ($P<0.01$) than the quantity of soft faeces collected within the same period.

4.9.3 Comparison between the day and night period collections.

Results in tables 4.16 and 4.17 show that the mean quantity of hard faeces collected during the day period for cassava-based diet (38.81g) was close to the quantity of hard faeces collected during the night period (35.15g). Similarly the quantity of hard faeces collected during the day-period for maize-based diet (38.60g) was close to that collected during the night period (34.69g). Their differences were not statistically significant.

On the other hand, soft faeces collected during the night period on each dietary group was significantly higher ($P<0.01$) than those collected during the day period.

4.10 Protein content of hard and soft faeces of rabbits fed cassava and maize-based diets

4.10.1 Day and Night-period collections

The percentage crude protein from hard and soft faeces collected during the day and night-periods were shown in tables 4.18 and 4.19. The results show that the mean crude protein content of hard and soft faeces of rabbits fed on maize-based diet were slightly

Table 4.18 : Percentage protein in hard and soft faeces of rabbits collected during the day (Day Collection)

Week	Animals fed on cassava-based diet		Animals fed on maize-based diet	
	Hard faeces *Mean ± SE	Soft faeces Mean ± SE	Hard faeces Mean ± SE	Soft faeces Mean ± SE
1	12.87 ± 0.68	20.93 ± 7.07	12.18 ± 1.71	20.23 ± 6.77
2	13.54 ± 0.45	19.67 ± 6.68	11.15 ± 1.18	23.08 ± 7.46
3	13.75 ± 0.99	14.96 ± 8.64	11.92 ± 1.18	12.34 ± 7.23
4	10.82 ± 0.69	23.45 ± 1.03	10.62 ± 0.32	18.53 ± 6.42
5	11.93 ± 1.66	26.56 ± 1.09	10.92 ± 0.81	26.48 ± 0.78
Mean±SE	12.58 ± 0.61[a]	21.15 ± 2.16[b]	11.36 ± 0.33[a]	20.13 ± 2.65[b]

*Mean ± SE = Standard error of means for 4 animals per group. One collection per week.

Means in the same row with different superscript differs significantly at P/0.05

Table 4.19 : Percentage protein in hard and soft faedes of rabbits collected during the night (Night Collection)

Week	Animals fed on cassava-based diet		Animals fed on maize-based diet	
	Hard faeces *Mean ± SE	Soft faeces Mean ± SE	Hard faeces Mean ± SE	Soft faeces Mean ± SE
1	14.92 ± 0.57	23.48 ± 1.23	9.59 ± 2.55	24.23 ± 2.45
2	11.39 ± 1.23	27.08 ± 2.34	11.20 ± 1.50	24.06 ± 1.42
3	12.77 ± 1.47	24.52 ± 2.50	11.39 ± 0.82	26.20 ± 1.64
4	11.10 ± 1.29	26.09 ± 2.50	7.51 ± 0.88	26.65 ± 2.35
5	10.60 ± 1.00	23.25 ± 1.29	10.81 ± 1.88	22.53 ± 1.55
Mean ± SE	12.16 ± 0.87[a]	24.88 ± 0.83[b]	10.10 ± 0.80[a]	24.73 ± 0.84[b]

*Means ± SE = Standard error of means for 4 animals per treatment. One collection per week.

Means in the same row with different superscript differs significantly at P/0.05

higher than those on cassava-based diet. The differences were not statistically significant.

These results also show that there was no significant difference between the percentage crude protein content of hard and soft faeces collected during the day and those collected during the night on each dietary group. The percentage crude protein in soft faeces were significantly ($P<0.05$) higher than that in hard faeces in each dietary group.

4.11 Vitamin B-levels in hard and soft faeces excreted by rabbits placed on cassava and maize-based diets.

4.11.1 Day and night-period collections

Table 4.20 show the calculated levels of B-vitamins collected during the day in hard and soft faeces collected during the day from rabbits placed on cassava and maize-based diets. Niacin, riboflavin pantothenic acid and vit. B_{12} levels in hard and soft faeces of rabbits placed on maize-based diet were higher than those from cassava-based diet. The differences were not statistically significant. The results also show that, within each dietary group, the vitamin levels in soft faeces were significantly higher ($P<0.01$) than the levels in the hard faeces.

Results . in table 4.21 show that the levels of niacin, riboflavin, pantothenic acid and vitamin B_{12} in hard faeces of rabbits fed on maize-based diet

Table 4.20 : *B-vitamin levels in hard and soft faeces of rabbits collected during the day

Vitamin (µg) in total collection	Animals fed on cassava-based diet		Animals fed on maize-based diet	
	Hard faeces	Soft faeces	Hard faeces	Soft faeces
Niacin	1602.07	1411.35	1610.70	1666.01
Riboflavin	378.28	389.35	380.34	459.59
Pantothenic acid	386.00	610.88	388.10	721.08
Vit B_{12}	36.01	19.48	36.21	22.99

* Calculated values (adapted from Kulwich et al. 1953)

Table 4.21 : *B-vitamin levels in hard and soft faeces of rabbits collected during the Night (Night collection).

Vitamin (µg) in total collection	Animals fed on cassava-based diet		Animals fed on maize-based diet	
	Hard faeces	Soft faeces	Hard faeces	Soft faeces
Niacin	1439.79	3966.45	1458.15	3521.32
Riboflavin	340.00	1094.01	344.45	971.23
Pantothenic acid	346.94	1716.45	351.48	1523.83
Vitamin B_{12}	32.37	54.73	32.80	48.59

*Calculated values (Adapted from Kulwich et al, 1953)

collected during the night were slightly higher than those in cassava-based diet (night collection). The vitamin levels in soft faeces of rabbits fed on cassava-based diets were found to be higher than those on maize based diet during night collection.. The differences did not show any significant difference.

Comparing the day and night collections (Tables 4.20 and 4.21) the vitamin B levels in hard and soft faeces collected during the night-period were significantly higher ($P \leq 0.01$) than the levels in hard and soft faeces collected during the day-period within each dietary group. This must be as a result of greater amount of soft faeces collected at night.

Table 4.22 : Fat, dry matter and thiocyanate contents of hard and soft faeces from male rabbits

Constituents	DIET			
	Cassava		Maize	
	Hard faeces	Soft faeces	Hard faeces	Soft faeces
Fat* %	1.20	1.23	0.79	0.77
Dry matter* %	53.78	35.26	55.86	35.09
Thiocyanate (µg/g)**	0.98	ND	ND	ND

* Result on dry matter basis
** Result on wet weight basis

ND Not detected.

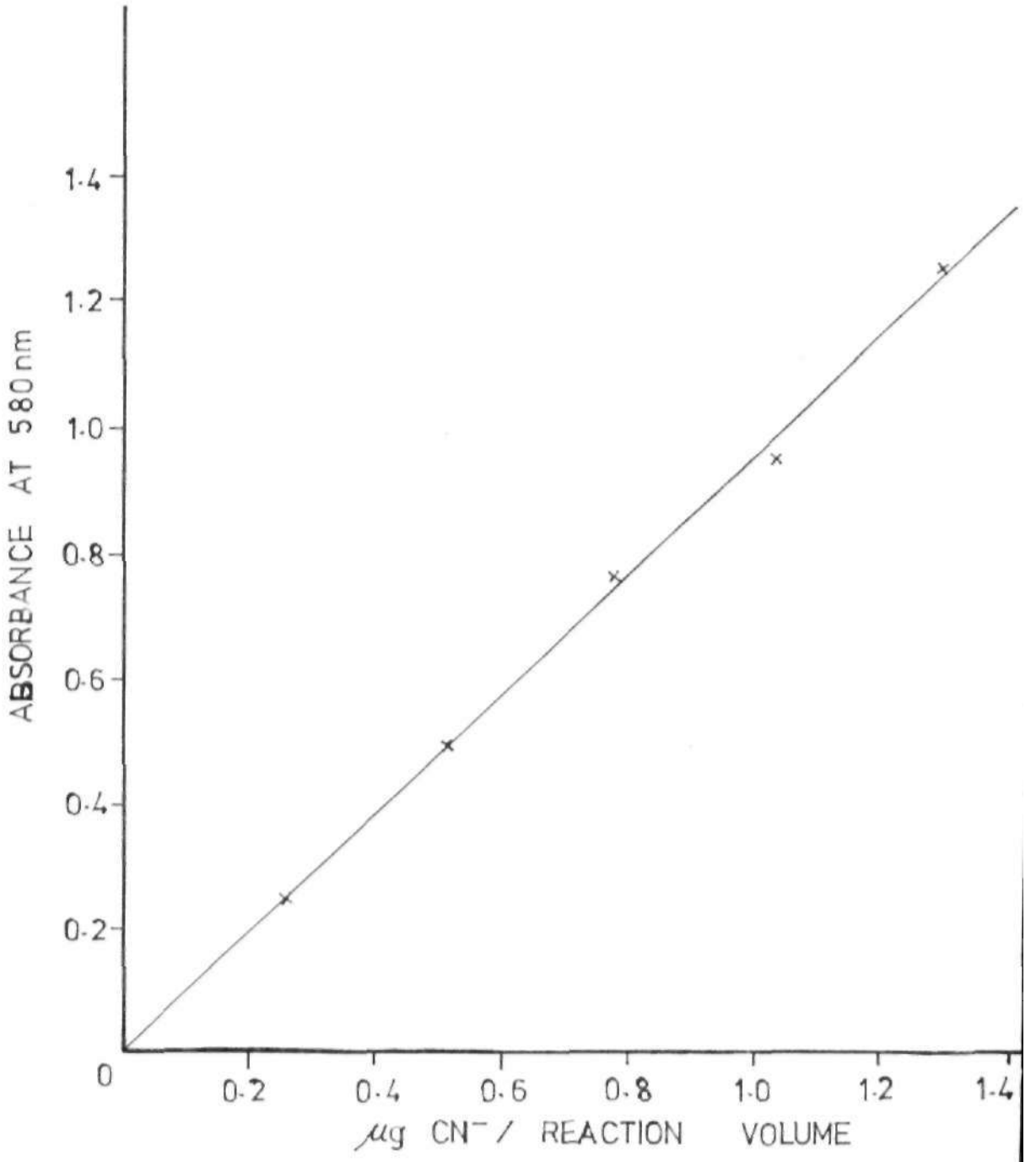


FIG. 4.4 STANDARD CURVE FOR CYANIDE ESTIMATION

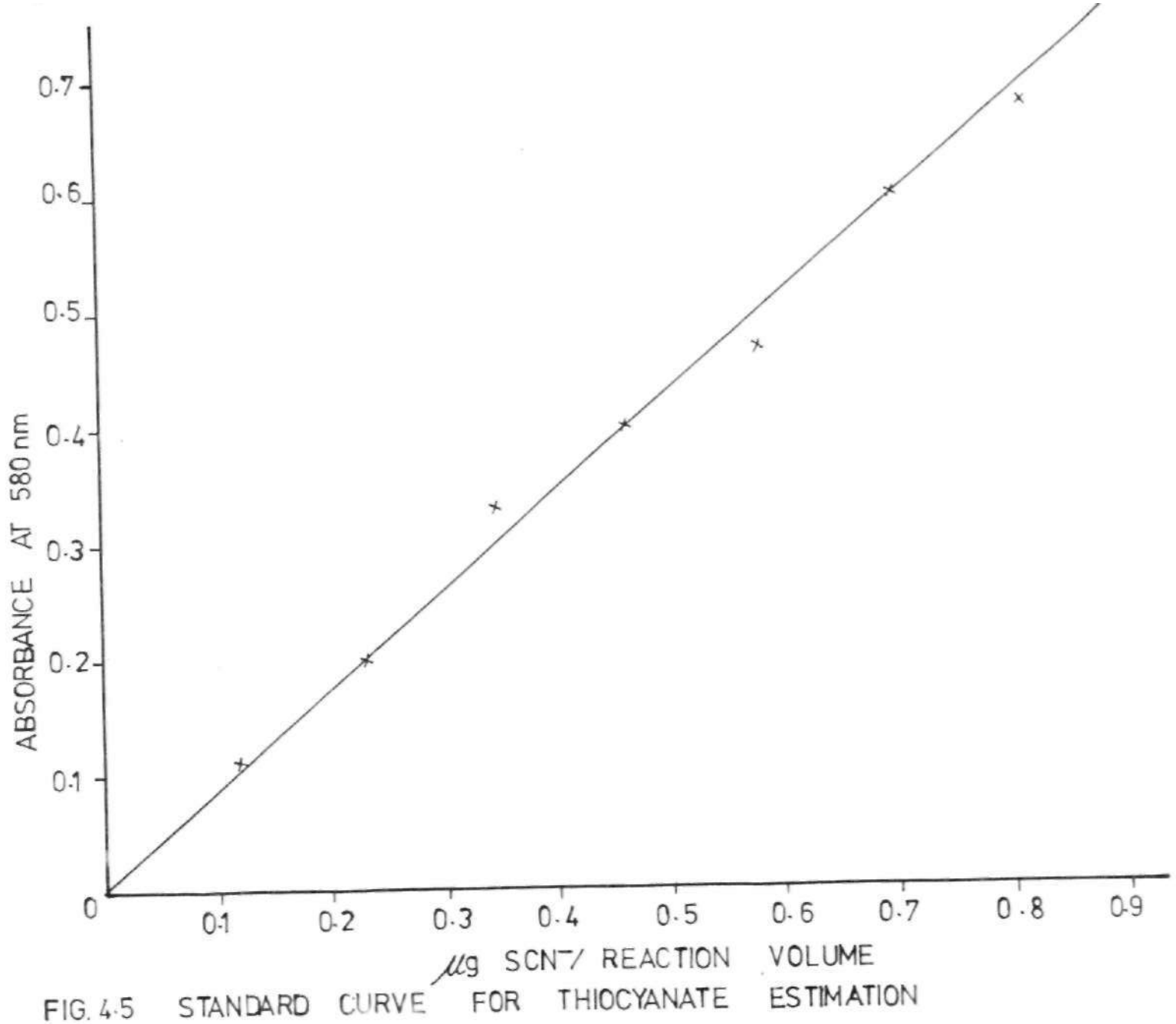


FIG. 4·5 STANDARD CURVE FOR THIOCYANATE ESTIMATION

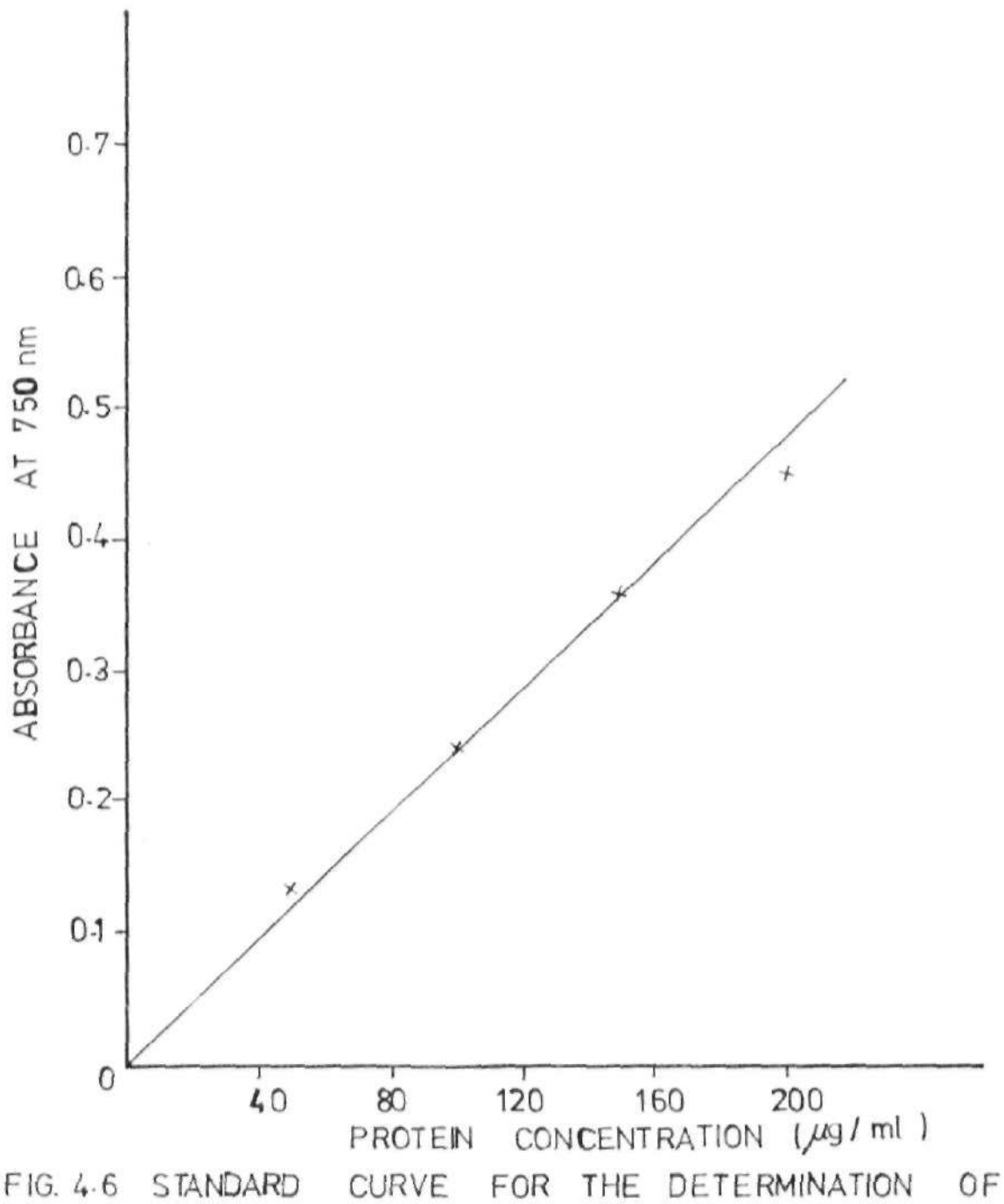


FIG. 4.6 STANDARD CURVE FOR THE DETERMINATION OF PROTEIN CONTENT

CHAPTER 5

DISCUSSION

5.1 Linamarase production and cassava processing

The four fungal isolates grown in sorghum extract liquid medium produced β-D-glucosidase which had earlier been regarded by some workers as linamarase (Onyike 1982; Ikediobi and Ogundu 1985; Ikediobi et al. 1987). Wiserman (1979) had earlier shown that these fungi among others were capable of amyloglucosidase production. The result shown in tables 4.1 and 4.2 indicate that this enzyme was able to hydrolyse PNP-glucoside; a synthetic analogue of linamarin, with the enzyme from *Fusarium oqluseti* showing the highest specific activity. This is in agreement with the work of Ikediobi and Ogundu (1985) who had shown that F. eqiuseti among other fungi, showed the highest specific activity for the enzyme.

Although the fungal enzyme was able to hydrolyse the PNP-glucoside, the results in table 4.5 show that it was unable to hydrolyse the crude natural linamarin in cassava. On the other hand, the enzyme from cassava cortex was able to hydrolyse both PNP-glucoside and crude linamarin in cassava while the bacterial enzyme did not produce the enzyme. It is however noted that Okafor and Ejiofor (1985) had found some bacterial isolates that could produce linamarase when linamarin was incorporated in its growth medium and the ability of some bacterial isolates to break down linamarin in fermenting cassava pulp (Ejiofor and Okafor, 1984); but the bacterial isolates used in this

work failed to produce linamarase with or without linamarin in the growth medium.

Although the work of Ikediobi et al. (1987) showed that the partially purified enzyme from F. equiseti had some activity on purified linamarin, the observation in this work has revealed an enzyme-substrate specificity whereby the crude fungal enzyme was able to show activity with the synthetic analogue of linamarin only, while the crude cassava enzyme showed activity with both the natural linamarin and its synthetic analogue, PNP glucoside. It thus seems reasonable to suggest that the fungal enzyme is not the true linamarase and could therefore not be of much benefit in cassava processing if the enzyme and linamarin must be in pure form to show any hydrolysis. The cassava enzyme could therefore be referred to as the true linamarase since it has high hydroltic activity on the crude cassava linamarin. It would be more realistic to use cassava linamarase to achieve total or partial hydrolysis of bound cyanide in cassava tubers though it has an economic disadvantage if it should be applied on industrial scale for cassava processing. The work of Ikediobi and Onyike (1982a) had earlier shown that the cassava linamarase could cause cyanide reduction when added exogenously in fermenting cassava pulp. This shows the ability of cassava linamarase to hydrolyse crude linamarin as obtained in this work.

To ascertin the concentration of the cassava linamarase that would cause a total hydrolysis of crude cassava linamarin and the duration of incubation, results in table 4.4 and fig 4.1

showed that 3 units of enzyme activity caused total hydrolysis of bound cyanide per gramme of cassava tuber. This result agrees with that obtained by Rao and Hahn (1981) which was also 3 units but lower than that reported by Ikediobi et al. (1980) which was 30 units. The work of Cooke (1978) showed that 0.25 - 0.3 units of exogenous linamarase could cause 95±2% linamarin recovery from cassava parenchyma, peel or leaves. The apparent differences in these results could be due to the sensitivity of the different assay methods used. Linamarin and other contaminants could affect cyanide availability (Cooke et al. 1978). Higher units of enzyme activity did not cause more hydrolysis rather there was a decline in the level of free cyanide in the medium available for assay (Table 4.4 and Fig 4.1). This characteristic behaviour agrees with the reports of Cooke, (1978) and Ikediobi et al. (1986), which suggest that linamarase or a contaminant of the enzyme could sequester some of the free cyanide in the reaction medium.

The duration of incubation required for total hydrolysis was found to be between 10-30 minutes beyond which there was a decline. This result agrees with the work of Cooke (1978) and Ikediobi et al. (1980), who used 15 and 10 minutes respectively. The decline is also attributable to cyanide sequestration by the enzyme.

The effect of processing on cyanide reduction in cassava tubers show that exogenous addition of linamarase from cassava source had a very high rate of detoxification in fermenting cassava pulp (Table 4.8). An addition of

1.40 units of linamarase per gramme of gratted fresh cassava tuber fermented for 8 days resulted in the highest detoxification rate of 98.11% compared to the rate of 93.77% obtained after 8-days of fermentation without exogenous enzyme addition and [illegible] by 3 days of sundrying of the cassava chips (all from the same source).

The use of exogenous linamarase to assist autolysis had been reported by some workers (Wood, 1966; Tewe, 1975; Ikediobi and Onyike, 1982a). Ikediobi and Onyike obtained 87.5% detoxification after 5 days of fermentation. This value is lower than the one obtained in this work (91.88%). The difference could be as a result of different methods of enzyme addition during fermentation and for the fact that they used higher units of enzyme activity which could cause cyanide sequestration. On the other hand, the higher percentage detoxification obtained in this work for the 8 days fermentation with exogenous enzyme addition was an indication that hydrolysis was not complete within 3-4 days used in traditional processing. The result obtained in this work compare favourably with that reported by El-Tinay et al. (1984) who obtained 83-91% detoxification in 8 days of fermentation without exogenous enzyme addition. The result obtained after 3-days of sundrying compares favourable with that reported by Maduagwu and Adewale (1980) on the loss of HCN and its derivatives during sun drying of cassava. They obtained a loss of about 85% of bound cyanide in 3 days using the low cyanide-containing cassava variety.

A close examination of the results in table 4.8 therefore indicates that despite the fact that enzyme treated grated cassava fermented for 8-days had the highest percentage cyanide reduction compared to the batch fermented for 8 days without enzyme addition and the cassava chips sundried for 3 days, the difference of 4.34% is insignificant to justify its use in industrial processing of cassava for animal feed due to labour, gratting and enzyme cost as well as long period of fermentation. It therefore seems more realistic to suggest that 3 days of sundrying at an average temperature range of 21-31°C would provide an effective and economical method for cassava processing for animal feed provided the chip sizes are small. This would provide a proper penetration of heat from the sun and allows the endogenous enzyme to act at its optimum rate (Ogundu 1984), for a long period of time before the chips dry up. This gives a better detoxification than oven drying at higher temperatures which no doubt removes water from the cassava chips in a shorter period of time but allows the enzyme little time to act on the cyanogenic glucoside. Earlier reports had shown that oven drying at higher temperatures caused lower percentage detoxification. (Nambisan and Sundaresan, 1985). The works of these authors also suggest that the most effective method for cyanide removal from cassava was by crushing fresh tubers and subsequently sundrying it whereby more than 95% of the cyanogenic glucoside would be removed.

The pH profile of the fermenting cassava pulp showed a sharp decrease from 6.1 to 4.2 within 2 days of fermentation with or without enzyme. This compares favourably with the reports of other workers who found similar drops in pH. Ikediobi and Onyike (1982a) reported a drop from 6.0 to 4.2 within 3 days of fermentation while El-Tinay et al (1984) reported a drop to 3.9 within 5 days. Such a drop it has been suggested would tend to limit the duration of endogenous linamarase activity (Ikediobi and Onyike, 1982a).

5.2 Performance of rats fed on cassava-based diets, Thiocyanate and Cyanide levels in their serum, urine and gastro intestinal tract.

The performance of rats in terms of feed efficiency and weight gain did not show any significant difference among the diets (Table 4.11). These results indicate that when cassava is processed to eliminate more than 93% of its cyanide, its utilization by animals would compare favourably with that of animals fed on maize-based well-balanced diet. This also shows that cassava-based diets with very low cyanide levels of 1 ppm will totally replace cereals in animal feed without any deleterious effects. The slight differences obtained in performance showed an inverse relationship with the cyanide content of the cassava incorporation into the diet. In other words the more the cyanide content of the cassava the less the feed efficiency and daily weight gain. With this therefore, the enzyme processed cassava that provided the best method of cyanide reduction showed the best performance compared to other cassava-based diets.

The palm oil-supplemented cassava-based diet which had the same cyanide level as the sundried-cassava-based diet showed a better performance than the latter. This agrees with the suggestion of Fomunyan et al. (1984) that palm oil could result in a better utilization of cassava meal by delaying the decomposition of acetone cyanohydrin; an intermediate metabolite in the hydrolytic release of cyanide. They also suggested the possibility of palm oil or its components modifying the enzyme systems that hydrolyse and metabolize linamarin most probably via glucoronides.

Although the differences in performance of rats did not show any significant difference among the diets due to very low levels of cyanide fed which resulted from the modes of detoxification, the trend of the result was in agreement with the findings of many workers that higher levels of cyanide in cassava-based diets would depress feed efficiency and the rate of growth (Oyenuga, 1961, Enriquez and Ross 1969, Pido and Adeyanju 1978). The reasons postulated for this reaction include the presence of the cyanogenic glucosides;

linamarin and lotaustralin which release hydrogen cyanide, a deadly poison, upon hydrolysis.

Free and bound cyanide (linamarin) were not detected in the faeces, gastrointestinal tract, serum and urine of these rats fed cassava-based diets containing linamarin in its natural form. Failure to find linamarin in faeces agrees with the observation of other workers (Hill, 1977; Barret et al. 1977; Umoh et al. 1986) who fed rats with pure synthetic linamarin. It could mean that the ingested linamarin was either completely absorbed intact or partially hydrolyzed and the products yet unidentified absorbed or excreted in the faeces (Hill, 1967; Barret et al. 1977). The above reasons could also be advanced for the inability to find linamarin or free cyanide in the gastrointestinal tract content and wall. The failure to detect linamarin and free cyanide in serum and urine could be that the linamarin was completely metaolized and the free cyanide converted to thiocyanate. This might be as a result of the fact that the level of linamarin ingested in the cassava-based diet was very low and over a long period of time (21 days).

Umoh et al. (1986) also did not detect linamarin in the blood of rats fed pure synthetic linamarin and therefore, suggested the possibility of quite diluted linamarin in the blood or its temporary binding with blood protein in a form not measurable by the analytical method used. These workers on the other hand detected linamarin in urine of both well and malnourised rats. This could be as a result of the massive dose of linamarin they used which was consumed as a single dose as against the low levels in cassava-based diet used in this work which was consumed over a long period of time (21 days). The work of Olugboji (1987) with dhurin showed that the small amount of this cyanogenic glycoside given to experimental rats as a single dose of malted sorghum was not completely hydrolyzed. There is therefore the possibility that the animals in this work improved their ability to hydrolyse linamarin under condition of chronic intake.

The detoxification of HCN by the enzyme rhodanese release thiocyanate, a goitrogen (Oke, 1978). Thiocyanate, the main detoxification product of cyanide in animals (Boxer and Rickards 1952) was found in serum, urine, stomach, small intestinal and large intesinal content and wall of rats fed on the diets. The thiocyanate obtained in any of the above tissues showed a linear relationship with the amount of cyanide consumed by the rats. The higher the level of cyanide the higher the thiocyanate recovered from the tissues in addition to the body's thiocyanate pool (tables 4.12 - 4.14). The thiocyanate level in serum was very small compared to the levels in urine which

compared favourably with the levels in the gastro intestinal content and wall with the latter being higher. These results show that urine was the major route of excretion of thiocyanate since there was none detected in the faeces (Umoh et al. 1986, Okoh and Pitt 1982).

The highest level of thiocyanate in the gastro intestinal tract was found in the stomach content which accounts for 46-63% of the total thiocyanate found in the gastro intestinal tract. The stomach wall accounted for 25-33%. The stomach content and wall collectively accounts for 77-88% while the small intestinal content and wall accounted for 5-11% and large intestinal content and wall, 5-12% of the thiocyanate in gastro intestinal tract. Generally the gastro-intestinal tract content contain more thiocyanate than the wall. This distribution pattern agrees with earlier findings both with acute and chronic administrations of radioactive cyanide to rats. Graley and Goddard (1977) showed that the distribution of thiocyanate was independent on the route of administration while Okoh and Pitt (1982) observed the highest uptake of radioactivity in gastro intestinal tract especially in the stomach. These workers suggested that since the radioactivity was largely associated with the contents, it was very likely that the initial concentration of this activity occured in the stomach and that the activity in the lower region was due to the movement of the stomach content down the gut.

It has long been known (Logothetopoulos and Myant 1956) that thiocyanate is concentrated into the gastric juice of animals. It is also well known that thiocyanate is the main detoxification product of cyanide in animals (Boxer and Rickard 1952) and that it is largely excreted in the urine (Boxer and Rickard 1952). The thiocyanate excreted into the stomach must be reabsorbed in the lower part of the gut into the body fluid to be partly excreted in the urine and partly resecroted in the gastric juice. It has therefore been ascertained that in the rats, there is a gastro intestinal circulation of thiocyanate linked with the body thiocyanate pool, (Okoh and Pitt 1982).

Thus the presence of high amount of thiocyanate in the stomach contents of rats on cassava based diet which is not excreted in the faeces confirm that the ingestion of cyanide in the bound form as cyanogenic glucoside does not affect gastro intestinal circulation of thiocyanate in animal. On the basis of this finding, it is concluded that the reported gastro intestinal circulation of thiocyanate (Okoh and Pitt, 1982) is an important feature in the metabolic elimination of this major detoxification product of cyanide and this is illustrated in fig. 5.1.

Ingested free cyanide or that arising from the in vivo hydrolysis of ingested bound cyanide is mainly converted to thiocyanate in the animal tissue, leading to a substantial elevation of the level of this radical in the plasma. The occurrence of acute toxicity (commonly leading to death) depends on the level of free cyanide ingested and on the rate

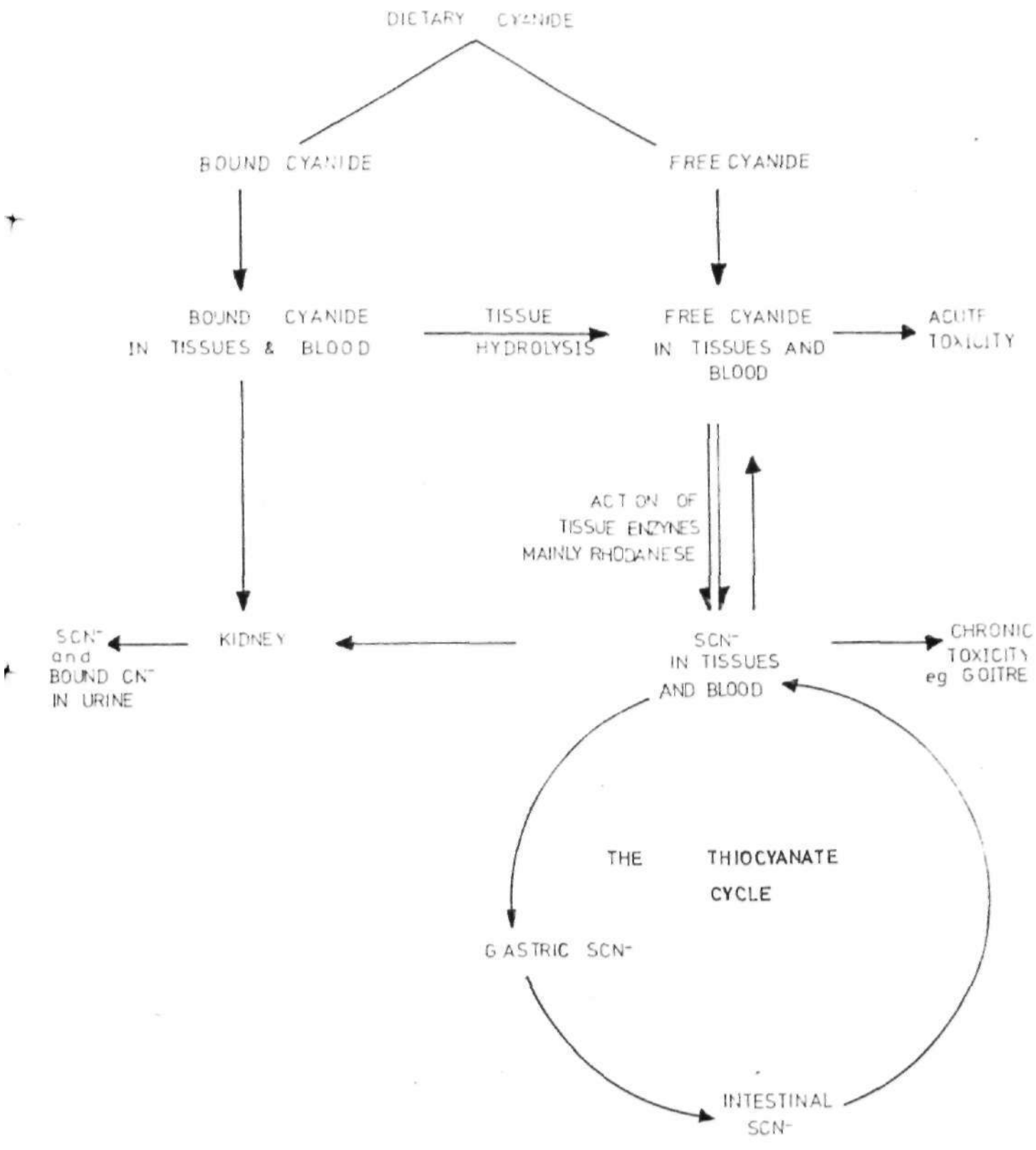


FIG 51 THE METABOLIC FATE OF FREE AND BOUND CYANIDE IN THE RAT.

OKON, P. N. (1988) TO BE PUBLISHED

of hydrolysis of bound cyanide in vivo. With a nonlethal dose of cyanide, respiratory arrest does not occur and the animal is able to form relatively large amounts of thiocyanate by the action of rhodanese in tissues, mainly liver and kidney. Thiocyanate itself is toxic at high concentration but it is secreted in large amounts into the stomach contents. The thiocyanate in the stomach is carried down the lower part of the gut along with gastric contents. In the intestine, the thiocyanate is largely reabsorbed into the body fluid to be partly excreted in the urine and partly resecreted into the gastric contents. The biological importance of this gastro intestinal circulation of thiocyanate in the rat is not clear. It is tempting however, to speculate that animals may have evolved the gastro intestinal circulation of thiocyanate as a means of removing this radical from tissues where it might be more toxic, while allowing time for its elimination in the urine. However, the possibility exists that this gastro intestinal circulation of thiocyanate may have a physiological role in the animal especially as it is known to inhibit gastric HCL secretion.

A number of workers (Boyland and Walker, 1974; Ruddell et al. 1977) have shown that thiocyanate was present in stomach of humans, and that large amounts are found in smokers compared to non smokers. Ruddell et al. (1977) concluded that the thiocyanate in the gastric juice was exogenous and probably salivary in origin. As Logothetopoulos and Myant (1956) found that other species besides the rat can secrete thiocyanate into the gastric juice, it seems probably that part of the thiocyanate found in the

stomach of humans may be due to gastro intestinal circulation of this radical.

The apparent danger in the high thiocyanate content in the stomach would be the possibility of nitrosamine formation in the stomach. Nitrosamines have been shown to be carcinogenic in a number of laboratory animals (Mayes and Barnes 1956 , 1967). Their formation is catalyzed by several anions including thiocyanate (Ridd 1961). Since secondary amines are present in a number of food sources (Lijinsky and Epstein 1970), there could be a possibility of nitrosamine formation *in vivo* particularly in the stomach and hence give rise to tumours in individuals not apparently exposed to nitrosamines *per se* (Sander *et al.* 1968; Ruddell *et al.* 1977). The thiocyanate in the stomach could act as a catalyst for the formation of carcinogenic nitrosamines from amines and nitrite. To avoid this incidence, a partial or total cyanide-free-diet should be fed to animals after appropriate detoxification methods as shown in this work.

5.3 Effect of cassava-based diet and day period on the quantity and quality of rabbit cecotropes.

Results in tables 4.16 and 4.17 show that rabbits produce cecotropes both at night and day, contrary to the earlier assumption (Thompson and Worden 1956) that rabbits produce cecotropes only at night and hence was termed night faeces. This agrees with the finding of Harris (1983) who showed that rabbits produce cecotropes both at night and day. The quantity of cecotropes collected at night-period was significantly ($P<0.01$) greater than the quantity produced at day-periods. This situation

was brought about by failure of some rabbits to produce soft faeces on some day-periods, while all rabbits produced soft faeces on all night-periods.

The result of the experiment indicates that a well balanced diet containing up to 22.75% cassava meal can replace a balanced diet containing 22.25% maize without affecting the quantity of cecotropes produced by rabbits. Further more, the chemical composition of the soft faeces is not adversely affected by the feeding of cassava diet.

In the two groups of rabbits, the percentage crude protein content of both hard and soft faeces were not affected by the diet. The protein content in the soft faeces was generally higher than that in the hard faeces. Day or night period did not affect the protein content of either the hard or soft faeces. This compares favourably with the work of Fekete and Bokori (1984) who obtained 15.4% in hard faeces and 25.7% in soft faeces.

The calculated amount of vitamin B-levels (adapted from Kulwich et al. 1953) based on the total amount of soft or hard faeces excreted, show that the amount of each B-vitamin in soft faeces which the rabbit consumes back is very much higher than their nutritional requirement (Lebas 1980). The excess from the consumed soft faeces which the body was unable to absorb from the small intestine could be responsible for the quanity of these vitamins obtained in the hard faeces. This high level of B-vitamins in soft faeces results from the fermentation in the cecum and large intestine and is responsible for the practice

of coprophagy by the rabbits to ensure that the animals are supplied with the necessary amounts of most B-vitamins to meet the body requirement. The ingestion of the soft faeces is necessary to ensure that their B-vitamin content were absorbed in the small intestine since their site of production was post - absorptive. This possibly provides insurance against an essential amino acid deficiency and permits further digestion of fibre and other nutrients by the second passage through the digestive tract (Thompson and Worden, 1956). This efficiency of the rabbit accounts for the ability of this animal to thrive on poorer quality diets such as cassava compared to poultry and swine.

The percentage dry matter of the hard faeces was consistently higher than the percentage dry matter of the soft faeces. This agrees with the findings of Fekete and Bokori (1984) who earlier gave the percentage dry matter of hard and soft faeces as 52.7 and 38.6 respectively.

Fekete and Bokori (1984) also showed that soft faeces contained higher amount of ether extract compared with the hard faeces. In this study, there was no remarkable difference in the ether extract contents of both hard and soft faeces in the two dietary groups. However, the rabbits on cassava-based diet excreted higher amount of fat in both the dry and soft faeces compared with the maize group.

The presence of low level of thiocyanate in hard faeces of the rabbits fed cassava-based diet must be as a result of contermination by urine which is probably passed at the

sometime as the hard faeces is being expelled. This is supported by the fact that the thiocyanate normmly secreted into the stomach of animals ingesting cyanide is largely reabsorbed in the distal parts of the gut resulting in the excretion of either none or trace amounts in the faeces (Okoh and Pitt, 1982;

Failure to detect thiocyanate in the soft faeces might mean that the rabbit is conscious of the fact that it has to ingest its own soft faeces and hence fails to pass urine anytime it is being expelled or deliberate direction of urine away from soft feaces.

CONCLUSIONS

The inability of the β-D-glucosidase from fungal source to hydrolyze linamarin in cassava tuber while similar enzyme from cassava cortex hydrolyzed the linamarin showed that the source of the enzyme is of great importance in achieving partial or total hydrolysis of linamarin which in this work has shown enzyme-substrate specificity.

When cassava (low cyanide variety) was processed to achieve 93% or more cyanide detoxification, the performance of rats fed on this cassava-based diet (well balanced) compared favourably with maize-based diet. Though the addition of exogenous linamarase to the fermenting pulp provided the best method of cyanide detoxification than 3-day sundrying, the difference was insignificant to justify the use of exogenous linamarase for cassava processing due to cost of the enzyme production, labour of grating and the long period involved in fermentation. Sundrying cassava for 3 days under appropriate temperature range would therefore provide the best method of cyanide detoxification in cassava for animal feed.

Though rats fed palm oil supplemented cassava-based diet showed better performance over those fed cassava-based diet without palm oil supplementation, the difference was insignificant to justify the use of palm oil since the processed cassava already contains very low levels of cyanogenic glucoside.

Furthermore, the inability to detect free and bound cyanide in serum, urine, faeces and gastrointestinal tract showed that the rats under chronic intake of low levels of linamarin in processed cassava would develop the ability to totally hydrolyze

this cyanogenic glucoside and the free cyanide so produced was converted to thiocyanate, a less toxic substance. The thiocyanate is partly excreted in the urine and partly undergoes a gastro intestinal circulation with the bodys thiocyanate pool to allow its gradual elimination in urine.

Feeding sundried cassava chips to rabbits did not affect both the quantity and some quality parameters of the cecotropes produced when compared to a maize-based diet and could therefore replace cereal in the feeding of rabbits without any deleterious effects.

APPENDCIES

APPENDIX 1 : CALCULATION OF LINAMARASE ACTIVITY

Appendix 1.1. Using P-nitrophenyl β-D glucoside as substrate

A = ECL

where A = Absorbance at 400nm

C = Concentration

L = Path length of cuvette

E = Molar extinction

$$\text{Enzyme unit} = \frac{A \times V \times 10^6}{E \times t \times 1000} \text{ micromoles/min}$$

where A = Absorbance at 400nm

V = Reaction volume = 4ml

t = Incubation time = 60 mins

E = Molar extinction coefficient = 1.8×10^4 Mol^{-1} cm^{-1}

1 unit of linamarase activity is defined as that which catalyze the formation of 1 micromole of P-nitrophenolate ion from p-nitrophenyl β-D-glucoside per minute under the described assay condition (Appendix fig. 1)

Total Activity = Total Volume x Activity/ml

$$\text{Specific Activity} = \frac{\text{Activity (units/ml)}}{\text{Protein content (mg/ml)}}$$

Appendix 1.2 : Using Linamarine as substrate

Micromoles of CN^- produced/min

$$= \frac{x}{t \times 26}$$

where x = Micrograme CN^- per reaction mixture from the standard curve.

t = Incubation time = 20min and molecular weight of CN^- = 26

1 unit of linamarase activity is defined as that which will catalyze the breakdown of 1 micromole of linamarin per minute under the described assay conditions.

This unit of enzyme activity can either be expressed in terms of the rate of product formation or that of substrate degradation.

Appendix table 1 : Standard curve for cyanide estimation

Cyanide concentration ($\mu g\ CN^-$/reaction mixture)	Absorvance at 580nm
0.26	0.24
0.52	0.49
0.78	0.76
1.04	0.95
1.30	1.25

Appendix table 2: Standard curve for thiocyanate estimation

Thiocyanate concentration (µg/SCN^-/Reaction mixture)	Absorbance at 580 nm
0.116	0.11
0.232	0.20
0.348	0.33
0.464	0.40
0.580	0.47
0.696	0.60
0.812	0.68

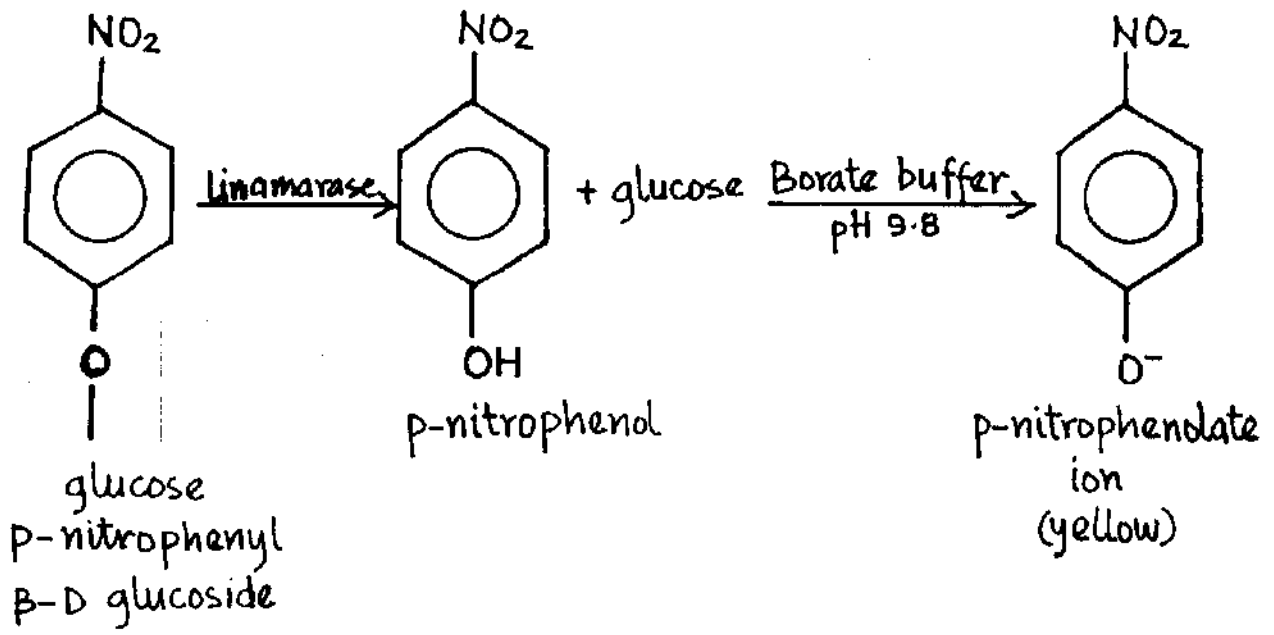
Appendix Table 3 : Nutrient requirements of Rabbits

Nutrient	Unit	Class of Rabbit			
		Growing 4–12 Weeks	Lactation	Gestation	Maintenance
Crude protein	%	15	18	18	13
Amino acids					
Sulfur	%	0.5	0.6	-	-
Lysine	%	0.6	0.75	-	-
Arginine	%	0.9	0.8	-	-
Threonine	%	0.55	0.7	-	-
Tryptophan	%	0.18	0.22	-	-
Histidine	%	0.35	0.43	-	-
Isoleucine	%	0.60	0.70	-	-
Valine	%	0.70	0.85	-	-
Leucine	%	1.05	1.25	-	-
Phenylalanine	%	1.20	1.40	-	-
Crude fiber	%	14	12	14	15–16
Indigestible fiber	%	12	10	12	13
Digestible energy	Kcal/kg	2500	2700	2500	2500
Metabolizable energy	Kcal/kg	2400	2600	2400	2120
Fat	%	3	5	3	3
Minerals					
Calcium	%	0.5	1.1	0.8	0.6
Phosphorus	%	0.3	0.8	0.5	0.4
Potassium	%	0.8	0.9	0.9	-
Sodium	%	0.4	0.4	0.4	-
Chlorine	%	0.4	0.4	0.4	-
Magnesium	%	0.03	0.04	0.04	-
Sulfur	%	0.04	-	-	-
Cobalt	ppm	1	1	-	-
Copper	ppm	5	5	-	-
Zinc	ppm	50	70	70	-
Iron	ppm	50	50	50	50
Manganese	ppm	8.5	2.5	2.5	2.5
Iodine	ppm	0.2	0.2	0.2	0.2
Vitamins					
Vitamin A	IU/kg	6000	12,000	12,000	-
Carotene	ppm	0.83	0.83	0.83	-
Vitamin D	IU/kg	900	900	900	-
Vitamin E	ppm	50	50	50	50
Vitamin K	ppm	0	2	2	0
Vitamin C	ppm	0	0	0	0
Thiamine	ppm	2	-	0	0
Riboflavin	ppm	6	-	0	0
Pyridoxine	ppm	40	-	0	0
Vitamin B_{12}	ppm	0.01	0	0	0
Folic acid	ppm	1	-	0	0
Pantothenic acid	ppm	20	-	0	0

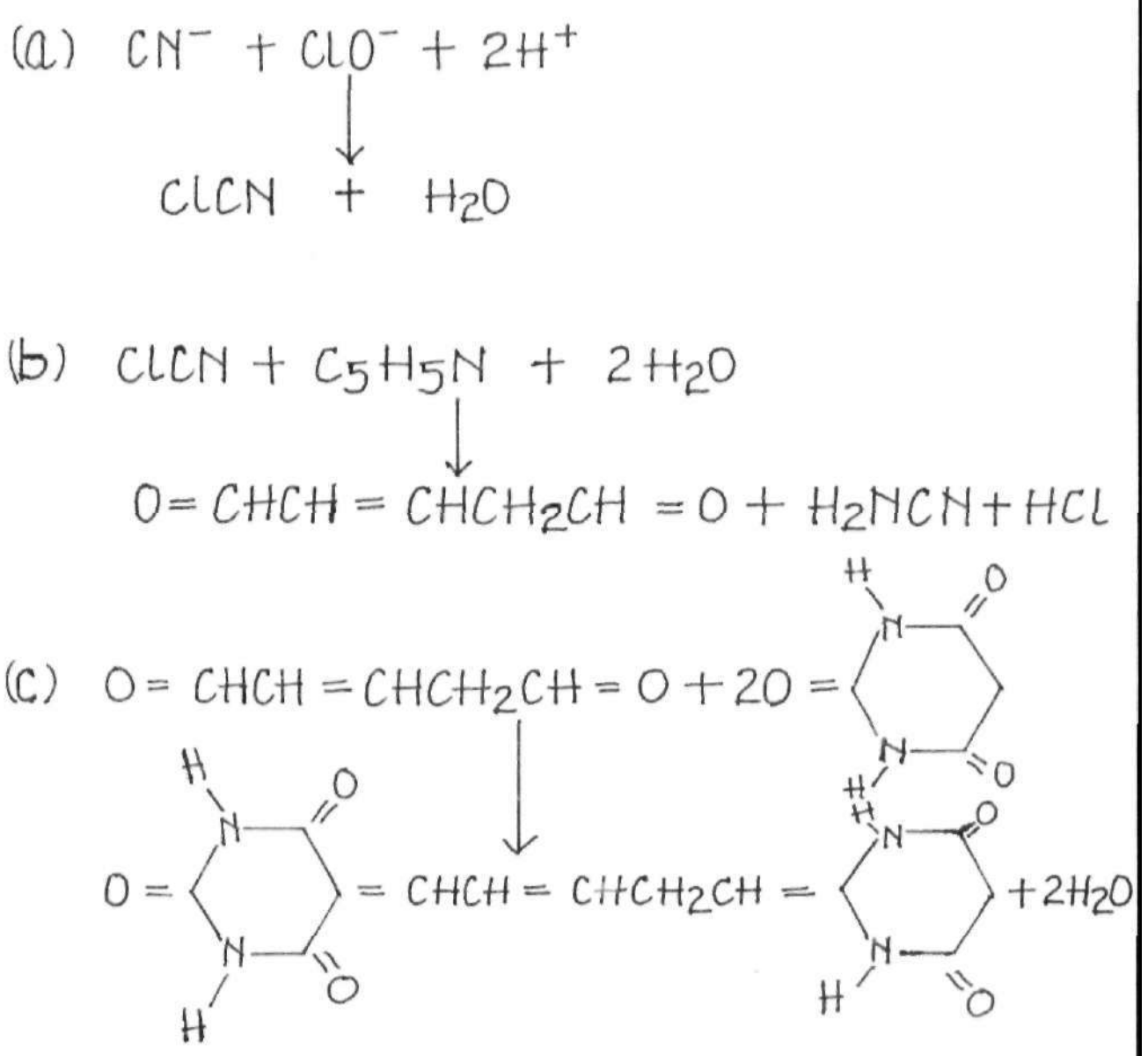
Source: Lebas, F. The journal of Applied Rabbit Research, Vol. 3 No. 2 (1980), P. 15.

Appendix Fig. 1

Enzymatic hydrolysis of p-nitrophenyl β-D glucoside



Appendix fig. 2: The Konig reaction for determination of cyanide.



Cyanide is first converted to cyanogen chloride by hypochlorite(a). Cyanogen chloride then reacts with pyridine to produce glutaconic aldehyde(b), which finally is coupled with barbituric acid yield to a chromogen (c). (Lundquist et al. 1985).

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