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Neurological disorders have been reported from parts of Africa with protein-deficient populations and attributed to prolonged dietary cyanide (CN⁻) exposure from cassava consumption. Cyanide is principally detoxified to thiocyanate (SCN⁻), a moiety lacking neurotoxic properties. However, in protein-malnourished subjects, in whom sulfur amino acids (SAA) are deficient, a minor detoxication pathway from CN⁻ to the neurotoxic moiety cyanate (OCN⁻) might be favored. This hypothesis is investigated in relation to: (i) the metabolism of sulfur, (ii) the metabolism of CN⁻ to OCN⁻ and (iii) the effect of OCN⁻ on glutathione (GSH) in rats maintained on either a balanced diet (BD) or a SAA-free diet. In both groups, there was a time-dependent increase in plasma cyanate, with a marked increase in SAA-deficient animals. A positive linear relationship between blood cyanide and plasma cyanate was observed in SAA-free animals but not in animals on BD. Urinary SCN⁻ levels increased approximately ten-fold over baseline values in both groups. Urinary inorganic sulfate in SAA-deficient animals fell close to zero while levels increased in BD animals. To elucidate the mechanism of OCN⁻ neurotoxicity, the effect of sodium cyanate (NaOCN) on GSH, a tripeptide involved with detoxification of xenobiotics, was studied in rat brain in vitro and in vivo. NaOCN reduced GSH levels and inhibited glutathione reductase activity in a dose- and concentration-dependent manner, respectively. The ratio of reduced to oxidized glutathione was selectively decreased in the striatum of animals dosed with NaOCN. Taken together, rats maintained on a SAA-free diet may model the
pathophysiology of protein-malnourished individuals. Under these conditions, cyanide may be preferentially converted to cyanate, an established cause of neurological disorders in humans and animals.
Cyanide Metabolism in Sulfur Amino Acid Deficiency: Relevance to Cassava-Related Neurodegenerative Diseases

by
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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

John Tor-Agbidye
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Dedicated To The Memory of My Father
Polycarp Tor-Agbidye and Brother Samson Tor-Agbidye
CHAPTER 1: GENERAL INTRODUCTION

The occurrence of neurodegenerative diseases poses major social and medical problems throughout the world. In sub-Saharan Africa, neurological disorders are an important cause of morbidity and mortality. While some neurological diseases in Africa are similar to those in the West, there are some striking differences which are due to special environmental, genetic, and socioeconomic factors (Cock, 1985; Rosling, 1996). Neurological syndromes, dominated by signs of myelopathy, have been reported from many populations in tropical countries (Roman et al., 1985). Most of these syndromes consist of various combinations of peripheral polyneuropathy and signs of spinal cord involvement. Roman et al. (1985) used the term “tropical myeloneuropathies” to group these disorders of unknown etiology and distinguished two clinical groups: tropical ataxic neuropathy (TAN), with prominent sensory ataxia, and tropical spastic paraparesis, or konzo with predominantly spastic paraplegia and minimal sensory deficit.

This Doctoral research thesis describes biochemical studies aimed at illuminating the mechanisms of two types of neurodegenerative diseases known as konzo and TAN. Konzo is a condition of growing importance among socio-economically deprived citizens of several central African countries. Konzo is the local name given to an irreversible spastic disease found in epidemic proportions among communities dependent on cassava (Manihot esculenta). Cassava roots diet is poor in protein and rich in carbohydrates, but unfortunately also contains neurotoxic principles if the roots of bitter cassava is not processed adequately. TAN is a neurological syndrome dominated by loss of sensation in the lower limbs and optic atrophy and was first reported in Nigeria.

While there is no animal model of cassava-induced spasticity, and there are a number of food-related and viral condition that resemble the clinical features of konzo, there nevertheless is strong circumstantial evidence from Mozambique, Central African Republic (CAR) and Democratic Republic of Congo, DRC (Zaire) of a cause and effect relationship between dietary dependence on cassava and neurodegenerative disease. The present study addresses the effects of protein malnourishment and the neurotoxic principle in cassava, namely cyanide-releasing moieties.

Given the remarkable clinical similarities between cassava-related neurological diseases and other food-and viral-induced disorders, there may exist a relationship in the pathogenesis of neuronal/axonal degeneration. For this reason, the thesis begins with a
discussion of the spastic neurodegenerative disorders associated with over-consumption of the grass pea (*Lathrus sativus*) and exposure to human T-Cell Lymphotrophic Virus I and II (HTLV-I, II) before discussing the neurological conditions associated with cassava dependency.

**LATHYRISM**

Lathyism is a degenerative disorder of the central motor pathway caused by excessive consumption of the grass pea or the seeds of other potentially neurotoxic *Lathyrus* species (Spencer, 1989). These legumes typically make up a component of the diet of poor people in regions endemic for the disease, but cases of lathyism usually appear when the adverse environmental conditions result in increased dependency on the grass pea for food. Epidemics of lathyism commonly follow periods of extreme weather, resulting in flood or drought, or disrupted social conditions such as war, pestilence, or famine.

Ancient and Recent History

Lathyism was known to ancient Hindus, and to Hippocrates (460-377 BC). An Italian, Cantani (1873), coined the name ‘lathyrismus’ to describe the disease. During the 18th, 19th, and 20th centuries, outbreaks of lathyism occurred throughout Europe, Northern Africa, the Middle East, Afghanistan, Russia, and India (Barrow et al., 1974). Thus, over the ages, lathyism has affected human or animal populations on at least three continents. Human cases still occur in parts of India, China, Africa (Ethiopia and Eritrea), and subjects with long standing lathyism live in parts of Europe (Hugon et al., 1993). During World War II, a group of Rumanian Jews developed lathyism in a German hard-labor camp in the Ukraine; these subjects currently reside in Israel (Cohn and Streifler, 1981a). Cases of lathyism were also identified among Greeks (Paissos and Demopoulos, 1962) following the Second World War, and it would be no surprise if, in the 1990s, cases occur among residents of the former Yugoslavia.

Human Disease

Lathyism is basically a pure form of central motor system disease featured by irreversible spastic paraparesis and corticospinal tract degeneration. Weakness of the legs characteristically appears acutely after a period (weeks to months) of heavy consumption of grass pea. Subacute and insidious presentations are also recognized. Lathyism is said to
affect both the well-nourished and the undernourished. Individuals across the span of postnatal life (2-70 years) have succumbed to lathyrism, although the disorder most commonly affects young adults (Spencer et al., 1984). Males are more commonly and more severely affected than females, and this differential gender sensitivity may be related to factors other than grass pea intake. In females, the disease tends to develop before puberty, during pregnancy, or after menopause (Dwivedi, 1989). The disease manifests in (minimally or malnourished) subjects who consume 400 g *L. sativus* for weeks or months; this corresponds to an estimated daily intake of 1-150 mg/kg/d β-N-oxalamino-L-alanine (BOAA) (Paissos and Demopoulos, 1962; Cohn and Streifler, 1981b).

**Etiology**

Lathyrism has been exploited as a model motor-system disorder in the experimental laboratory. Principal focus has been placed on the role of BOAA, a free excitatory amino acid in the grass pea that appears to be the culpable neurotoxic agent in those species of lathyrus that are associated with human lathyrism. But for the presence of the potentially neurotoxic amino acid BOAA, the grass pea has many features and properties that make it extremely attractive as a food and fodder crop. Not only does the legume show a luxuriant growth in poorly prepared soils, it is exceptionally tolerant of drought and water-logging and fixes nitrogen, thereby enriching the soil; it also has few pests, an appealing taste, and is an excellent source of protein and calories. The protein composition of grass pea compares favorably with that of the chick pea (*Cicer arietinum*) (Roy et al., 1989), the seed of which is widely consumed in the West.
Clinical Presentation

Lathyrism displays a constant clinical picture consisting of varying degrees of spastic paraparesis involving the lower limbs (Ludolph et al., 1987; Tekle-Haimanot et al., 1990). One of the best early descriptions of the disease is by Stockman (1929):

"In man the symptoms usually begin suddenly and seem often to be precipitated by exposure to cold and wet and fatigue, this being the reason assigned for the comparative immunity of children and elderly men. Sometimes there are prodromal sensory symptoms of pain, pricking, numbness and cramps, but most commonly the victim quite suddenly feels the legs and loins weak and heavy, the muscles are tremulous when weight is put on them, there is dragging of the legs, increased reflexes and more or less inability to walk. This may go on to a further stage of extreme spasticity and rigidity of the leg muscles; the gait becomes jerky with short steps taken on the balls on the feet and with the knees slightly flexed; ankle clonus and adductor spasm of the thigh muscles are easily excited and account for the jerky cross-legged walk, in which the limb is raised up with an effort and a heave of the corresponding shoulder; the foot is turned in with the great toe tending to scrape the ground and the heel drawn up by gastrocnemius spasm. In the slighter cases the patient can get about with or without the aid of a stick, but often the disability is very severe and he finds himself unable to walk or stand, or only able to do so with the support of one or two long sticks which he grasps high up. In extreme cases he is driven to progress on his hands and feet in a sitting posture. There may be no sensory disturbances and no muscular wasting and almost invariably the lower limbs are affected. The lesion in chronic poisoning is evidently a partial degeneration of the motor tracts in the cord. Babinski’s sign is present as well as the exaggerated deep reflexes. But in many cases other symptoms have been recorded which point to a wider implication of the spinal cord. Loss of control of the bladder and rectum and impotence are quite common. Tingling, formication, lightening pains, pains around the waist, diminution of tactile sensation, complete loss of sensation to heat and pain, and marked muscular wasting have been observed. In a few cases the arms have been affected like the legs. It is said to be sometimes ushered in by convulsive movements of the upper and lower limbs and painful contraction of the muscles—apparently somewhat similar to the seizures I have described in monkeys. Early cases sometimes recover, others never”.

The clinical profile at disease onset is of considerable interest because it may well reflect the differential vulnerability of nerve cells to the excitotoxin BOAA, an $\alpha$-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid agonist, and thus to the characteristics of the subclass of glutamate receptors associated with those cells. The onset of spastic paraparesis may be preceded or accompanied by prodromal motor symptoms (Spencer and Dastur, 1989). A sensation of leg weakness, heaviness, or stiffness is frequently reported. Muscle cramping may occur; this is usually confined to the calf musculature. Tremulousness with and without tremor are reported at disease onset. Fasciculation and myokymia also have been noted in some cases. Paresthesias, numbness, and formication
attacking the legs, muscle aching occasionally reported in the legs and back are some of the sensory signs that have been reported with lathyrism. Urological involvement is probably rare: reports of frequency and urgency of micturition, urinary hesitancy and dribbling, and loss of sensation of micturition are occasionally found (Spencer, 1995). Abdominal cramping, nausea, diarrhea, and spasm of the anal sphincter have been reported. Other prodromal signs reportedly include dizziness, dry mouth and excessive thirst, fever, sweating, loss of short-term memory, and frequent and intensive nocturnal dreaming.

The key features of the clinical profile are briefly: (a) reversible leg dysfunction if consumption of the offending agent is discontinued in the prodromal phase; (b) irreversible and increasingly severe spastic paraparesis with continued grass pea consumption; and (c) largely selective involvement of the central motor system regulating the extremities, generally only the legs. Severity of disease may be classified as follows (deficits more or less symmetrical in distribution): (a) stage 1 (mild or 'no stick' cases), mild spastic gait with no use of a stick, complaints of increased leg stiffness, brisk deep tendon reflexes at ankle or knee, and Babinski sign equivocal or present; (b) stage 2 (moderate or 1-stick case), spastic gait requiring the use of a stick for support during ambulation, mild rigidity and increased deep tendon reflexes, and ankle clonus and Babinski sign present; (c) stage 3 (moderately severe, 2-stick case), severe spastic gait requiring the use of 2 sticks for support during ambulation, crossed adductor gait, markedly exaggerated deep tendon reflexes with clonus at ankle and knee, and Babinski sign present; and (d) stage 4 (severe, crawler stage), crawling, wheelchair-bound, or bed-ridden state, total loss of leg use, extreme muscle rigidity with contracture and disuse atrophy, brisk tendon reflexes in the upper extremities, and the Hoffmann sign is present.

Epidemiology

Epidemiological studies of lathyrism have been carried out in Bangladesh, China, Ethiopia, and India, where the disease was first recorded in 400 BC. In India, Acton (1922) estimated the presence of 60,000 cases in North Rewa (part of Madhya Pradesh). In recent times, the disease has been mainly confined to Uttar Pradesh, Madhya Pradesh, and Bihar and is seen among small landholders and farmers who practice grass pea cultivation because of lack of irrigation on their fields. Disease prevalence in endemic areas fell from 14.6/1000 in 1950-1959 to 9.1/1000 in 1981. In Ethiopia, disease prevalence of 0.5-2.3% was reported around Lake Tana.
The largest epidemiological study of lathyrism was undertaken between 1988-1990 in Ethiopia as part of a multidisciplinary approach to study the medical, agricultural, and nutritional aspects of this disorder preparatory to efforts at disease control (Tekle-Haimanot et al., 1990; 1993; 1994). Both Ethiopia and nearby Eritrea are periodically hit by epidemic lathyrism. A survey of over 1 million subjects residing in northwest and central Ethiopia revealed lathyrism prevalence rates of 1-75/10,000. The affected population consisted of very poor peasants who rely on subsistence farming using primitive agricultural practices. Overall, male subjects with lathyrism were more common than females (2.6:1), but significantly more females (69.2%) than males (47.7%) experienced disease onset before age 20. A similar trend was noted in the Bangladesh population with lathyrism (Kaul and Combes, 1986).

Diagnosis

There are no specific clinical or laboratory tests for lathyrism. While sensitive methods exist for the accurate quantitation of BOAA (Abegaz et al., 1989; Kisby et al., 1989; Khan et al., 1993), measurements are only performed for research purposes. The clinical chemistry profile of lathyrism is not reported and is not of significance in clinical diagnosis of the disease. Routine and research electrophysiological studies have been performed, but none is needed for diagnosis (Spencer, 1995). Contemporary in vivo imaging of the brain and spinal cord has yet to be carried out. X-rays of the pelvis and long bones of subjects who consumed Lathyrus spp. during developmental maturation may display mild growth defects indicative of osteolathyrism (Paissos and Demopoulos, 1962). Osteolathyrism is the name given to an experimentally induced disorder in animals given lathyrus seedling components containing P-aminopropionitrile, 2-cyanoethyl-isoxazolin-5-one that appear to lack neurotoxic properties but disrupt collagen metabolism (Martin et al., 1961). Lathyrism is distinguishable from other forms of spastic paraparesis primarily by history and, to some extent, on clinical grounds (Roman et al., 1985). Several family members may be affected in a similar time frame, in contrast to hereditary forms of spastic paraparesis where walking disability affects up to half the family members from their childhood onwards.

In Africa, epidemic lathyrism may be mistaken for outbreaks of mantakassa or konzo, another form of acute-onset epidemic spastic paraparesis associated with a potentially neurotoxic diet, the cyanogenic tuber and leaves of cassava (Ministry of Health Mozambique 1984; Howlett et al., 1990). Whereas African nationals with lathyrism usually reside in the Horn of Africa, those with konzo originate from southern African
nations (Angola, Central African Republic, Mozambique and Tanzania). Pyramidal signs in the upper extremities are more common in konzo and a history of cranial nerve involvement may be reported at the onset. African and other subjects with spastic paraparesis attributed to the HTLV-I retrovirus are usually distinguished from lathyrism by the presence of objective sensory loss in the lower extremities, signs of amyotrophy, subtle changes in mental status, and the progressive course of the viral disease (Roman et al., 1985). Lathyrism should also be distinguished from other disorders associated with spastic paraparesis, including multiple sclerosis, cervical spondylotic myelopathy, cerebral palsy, and hereditary spastic paraplegia.

Course and Prognosis

Patients with lathyrism have been followed for more than 40 years after disease onset. There may be slow progression of motor deficits in the central nervous system of patients (Gimenez-Roldan et al., 1994).

Treatment

Attempts to treat lathyrism have been aimed at reducing muscle spasm. Dorsal rhizotomy or incomplete surgical transection of the thigh adductor muscles has been tried with some occasional success. Little relief is offered by chemical spasmolytics, and continued treatment of impoverished subjects in remote rural settings is impractical. Claims that vitamin C can prevent the onset or reverse the clinical deficits of lathyrism are subjective.

Neurophysiology

Hugon et al. (1993) studied the integrity of the central motor pathway and reported that central motor conduction time was increased markedly in patients with severe (grade 3 or 4). Subjects showed brisk tendon reflexes and the Hoffmann sign. A sensorimotor pattern of deficits indicative of mild peripheral neuropathy has been reported in male subjects with lathyrism (Hugon et al., 1990).
Neuropathology

Lack of a complete neuropathological study using contemporary histopathological techniques has made comprehension of lathyrism more difficult. Studies of the brain are lacking, save for one case of a subject who developed the disorder 31 years prior to death and in whom loss and shrinkage of large (presumably pyramidal) neurons in the upper part of the precentral gyrus were noted (Spencer, 1995). Neuropathological studies on the spinal cord show predominantly distal symmetrical degeneration of lateral and ventral corticospinal tracts, sometimes with distal degeneration of spinocerebellar and gracilus tracts (Streifer et al., 1977). Distal axonal degeneration of the fasciculus gracilus and spinocerebellar pathways have been reported (Hirano et al., 1976). Spencer et al. (1984) proposed that lathyrism is a central distal axonopathy marked with degeneration of the longest corticospinal tracts, with lesser involvement of the shorter pyramidal pathways serving the upper extremities of the most severely compromised individuals. This interpretation is consistent with the neurophysiological and neuropathological findings except perhaps the loss of upper motor neurons. The cortical neuron loss as a primary event (neuronopathy) provides an alternative and more attractive explanation that would fit well with evidence that BOAA is an excitotoxin capable of inducing acute neuronal degeneration.

Prevention

Prevention of lathyrism and promotion of nutrition through the development and use of a nontoxic strain of the grass pea have attracted considerable scientific interest in recent years (Kaul and Combes, 1986; Spencer, 1989). The International Network for the Improvement of *Lathyrus sativus* and Eradication of Lathyrism (INILSEL) consists of a network of scientists of various disciplines seeking to exploit the valuable properties of the grass pea for use as a safe and nutritious food and fodder crop in rainfed areas of the world. For many centuries, the grass pea and related species have formed an inexpensive component of the human diet of poor people. In countries like Bangladesh and Ethiopia, the grass pea is predominantly eaten by the poor. Additionally, in times of flood and drought, when other food crops are destroyed, the hardy grass pea becomes a survival food for humans, cattle, and horses. A question of immense importance to the public health of countries endemic for lathyrism is the level of consumption that produces no adverse health effects in both the short-and the long-term. As grass pea production increases to meet the demands of enlarging populations, accurate data on BOAA intake and toxicity levels are needed to assess health risks and advise population accordingly. The
establishment of a no-adverse-effect level could be obtained from a study of populations which consume legumes contaminated with small concentrations of the neurotoxin during various cooking procedures. Aqueous leeching of the dehusked seed in hot water for several hours or boiling the seeds in water and draining the fluid reportedly reduces the amount of BOAA by 70-80% (Mohan et al., 1966).

Animal Studies

Lathyrism has been exploited as a model motor-system using laboratory animals as well as primates (Spencer, 1995). However, the successful development of animal models of human lathyrism has proven to be a major challenge and the reason is unclear. The disease is reported in different animal species such as in avian, swine, bovine, ovine, equine and elephants (Spencer, 1995). In the horse, supposedly the most susceptible species, manifestation of clinical signs was evident after 10 days on a diet composed exclusively of *L. sativus*. Animals fed only 1-2 quarts per day developed clinical signs of lathyrism after 2-3 months. Neurological signs are said to resemble stringhalt and may be accompanied by dyspnea and roaring, the latter suggestive of recurrent laryngeal nerve involvement.

There have been several attempts to model the disease in primates (Rao et al., 1967; Parker et al., 1979). Rao et al. (1967) reported that continuous exposure to grass peas produced spastic paraparesis in monkeys fed *L. sativus* for periods up to 15 months. In a study of well-nourished cynomolgus monkeys fed a grass pea diet, Spencer et al. (1986; 1988) reported that: (a) prolonged exposure to BOAA induces a pattern of motor-system dysfunction reminiscent of human lathyrism, (b) clinical signs consistent with neuroexcitation (myoclonus but not grand mal seizures) occur in primate lathyrism, as in the human disease, (c) early leg weakness is reversible in both species, and concluded that (d) BOAA is the likely culpable agent in human lathyrism, but (e) in optimally-nourished primates, BOAA alone is insufficient to induce irreversible spasticity with pyramidal degeneration. Studies with BOAA-dosed animals subjected to malnourishment or excessive physical exercise have not been performed.

Molecular Mechanisms

Different nomenclatures have been used to describe the excitatory and neurotoxic amino acids present in species of lathyrus associated with clinical lathyrism: (a) β-N-oxalylamino-L-alanine (BOAA), (b) L-3-oxa1yl amino 2-aminopropionic acid, (c) β-N-oxalylamino-α, β-diaminopropionic acid, and (d) 3-N-oxalyl-2,3-diaminopropanoic acid
ODAP, the term currently favored by chemists. BOAA was isolated from the grass pea in 1964 (Murti et al., 1964) and synthesized thereafter (Rao, 1975). Recent studies suggest that BOAA is formed by enzymatic opening of β-(isoxazolin-5- on-2-yl)-alanine (BIA), a heterocyclic compound found in lathyrus (Lambein et al., 1993). Experimental studies have shown that L-BOAA disrupts a number of systems in mammalian CNS tissue but the molecular mechanisms responsible for the neurotoxic disease have yet to be identified with certainty. Recently, research efforts have been directed to a molecular explanation for the apparently peculiar sensitivity of cortical motor neurons.

One line of inquiry recognizes that L-BOAA is an excitatory amino acid of motor neurons (Watkins et al., 1966) which exerts its neurotoxic effects directly through overstimulation of synaptic mechanisms that utilize glutamate, aspartate, or cysteate as neurotransmitters (Olney et al., 1976). Receptors for these endogenous amino acid neurotransmitters are classified pharmacologically according to their most potent agonists: viz. (a) N-methyl-D-aspartic acid (NMDA) and two non-NMDA receptors, (b) α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and (c) kainic acid. BOAA preferentially activates non-NMDA receptors (MacDonald and Morris 1984; Weiss et al., 1989), especially the AMPA receptor (Ross et al., 1989; Allen et al., 1990).

Overstimulation of neurons with receptors that selectively respond to L-BOAA or other excitatory acidic amino acids has been proposed to cause (a) an excessive influx of sodium ions (Na+) and chloride ions (Cl−), resulting in membrane depolarization, (b) increased activity of the Na+ + K+-ATPase membrane pump, which diminishes ATP as the pump attempts to restore the transmembrane ionic balance, and (c) failure of the cell to reestablish ionic homeostasis, leading to cascade of events that culminate in cell death. These pathophysiological events, which may develop within seconds of the application of L-BOAA (10 μM) to mouse CNS explants in vitro, constitute the excitotoxic theory of neuronal death as advanced by Olney (1980). A second delayed mechanism of neuronal demise is proposed to result from an increase in free intracytoplasmic calcium ions (from extracellular or intracellular sources) which activate Ca2+-dependent enzymes, with consequent dissolution of cytoplasmic structure (Choi, 1987). It has been shown that non-NMDA antagonism also blocks dose-dependently a variety of convulsive behaviors seen in mice following intracerebroventricular administration of L-BOAA (Ross and Spencer, 1987). The demonstration that glutamate antagonists block cortical motor neuron degeneration in a bona fide animal model (for irreversible BOAA-induced spastic paraparesis) will provide conclusive evidence that perturbation of neuroexcitation is responsible for motor neuron loss in lathyrism (Spencer, 1994, 1995).
Pathogenesis

Signs of impending lathyrism in the prodromal period probably result from widespread BOAA-induced synaptic overexcitation of CNS motor neurons. This accounts for the myoclonus, tremor, global muscle spasm, and involuntary voiding reported by subjects who subsequently develop severe spastic paraparesis. During the initial degenerative period, the most exquisitely sensitive neurons (likely neurons in motor cortex) are lost, while others survive and recover from their altered pharmacological state. If consumption of the grass pea ceases and BOAA is excreted, the subject is left with a clinical deficit (spastic paraparesis) resulting from degeneration of the central motor pathway. The legs are affected earlier and to a greater degree than the arms. In the recovery period, the degree of neurological deficit subsides with discontinuation of grass pea consumption (presumably because pharmacological effects of BOAA disappear), but varying degrees of spastic paraparesis remain. Central motor deficits may slowly advance with the passage of time.

HUMAN T-CELL LEUKEMIA VIRUS (HTLV) TYPES -I AND -II AND ASSOCIATED DISEASES

HUMAN T-CELL LEUKEMIA VIRUS TYPE-I (HTLV-I)

Originally isolated from a patient with a cutaneous lymphoma, the human T-Cell leukemia/lymphoma virus type I was the first pathogenic retrovirus to be discovered in man. In contrast to the human immunodeficiency virus (HIV), HTLV-I causes disease in only about 5 percent of infected people. However, the virus is of special interest and importance because it is associated with two quite different conditions: adult T-cell leukaemia/lymphoma and tropical spastic paraparesis, also known as HTLV-I-associated myelopathy. Associations between HTLV-I infection and other conditions are now being reported (see below); a suggested association with multiple sclerosis has been refuted (Bangham and Nightingale, 1996).
Epidemiology

The epidemiology of HTLV-I is unusual. It is endemic in many countries in the tropics, particularly the Caribbean, West Africa, also East Africa, Seychelles Island parts of South America, and Papua, New Guinea, where the seroprevalence is between 1 and 20 percent of the population. Elsewhere it is found in certain aboriginal groups in North America, Australia, and northern Japan; however, the largest known area of high endemicity is in southern Japan, on the island of Kyushu, where the seroprevalence in adults aged over 25 years exceeds 20 percent in certain prefectures. The distribution of the virus, even in highly endemic areas, is uneven, probably because of its poor transmissibility. In the United Kingdom, HTLV-I is found in 1 to 4 percent of people of Caribbean origin. In North America and Europe, it is also found in some communities of intravenous drug abusers (Bangham and Nightingale, 1996).

Mode of Transmission

There are three important modes of transmission: perinatal and neonatal infection from a seropositive mother, in which breast-feeding is a significant factor; sexual transmission, particularly from males to females; and transmission by infected blood, either by transfusion or by sharing of needles among drug abusers. Transmission of the virus depends on transfer of cells from infected people, for cell-free virus is rare and poorly infectious.

HTLV-I is closely related genetically to leukemia viruses in monkeys and cattle; its relationship to HIV is distant. It contains the three genes usually found in retroviruses, encoding the core protein Gag, the receptor-binding protein Env, and the polymerase Pol. The polymerase is a reverse transcriptase (RNA-dependent DNA polymerase); this is responsible for converting the viral genome into a double-stranded DNA molecule, called the provirus, which becomes integrated into the host cell DNA. In addition, HTLV-I makes at least two proteins, Tax and Rex, that regulate the transcription of the virus. Tax stimulates the production of new viral RNA, and Rex controls the splicing of viral mRNA.

Although it can infect a wide variety of cell types in vitro, HTLV-I replicates efficiently only in CD4+ (helper) T cells; these are the cells that are transformed in adult T-cell leukaemia/lymphoma.
Diagnosis

Those infected with HTLV-I, with rare reported exceptions, develop antibodies that recognize the viral proteins. Diagnosis of HTLV-I infection is based on detection of these antibodies in a particle agglutination assay, or the more specific enzyme immunoassay and Western Blot. Reliable estimates of the median interval between infection and seroconversion are lacking; this interval can be several years, especially in those infected during childhood. Rare individuals carry HTLV-I nucleic acid but lack antibodies or T-cells specific to the virus; the significance of this is not yet understood. Antibody to the Tax protein is present in about half of infected people, and is associated with a higher antibody titre and greater infectivity. About 1 percent of seronegative individuals in a highly endemic area in Japan were found to have antibody to Tax alone, undetected by the other assays: again, the significance of this awaits clarification. High proportion of HTLV-I infected individuals have chronically activated cytotoxic T-lymphocytes that recognize the Tax protein; the possible pathogenetic role of these cells in tropical spastic paraparesis is under investigation.

TROPICAL SPASTIC PARAPARESIS

Epidemiology

The association between HTLV-I and tropical spastic paraplegia was first reported in the Caribbean by Gessain (1985), and in Japan, where it was called HTLV-I-associated myelopathy (HAM), by Osame (1986).

Mode of Transmission

Tropical spastic paraparesis mainly affects those living in, or having emigrated from, areas of endemic HTLV-I infection, but also occurs in others infected by intrauterine (mother-child) transmission, sexual activity, intravenous drug abuse, or blood transfusion. The lifetime risk of tropical spastic paraparesis in infected individuals, based on Japanese prevalence data, is about 0.25 percent. It is more common in women (90 percent), beginning in the third to sixth decade of life.
Clinical Presentations

Progressive spastic paraparesis with gradual onset (rarely rapid) is the major feature, and most patients experience severe difficulty walking or are confined to a wheelchair within 10 years. Minor distal lower-limb sensory symptoms are common and may precede the onset of weakness by some months, but sensory signs are few. Most patients experience back or leg pain, which may be an early feature. Bladder involvement is common, producing frequency, urgency and incontinence; constipation is also a frequent problem. Other less common features include spastic weakness of the arms, a sensory level deficit on the trunk or Brown-Sequard syndrome, male impotence, sensorineural deafness, and optic atrophy. Rarely, lower motor-neuron signs such as prominent wasting, fasciculation, and areflexia are observed. The severity of the paraparesis may fluctuate and eventually stabilize, but in contrast to multiple sclerosis, tropical spastic paraparesis does not result in acute relapsing and remitting disease in the spinal cord or elsewhere in the central nervous system.

Diagnosis

Patients with tropical spastic paraparesis are seropositive for HTLV-I, usually at titres higher than healthy carriers of the virus. However, progressive paraparesis in an HTLV-I-positive patient should not be assumed to be due to tropical spastic paraparesis, as other spinal disorders may occur in otherwise healthy asymptomatic carriers of HTLV-I. A myelogram or magnetic resonance imaging (MRI) scan are usually required to exclude spinal-cord compression, syringomyelia, or intrinsic cord tumor. Serum vitamin B-12 estimation, and Treponema and Borrelia serology are needed to exclude metabolic or infective myelopathies.

Examination of cerebrospinal fluid usually shows a lymphocytosis of five or more cells/µL, sometimes as high as 50 cells/µL. The cerebrospinal-fluid protein may be elevated to as high as two or three times normal. Positive treponemal serology due to yaws is common among residents of, or emigrants from, the West Indies. But if treponemal serology of cerebrospinal fluid is positive, neurosyphilis should be considered, particularly if there is a history of infection or if other clinical features of syphilis are present. Visual, auditory, and lower-limb somatosensory-evoked potentials may show minor abnormalities, but electromyography and peripheral nerve conduction are usually normal. MRI scanning of the brain may show scattered lesions in the white matter similar to, though usually less extensive than, the lesions seen in multiple sclerosis.
Neuropathology

The pathology of the spinal cord consists mainly of perivascular infiltration of mononuclear cells with proliferation of microglia and reactive astrocytic gliosis. There may be demyelination as well as capillary proliferation. The degree of inflammatory response is variable from patient to patient.

Treatment

There is no specific treatment for tropical spastic paraparesis. Some patients have shown a minor response to corticosteroid treatment, but disease progression cannot be prevented. Physiotherapy and occupational therapy are important in the management of the progressive disability. Urodynamic investigation may help to select appropriate drugs, such as oxybutynine for detrusor instability, or urological surgery, such as ileal conduit (Bangham and Nightingale, 1996). Spasticity of the legs can be treated with baclofen or dantrolene (Bangham and Nightingale, 1996).

OTHER DISORDERS ASSOCIATED WITH HTLV-I

Where HTLV-I is endemic, many disorders may occur in asymptomatic carriers of the virus. The etiological relation of HTLV-I to tropical spastic paraparesis is accepted, but the disorders for which HTLV-I is responsible are uncertain. A list of disorders that have been associated serologically with HTLV-I is found in (Table 1.1), the associations are strongest with tropical spastic paraparesis.
Table 1.1 Disorders associated with HTLV-I

<table>
<thead>
<tr>
<th>Disorder</th>
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<tbody>
<tr>
<td>Adult T-cell leukaemia/lymphoma (ATLL)</td>
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<tr>
<td>Tropical spastic paraparesis or HTLV-I-associated myelopathy (TSP/HAM)</td>
</tr>
<tr>
<td>Polymyositis</td>
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<tr>
<td>Chronic arthritis</td>
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<tr>
<td>Uveitis</td>
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<tr>
<td>Motor neuron disease-like disorder</td>
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<tr>
<td>Sicca syndrome</td>
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<tr>
<td>Lymphocytic alveolitis</td>
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<tr>
<td>Chronic infective dermatitis</td>
</tr>
</tbody>
</table>

Polymyositis (rarely in combination with tropical spastic paraparesis) which has been reported in association with HTLV-I, produces a gradually progressive weakness of proximal muscles, elevated creatine kinase and myopathic electromyogram. HTLV-I is sometimes associated with predominantly lower motor-neuron features such as weakness marked wasting, and denervation on electromyography. Rarely, this may result in a clinical picture resembling motor neuron disease. Inflammatory arthropathy and uveitis may be associated with endemic HTLV-I infection in Japan. Some patients with tropical spastic paraplegia also have sicca syndrome, a rheumatoid-like arthropathy or a lymphocytic alveolitis. In children, HTLV-I is associated with a chronic infective dermatitis.

In those at risk of HTLV-I infection, anti-HTV-I antibodies should be measured in cases of polymyositis, motor neuron disease, and progressive neurological syndromes in which spastic paraparesis is a prominent feature. It is likely that over the next few years the spectrum of HTLV-I-associated disorders will widen. An association between persistent strongyloidiasis and HTLV-I infection has been suggested but is not proven.

HUMAN T-CELL LEUKEMIA VIRUS -II (HTLV-II)

HTLV-II, a retrovirus closely related to HTLV-I, was first isolated from the tissue of a patient with hairy-cell leukemia. The virus occurs sporadically in West Africa. In intravenous drug abusers in Europe and North America, infection with HTLV-II is as common as HTLV-I. HTLV-II is also common in several native groups in both North and
Central America. Since the early 1980s, HTLV-II has been found in a small number of patients with an atypical form of hairy-cell leukemia. However, there is not yet sufficient evidence that the virus causes this malignancy. There are case reports of a paralytic syndrome similar to tropical spastic paraparesis in HTLV-II-seropositive individuals; in some cases, the paralysis is flaccid rather than spastic.

CASSAVA-RELATED NEURODEGENERATIVE DISEASES

TROPICAL ATAXIC NEUROPATHY

The first report of a prototype of the syndrome now commonly referred to as tropical ataxic neuropathy (TAN) was made in 1888 by Strachan among Jamaicans. Strachan believed the cause was malarial infection. He reported over 500 cases of "a form of multiple neuritis prevalent in the West Indies." The main symptoms were severe burning pain of the soles of the feet, dimness of vision, ataxia, and increased pigmentation of the skin. Syndromes similar to that described by Strachan (comprising lesions of the skin, mucous membranes and the nervous system) and putatively related to dietary deficiencies, toxins and infections have been described in communities with low standards of nutrition, especially in the tropics and in prisoner-of-war camps. TAN or variants thereof have been reported in different parts of West Africa (Senegal, Sierra Leone, Liberia, Ghana and Nigeria), North Africa (Egypt, Ethiopia and Eritrea), East Africa (Uganda, Tanzania and Kenya), Central and South Africa (Zaire, Zambia, Malawi and Natal), Guiana Jamaica and Trinidad in the Caribbean, and Sri Lanka, Malaya, South India, and the Philippines in South East Asia. No large area of the tropics appears to be free of these disorders (Osuntokun, 1968).

History of Occurrence of TAN in Nigeria

The earliest reports of TAN in Nigerians were by Moore (1937). He suggested that a specific item of diet, cassava, was the cause of TAN. In the 1930s, Moore described a syndrome of visual loss, glossitis, angular stomatitis and eczema of the scrotum in adolescent boys in an institution in Nigeria. Cassava was implicated because the condition improved when the cassava component of the diet was reduced.

Clark in 1935, first attributed the cause specifically to the high content of cyanogens in cassava. In 1936 Clark postulated that TAN resulted from chronic cyanide intoxication from hydrocyanic acid released on enzymatic or acid hydrolysis of the glycoside,
linamarin, found in high concentration in the integument of the root of cassava. Clark (1936) stated that the amount of thiocyanate contained in the urine and saliva of patients was greater than in control subjects but actual figures were not reported.

These observations were, however, not followed up until after nearly three decades, when there was a resurgence of interest in the role of chronic cyanide intoxication and cyanide metabolism in the pathogenesis of demyelination and in some degenerative disorders of the nervous system including multiple sclerosis, tobacco amblyopia, Addisonian subacute combined degeneration, and Leber's hereditary optic atrophy. Osuntokun carried out extensive clinical, biochemical and epidemiological studies in Nigeria between 1966 and 1980 and produced a wealth of circumstantial evidence to support the hypothesis that dietary cyanogens were the most important causal factor of chronic cyanide intoxication. He noted that the disease occurred among "poor laborers living entirely on gari---a culinary derivative of cassava".

**Etiology**

Considerable circumstantial evidence incriminates chronic cyanide intoxication of dietary origin as the major etiologic factor in TAN among some Nigerians (Osuntokun, 1981).

**Clinical Neurological Presentations**

The essential neurological components of the disease are myelopathy, bilateral optic atrophy, bilateral perceptive deafness and polyneuropathy (Osuntokun, 1968). Motor neuron disease, Parkinson's disease, cerebellar degeneration, psychosis and dementia have also been found in association with the disease. The initial and commonest symptoms consist of various forms of paraesthesia and dysesthesia usually starting in the distal parts of the lower limbs. The next commonest is blurring or loss of vision. Other symptoms in order of frequency are ataxia of gait, tinnitus, deafness, weakness and thinning of legs. As the disease progresses, defective perception of sensory modalities (often confined to the lower limbs), bilateral optic atrophy, ataxic gait, and impaired muscular coordination, bilateral perceptive deafness, weakness and wasting of the muscles (usually of the lower limbs) may be common. Deep tendon reflexes are absent in the lower limbs, and peripheral nerves reportedly showed segmental demyelination.
Diagnosis

The diagnostic criteria are myelopathy, bilateral optic atrophy, bilateral sensory neural deafness and symmetrical peripheral neuropathy. The diagnosis is made when two of these components are present (Osuntokun, 1968).

Epidemiological Evidence

TAN is a disease of poor people and, in Nigeria, has never been encountered among those in the high socio-economic groups (Osuntokun, 1981). Epidemiological studies show a correlation of disease prevalence with intensity of cassava cultivation, frequency of cassava meals, and plasma thiocyanate levels (Osuntokun 1968; 1981). The prevalence of the disease in one high cassava-eating village was 3% and, in the 50-60 year old age group, was 8%. In Nigeria, endemic foci of the disease recognized since the 1930's (Moore 1930; 1937) correspond with the areas where cassava is intensively cultivated and consumed as the major or the sole dietary source of carbohydrate. In one endemic focus studied, 43 families in which cases of TAN were found, cassava meals constituted 95% of a total of 417 meals/day compared with 60% of 279 meals/day in 25 neurologically intact families of comparable socio-economic status in the same focus.

The study of three Nigerian villages (two in an area where cassava formed the staple diet, and the other where yam was predominantly eaten) showed that the minimum prevalence of the disease in the high cassava-eating villages was about 18-26 per 1000 of the adult population (the prevalence in the 6th decade in one village was 80 per 1000) whereas the disease did not exist in the yam-eating area.

Pathophysiologic Changes

There have been no autopsy studies of the neuropathology of the disease. None of the Nigerian patients (where the disease was first reported) died from the disease. Studies of peripheral nerves obtained at biopsy have shown segmental degeneration (Osuntokun 1970). Nerve conduction velocity is reduced significantly in the lower limbs (Osuntokun, 1981). Williams and Osuntokun (1969) found that the demyelination of peripheral nerves induced in rodents by cyanide injection bore a striking resemblance to the lesions found in biopsy specimens of peripheral nerves of Nigerian patients who suffer from TAN. Some of the features of the disease; i.e. high frequency of certain forms of visual field disorders, optic atrophy, high association of motor neuron disease - parkinsonism - cerebellar
degeneration dementia complex indicate widespread neuronal degeneration and could be compatible with the effects of cellular toxin on parts of the nervous system highly susceptible to metabolic insult.

TROPICAL SPASTIC PARAPARESIS (KONZO)

Definition

Cassava-related-tropical spastic paraparesis (konzo or Mantakassa) is characterized by the abrupt onset of an isolated and symmetric spastic paraparesis which is permanent but non-progressive. Konzo is an upper motor neuron disease that has only recently been defined as a distinct disease entity. The disease has been reported only from poor rural communities in Africa (and must be distinguished from the lathyrisim and HTLV-I associated tropical spastic paraplegia) (Rosling et al., 1988a, Rosling and Tylleskar, 1995). Its name is derived from the local designation used by the Zairian population affected by the first reported outbreak, in 1936. In the Yaka language, konzo means 'tied legs', a good description of the resulting spastic gait.

Etiology

The etiology of konzo has not been established with certainty. While an infectious etiology is highly improbable, a toxico-nutritional etiology is strongly supported by both clinical and epidemiological studies. An infectious etiology was initially suspected in all outbreaks due to its epidemic occurrence and the familial clustering (Carton et al., 1986). However, konzo patients show no signs of infection, and laboratory tests for infection as well as virus isolation have proved negative. Konzo patients have no antibodies against HTLV-I or other human retroviruses (Tylleskar et al., 1996). A toxico-nutritional etiology is indicated by several epidemiological studies made in Democratic Republic of Congo (Zaire), Mozambique, Tanzania and the Central African Republic (Tylleskar et al., 1992; Cliff et al., 1995; Mlingi, 1991, 1995; Banea 1997). All revealed an association between konzo and dietary cyanide exposure from exclusive consumption of insufficiently processed bitter cassava. Cassavism (by analogy with lathyrisim) is an alternate name of the disease. Trolli (1938) was the first to suggest that high cyanide exposure from exclusive consumption of insufficiently processed bitter cassava is associated with neurological dysfunction. However, no attention was given to this suggestion until 50 years later when scientists showed interest following epidemics of konzo.
Epidemiology

*Konzo* was brought to scientific attention by two major epidemic outbreaks, each numbering more than 1,000 cases. The first was in the Bandundu Region in present-day Democratic Republic of Congo (Zaire) in 1936-37 and the second in Nampula Province of Northern Mozambique in 1981. Smaller outbreaks in rural areas have subsequently been reported from Zaire, Mozambique, Tanzania and Central African Republic (Figure 1.1).

![Map of Africa showing years and areas of konzo and TAN have been reported. Modified (Rosling, 1988b)](image)

Outbreaks of *konzo* have generally occurred in times of food shortage caused by drought, civil strife or other causes of population disruption. Cassava cultivation spread across Africa after its introduction from South America 300-400 years ago.

Sporadic cases of konzo also occur in affected areas, years after an extensive outbreak. The majority of *konzo* occurs during the dry season, especially in drought years.
Women of child-bearing age and children, 3-13 years old, are at greatest risk for *konzo* (Figure 1.2).

![Image of children affected by spastic paraparesis.](image)

**Figure 1.2** Children affected by spastic paraparesis. From (Rosling, 1986).

Lower extremities exhibit increased muscle tone, crossing of the legs, increased deep tendon reflexes and extensor planter reflexes. Upper extremities are less affected. Superficial sensation is usually intact. Cranial nerve function is rarely affected and mentation is clear. Neurobehavioral studies have not been undertaken.

Men are less frequently affected. No breast-fed children are known to have contracted *konzo*, which in affected communities means no child below the age of 2.5 years. Familial clustering is common. At present, a total of 3,700 *konzo* cases have been documented in studies and reports. Of these, more than 2,000 cases are from the Bandundu Region of the Democratic Republic of Congo (Zaire) (Banea et al., 1992).

**Clinical Presentations.**

The onset of *konzo* is sudden without prodromal signs. In 90% of cases the duration of onset is less than one day. As in lathyrisn a long walk or hard work seems to trigger the onset. The initial symptoms of *konzo* are described as trembling or 'cramping' in the legs, heaviness or weakness of the legs, a tendency to fall or an inability to stand. Symptoms such as low back pain and paresthesia in the legs are occasionally present at the onset but resolve within 30 days. During the first few days, most patients experience
generalized weakness and are usually bedridden for some days or even weeks before trying to walk. Initially patients have blurred vision and/or speech difficulties which typically clear during the first month, except in severely affected patients in whom such complaints may persist. No such changes are reported in lathyrism.

The most noticeable finding on clinical examination is symmetric spastic paraparesis indistinguishable from lathyrism and similar to HTLV-I spastic paraparesis. The reflexes of the lower limbs are exaggerated in all cases of konzo and extensor plantar responses can be elicited in most cases if done with the patient in recumbent position. Intellectual capacity, hearing, coordination, sensory function, and urinary, bowel, and sexual functions are all normal (Howlett et al., 1990). The severity of konzo varies from hyperreflexia in the lower limbs to a severely disabled, bedridden patient with spastic paraparesis and associated weakness of the trunk and arms, impaired eye movements, dysarthria and possibly visual impairment. Although the severity varies from patient to patient, the upper motor neurons with the longest axons are invariably more affected than the shorter ones. Thus a konzo patient with dysarthria always shows severe pyramidal signs in the legs and, to a lesser extent, the arms (Howlett et al., 1990).

Epidemiological Studies

Epidemiological studies of konzo indicate that a combination of dietary factors create the metabolic situation that is believed to cause a permanent neurodegenerative state (Cliff et al., 1985; Casadei et al., 1990). All konzo-affected areas are characterized by rapidly growing populations with insufficient household food security, economic stagnation and severe agro-ecological problems. Bitter cassava varieties are the dominant staple crop and women do most of the agricultural and post-harvest work. Epidemics coincide with food shortages that invite short-cuts in cassava processing. The short-cuts in processing allow large amounts of cyanogens to remain in the cassava consumed and hence a high dietary cyanide exposure. A concurrent protein-deficient diet results in low intake of sulfur amino acids, which in turn reduces the availability of the sulfur substrates needed for conversion of cyanide to thiocyanate, the main cyanide detoxification pathway in the human organism. A combined high cyanide/low sulfur intake has been linked with the onset of konzo in several epidemiological surveys (Cliff et al., 1985). Rosling (1986) reported that the affected population lost almost their entire production of protein-rich crops during the drought. Due to the low yield of cassava, most families were unable to barter cassava for fish, as they usually did in normal years. A typical answer in the interviews (Rosling,
1986) was: "We have cassava roots but not enough time to let them dry before we eat them and there is no other food to eat together with these bitter roots"

Diagnosis

The following criteria are used: (1) a visible symmetric spastic abnormality of gait while walking or running; (2) a history of onset of less than one week followed by a non-progressive course in a formerly healthy person; (3) bilaterally exaggerated knee or ankle jerks without evidence of sensory loss and (4) absence of grass pea consumption. While the first and third criteria are fulfilled by many types of spastic paraparesis, the history of a rapid onset and non-progressive course will leave only lathyrism as a differential diagnosis. HTLV-I associated myelopathy and other tropical myeloneuropathies are slowly progressive disorders. Depending on the degree of disability, konzo can be classified as mild when subjects do not require a walking aid, as moderate when one or two stick(s) or crutches are used as ambulatory aids, and as severe when patients are bedridden or unable to walk.

The diagnosis should be confirmed by careful clinical history and examination in a community-based survey. Quick involvement of qualified expertise may be worthwhile, as early preventive action can avoid much disability. Preferably, urine specimens from a sample of the population should be collected and analyzed for thiocyanate, the main metabolite of cyanide. A survey to identify all the affected subjects will help in assessing the magnitude of the problem. Unexpected changes in cassava processing have been made in the konzo-affected areas when people are suffering from food shortage. A health education message should be broadcast in the affected area, emphasizing: 1) the non-infectious nature of konzo to prevent irrational isolation of affected individuals (2) effective cassava processing, and (3) the importance of a varied diet. The common characteristics of konzo, tropical ataxic neuropathy, lathyrism and HTLV-I myelopathy are summarized in Table 1.2.
Table 1.2 The characteristic features of four tropical myeloneuropathies

<table>
<thead>
<tr>
<th></th>
<th>Konzo</th>
<th>Tropical ataxic neuropathy</th>
<th>Lathyrism</th>
<th>HLTV-I associated myelopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geographical area</td>
<td>Africa</td>
<td>Africa</td>
<td>Asia/Africa</td>
<td>Worldwide</td>
</tr>
<tr>
<td>Occurrence</td>
<td>epidemic</td>
<td>endemic</td>
<td>epidemic</td>
<td>endemic</td>
</tr>
<tr>
<td>Highest prevalence</td>
<td>3 %</td>
<td>3 %</td>
<td>3 %</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Familial clustering</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Type of onset</td>
<td>subacute</td>
<td>slow</td>
<td>subacute</td>
<td>slow</td>
</tr>
<tr>
<td>Course</td>
<td>permanent</td>
<td>insidious</td>
<td>permanent</td>
<td>progressive</td>
</tr>
<tr>
<td>High-incidence age</td>
<td>&lt; 40</td>
<td>&gt; 40</td>
<td>&lt; 40</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main neurological</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>findings:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gait abnormality</td>
<td>spastic</td>
<td>ataxia</td>
<td>spastic paraparesis</td>
<td>spastic paraparesis</td>
</tr>
<tr>
<td></td>
<td>paraparesis</td>
<td></td>
<td></td>
<td>paraparesis</td>
</tr>
<tr>
<td>Peripheral</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>common</td>
</tr>
<tr>
<td>neuropathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphincter</td>
<td>no</td>
<td>no</td>
<td>rare</td>
<td>yes</td>
</tr>
<tr>
<td>involvement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optic Atrophy</td>
<td>rare</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Deafness</td>
<td>no</td>
<td>common</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Etiology</td>
<td>(attributed to weeks of high cyanide exposure from cassava)</td>
<td>(attributed to prolonged, varying cyanide exposure from cassava)</td>
<td>(caused by weeks of high Lathyrus consumption)</td>
<td>(caused by chronic HTLV-I infection)</td>
</tr>
</tbody>
</table>
Treatment

There is no known cure for konzo. A good and varied diet, and immediate treatment with high doses of multivitamins, but especially vitamin B\textsubscript{12}, is recommended to avoid possible increased neurodamage due to concurrent vitamin B deficiency. Physical rehabilitation with crutches has proven successful in achieving independent locomotion.

Prevention

Konzo is not a major public health problem in Africa as a whole, but the condition may be epidemic in affected communities. There is a risk of konzo epidemics in parts of Africa where agro-ecological problems turn bitter cassava (usually of high cyanogenic potential) into the major source of calories; under these settings people attempt to undertake short-cuts in cassava processing. Konzo can probably be prevented by applying effective processing of the cassava root. There are three predominant cassava processing methods: (1) fermentation by soaking in water, followed by sun-drying or cooking, (2) grating and fermentation of fresh pulp under air exclusion followed by heat drying to make gari and (3) direct sun-drying of fresh roots. The first two procedures effectively reduce the toxic cyanogens to negligible levels if the procedure is allowed sufficient time. Soaking of cassava should continue until the roots are soft, which usually requires three days and longer if the water is cold. Fresh pulp fermentation should also be allowed sufficient time, at least two nights (Mlingi, 1995). Absolute desiccation (drying) is as important as adequate fermentation. Direct sun-drying is an inefficient way to eliminate the toxins, especially if the drying is rapid and incomplete. Food science competence is needed to elaborate locally applicable and effective advice on processing methods.

CASSAVA

ORIGIN AND DISTRIBUTION OF CASSAVA

Cassava, a native plant of tropical America, is a perennial, tropical woody shrub that grows 2 to 4 meters high (Figure 1.3). Cassava is widely grown in Latin America, Asia and Africa. It was introduced into Asia and South Pacific region in the 17th and 18th centuries (Carter, 1992).
The underground tubers are a major source of carbohydrate and the leaves contain some protein. Both may be used for food.

There is evidence that cassava was grown in Peru four thousand years ago (Okigbo, 1980) and consumption continues in South America to date. Cassava was introduced from South America into Africa in the 15th to 17th centuries by Portuguese traders (Jones, 1959). By the end of the 18th century, it was grown widely in southern and western Africa. At present, cassava is grown in all tropical and subtropical countries up to an altitude of 1500 meters between 30° N and 30° S of the Equator. This belt comprises all the continents except Europe and Antarctica (Figure 1.4).
Cassava is consumed by an estimated 500 million people in the tropics and subtropics. Neurological disease associated with cassava is reported exclusively from Africa where high dietary reliance, incomplete detoxification and protein malnutrition appears to be causal.

Cassava is eaten by some Hispanics and African Americans in the United States and in Europe, cassava products are incorporated into popular food items such as hamburgers. The plant is known by a variety of names: cassava in English-speaking Africa; manioc in Francophone Africa, yucca in Latin America, and tapioca in Asia.

**CASSAVA PRODUCTION AS HUMAN FOOD**

Cassava ranks fourth on the list of major food crops in developing countries, after rice, wheat, and corn (FAO, 1989). In sub-Saharan Africa, cassava is ranked second after corn (Nweke, 1994) and in central African countries such as Democratic Republic of Congo (former Zaire), it is a staple food. As such, cassava is the most important tropical root-crop. Its starchy roots are a major source of dietary energy for over 500 million people (Cock, 1982; 1985). In sub-Saharan Africa, cassava is mainly a subsistence crop grown by small-scale farmers. It feeds about 200 million people daily, nearly half of the continent's population (Madeley, 1993). Protein content of cassava root is low.
Cassava production has nearly doubled in Africa in the last two decades (de Bruijn and Fresco, 1989), although per capita food production has been declining. Current trends indicate that cassava cultivation is spreading to semi-arid areas and the consumption of cassava is increasing. The introduction of disease-and pest-resistant varieties released by the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, has increased cassava production in many African countries. Nigeria is the largest cassava producer in the world (FAO, 1989). Approximately 90% of the cassava produced in Africa is used for human consumption. In Thailand, however, a large proportion of the cassava produced is exported to the European community to be used in animal feed formulations.

Cassava has been widely adopted by African peasants because of its agricultural advantages. The main advantages are higher yields per acre as well as per unit of labor compared to other cereals under similar conditions. It is a better converter of solar energy to carbohydrates (Cock, 1985) than other cereals. Cassava adapts easily to traditional farming systems and is productive even on poor soils where other staple crops fail (Figure 1.5). It resists locust damage and is tolerant of many pests. It has no critical planting or harvesting periods. The roots can be kept in the ground (with limited need for weeding) and harvested when needed. Cassava is easily propagated by stem cuttings, i.e. the planting material is not edible and this adds to cassava’s advantages as a famine crop. Thus, cassava is of great importance for food security in Africa.

Figure 1.5 Cassava field where other staples will not grow. (IITA, Ibadan 1990).

The bitter varieties grow especially well in challenging environmental conditions. With modest growth in sandy soil type (left) and luxuriant growth in the Guinea Savannah vegetation zone (right). The underground tuber may be left in place and harvested when needed.
CASSAVA CYANOGENESIS

Despite its importance as a source of food for over 500 million people, cassava produces two cyanogenic glucosides, linamarin and lotaustralin in about 10 to 1 ratio (Bokanga, 1994a), that potentially will cause health problems. The glucosides are synthesized mainly in the leaves and transported to the roots (Bokanga, 1995, Du et al., 1995). The main glucoside, linamarin, is synthesized from the amino acid valine, while lotaustralin is synthesized from isoleucine (Nartey, 1981). The glucosides are restricted to the cytoplasm. Linamarase, a β-glucosidase capable of hydrolyzing the cyanogenic glucosides is located on the external surface of the cell wall (Bokanga et al., 1994). Breakdown of cyanogenic glucosides and the release of cyanide takes place only when the cell wall is ruptured. When cassava tissues are damaged and cellular structures disrupted, linamarin and lotaustralin come in contact with linamarase and are hydrolyzed. The hydrolysis of linamarin and lotaustralin leads to formation of glucose and cyanohydrins (Conn, 1969). If the pH is above 5, the cyanohydrins will spontaneously break down into ketones and hydrogen cyanide (HCN) (O’Brien, 1992), Figure 1.6.

![Cyanogenesis in cassava](image)

Figure 1.6 Cyanogenesis in cassava.

The cyanogenic glucoside linamarin, which is stored in the plant intracellularly is cleaved enzymatically when cell walls are broken (plant bruising, crushing). The same process occurs with lotaustralin which has an additional methyl group on the carbon with asterix. Acetone cyanohydrin is stable at low pH; at higher pHs, it will spontaneously break down and liberate hydrogen cyanide.
This breakdown may also be catalyzed by the enzyme hydroxynitrile lyase which is also present in cassava. Once HCN is produced, it will dissolve in water or evaporate into the air since its boiling temperature is 25.7 °C (O'Brien et al., 1992). The term cassava cyanogenesis refers to the process by which destruction of plant cells of cassava leads to release of cyanide from its natural content of cyanogenic glucosides (Rosling and Tylleskar in Press). The glucosides, cyanohydrins and hydrogen cyanide, are collectively known as cyanogens. Cyanogenic potential refers to the total concentration of all compounds that are converted to and measured as cyanide ions.

CASSAVA PROCESSING

The levels of glucosides vary 25-fold between different genetic varieties and adverse environmental factors such as drought and poor soil increase the levels of glucosides (Bokanga, 1994b). The high concentrations of glucosides in the bitter roots can be toxic to humans and animals if they are eaten in an insufficiently processed form (Hahn, 1989). Cassava cultivars that produce non-bitter roots with low glucoside levels can be eaten fresh or boiled directly. Such cultivars are usually referred to as 'sweet cassava'. Farmers prefer to grow the pest-resistant bitter variety in communities where cassava is the main staple food as this form is considered to provide better food security (Essers, 1992). Cassava roots rot within 3-4 days of harvesting and must be processed before transportation from rural to urban areas for marketing (Nweke, 1994).

There are three main reasons for processing cassava roots: to lengthen their shelf-life, to reduce their water content and thus facilitate transportation, and to reduce cyanogens to safe levels. Processing improves palatability and is important from a nutritional point of view. It can either reduce or increase the content of proteins, vitamins and minerals in cassava products (Mlingi, 1995). A wide range of processing methods for cassava is used in Africa (Ugwu, 1992). Bitter cassava roots are usually processed according to local customs, preferences, and the availability of resources such as water, firewood and sunshine (Figure 1.7).
Soaking of poisonous plant products to leach out toxic principles is used by a variety of non-industrialized human groups.

Each processing method includes a few of the following techniques: peeling, crushing, milling, slicing, grating, soaking, heaping, stacking, boiling, steaming, sedimentation, removal of water by pressing, decanting, sundrying, smoke-drying and frying (Nweke, 1994). Various combinations of these techniques result in different end-products. The final products obtained from processing are known by different local names; three groups are used in Nigeria: steam-boiled paste, known as akpu; flour form, known as alubo; and granules, known as gari.

Soaking of roots for three or more days followed by sundrying is the most common processing method for bitter cassava (Mlingi, 1995). During soaking, several microorganisms ferment the roots, thus softening the pulp; this is accompanied by increased hydrolysis of the cyanogenic glucosides by the endogenous linamarase. Traditional
soaking for more than three days and nights has been found to be the most effective way to remove cyanogens from bitter cassava roots (Oke, 1994).

EFFECTS OF EXPOSURE TO CYANOGENS FROM CASSAVA

Cyanide exposure from cassava has been associated with several health disorders in humans (Rosling and Tylleskar, 1995). Epidemiological studies suggest that exposure to cyanogens from cassava products with high levels of cyanogens is a major or contributory factor in diabetes Type III (Bell and Hockaday, 1996), iodine deficiency disorders (IDD) (Bourdoux et al., 1978), tropical ataxic neuropathy (TAN) (Osuntokun, 1972, Osuntokun, 1981), protein-energy malnutrition (PEM) (Vis et al., 1982), acute cassava poisoning (Mlingi, 1992; Akintowa, 1994) and konzo (Tylleskar et al., 1992b). The extent of cyanide exposure from cassava consumption depends on the content of cyanogenic glucosides in the fresh roots and leaves, the degree of removal of cyanogens during processing, the frequency of consumption of cassava products, and the amounts consumed at each meal. The effects also depend on the duration of exposure, the proportion of dietary cyanogens that decompose to cyanide in the gut, and the cyanide detoxification rate in the body. The occurrence of diseases and symptoms also depends on the presence of other nutritional deficiencies and unknown factors relevant to individual susceptibility. Consumption of cassava as a component of a varied nourishing diet has not been associated with any form of chronic disease.

ACUTE CASSAVA POISONING

The risk of acute poisoning from eating insufficiently processed bitter cassava is well known in all communities where bitter cassava is grown. Cases of acute poisoning are said to be rare despite high consumption of cassava roots that are bitter and toxic when harvested (Banea, 1997). There are few reports of acute cassava poisoning in the literature; blood cyanide level has been documented in only one report (Akintowa, 1994). The unspecified symptoms occur between 1-2 hours following a meal of insufficiently processed bitter cassava. General symptoms include malaise, dizziness, vertigo, vomiting, diarrhea, and weakness. In severe cases, there is collapse and death within hours; however, lethal cases are rare and all symptoms resolve within 12-24 hours leaving no sequelae (Mlingi, 1992). The typical delay between intake of cassava and onset of symptoms is compatible with the cyanide exposure resulting from release of cyanide in the gut from ingested cyanogens. It is not known if acute poisoning results mainly from
ingestion of glucosides, cyanohydrins, or cyanide itself. Outbreaks of acute poisoning from cassava ingestion have been reported mainly when a population that normally eats non-bitter cassava starts to eat bitter and toxic cassava roots without having learned safe processing. Even in populations that have been eating bitter cassava for generations, acute poisoning may nevertheless occur under special circumstances, such as when drought increases glucoside levels and, when shortcuts in processing are made, due to food shortage (Mlingi, 1992).

CYANIDE METABOLISM IN HUMANS

If cyanogens are not effectively removed during processing, the consumption of cassava may result in dietary cyanide exposure (Banea, 1997). The source of dietary cyanide from cassava may be ingested glucosides or cyanohydrins. These are then broken down to hydrogen cyanide in the gut. The fate of linamarin in the gastrointestinal tract depends on physico-chemical properties of the gut microflora and the activity of the hydrolyzing enzyme (β-glucosidase). Kamalu (1996) discussed possible mechanisms of linamarin toxicity in a recent review.

The human body has two well known defense mechanisms against cyanide (Way, 1983). When cyanide enters the blood stream from either the lungs or the gastrointestinal tract, it is trapped by the methemoglobin fraction in the red blood cells. This fraction normally constitutes about 1% of all hemoglobin, and it can reversibly bind about 10 mg of HCN as cyanomethemoglobin (Schultz, 1984). The second defense mechanism is the conversion of cyanide to thiocyanate (SCN) (Figure 1.8) catalyzed by the enzyme rhodanese (thiosulfate sulfur transferase EC 2.8.1.1) (Sorbo, 1953).
Figure 1.8  Formation of thiocyanate.

The conversion of cyanide to thiocyanate may follow two routes (i) enzymatic interaction with mercarptopyruvate (from cysteine via transamination reaction) to form thiocyanate (ii) by enzymatic interaction with thiosulfate (derived from mercarptopyruvate) via rhodanese and is the main pathway for cyanide detoxification in the body.

The rhodanese-catalyzed reaction requires a sulfane sulfur substrate. A sulfane sulfur is a divalent sulfur (-S-) covalently bonded to another sulfur atom. The substrate is provided from dietary sulfur amino acids through different metabolic pathways. Substrate availability is the rate-limiting factor (Lundquist, 1992) of rodanese activity. The detoxification process takes place in the liver and the kidneys. Thiocyanate is mainly found extracelluarly in blood, urine, saliva and in gastric juice (Ruddell et al., 1977). The thiocyanate ion is a pseudo-halide that is handled as chloride and iodide. It is rapidly filtered by the glomerulus and efficiently reabsorbed by the tubule (Schultz, 1984). Thiocyanate was used for controlling hypertension in the 1950s. Toxic manifestations, which include bone marrow depression, hypothyroidism, fatigue, anorexia and nausea occur at serum levels above 1,300-20,000 µmol/L (Barnett et al., 1951).

When cyanide exposure rates are greater than the conversion rate and cyanide saturates the methemoglobin pool, acute cyanide intoxication occurs. In this situation cyanide rapidly accumulates in plasma and attacks target organs such as the brain (Lundquist et al., 1985). Fatal toxicity of cyanide is believed to be due to energy failure of cells as cyanide inhibits cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport chain and blocks the oxidative phosphorylation (Pettersen and Cohen, 1993).
OTHER SOURCES OF CYANIDE EXPOSURE

There are several sources of cyanide exposure to humans beside consumption of insufficiently processed cassava. These include consumption of other cyanogenic plants such as linseeds and bitter almonds. In addition, fruit seeds such as apple and apricot contain cyanide. Fire gases from combustion of nitrogen-containing materials and tobacco smoke may also result in cyanide exposure (Hall et al., 1981). The anti-hypertensive drug sodium nitroprusside consists of 44% cyanide, which is liberated following administration of the drug (Schultz, 1984).

Hydrogen cyanide (HCN) and cyanogen bromide and chloride share many toxic properties. Cyanide as cyanyl chloride is used as a chemical warfare agent (Marrs and Maynard, 1995). Modern chemical weapons containing HCN have been produced and might be effective in the event of an effective strike in a restricted area. Hydrogen cyanide gas is used to fumigate ships and buildings and to sterilize soil. Other sources are the occupational exposures from alkyl-cyanides used as solvents or cyanide salts used industrially for metal cleaning and polishing. Because of its ability to form complexes with metals, cyanide is used in metallurgy, electroplating, and metal cleaning. In the home, cyanides are present in silver polish, insecticides, and rodenticides. The toxicity of laetrile, a once-popular "cancer cure", is due to its cyanogenic glycoside. Cytochrome P450-dependent monoxygenases liberate cyanide from organic nitriles as do glutathione S-transferases from organic thiocyanates (Klaassen, 1996). Combustion of nitrogen-containing plastics may result in release of HCN. Fire on board airlines killed 119 passengers in Paris in 1973 and 303 pilgrims in Riyadh, Saudi Arabia, in 1980 due to combustion of plastic material that produced HCN (Wegner, 1983). Cyanide was utilized for suicidal purposes by more than 900 religious cult members in Guyana in 1978 and for executions in gas chambers (Klaassen, 1996).

CLINICAL PRESENTATION OF CYANIDE TOXICITY

Cyanide is one of the most rapidly acting poisons; victims may die within minutes of exposure. Cyanide has a very high affinity for iron in the ferric state. When absorbed, it reacts readily with the trivalent iron of cytochrome c oxidase in mitochondria. Cellular respiration is thus inhibited, resulting in lactic acidosis and cytotoxic hypoxia. Respiration is stimulated because chemoreceptive cells respond to decreased oxygen. A transient stage of central nervous system (CNS) stimulation with hypernea and headache is observed. Finally hypoxic convulsions occur and death is due to respiratory arrest. Most people with acute exposure to cyanide usually die promptly or fully recover. However, cases of
neurological seqeulae including extrapyramidal syndromes, personality changes, and memory defects have been reported in survivors (Marrs and Maynad, 1995).

CYANIDE TOXICODYNAMICS AND MECHANISMS OF INTOXICATION

Cyanide intoxication is the result of a complex series of effects, with the primary sites of action in the CNS and cardiovascular system (Brierley et al., 1977, Hall et al., 1986). Several enzymes and cellular processes are altered by cyanide, and the manifestations of toxicity are the result of these multiple actions. Cyanide toxicity can be viewed from two perspectives: an acute life-threatening form and subacute or post intoxication sequelae (Table 1.3). In acute intoxication, cyanide produces a rapid inhibition of the enzyme activity of cytochrome c oxidase, resulting in an energy deficit within the target tissue (Isom and Way, 1974). Additionally, other enzymatic processes are inhibited and may contribute to toxicity (Ardelt et al., 1989). This includes the antioxidant defense enzymes (catalase, superoxide dismutase and glutathione peroxidase).

Table 1.3  Mechanisms of cyanide intoxication

<table>
<thead>
<tr>
<th>Acute life threatening</th>
<th>Subacute or post intoxication sequelae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of cytochrome oxidase</td>
<td>Prolonged energy deficit</td>
</tr>
<tr>
<td>Inhibition of multiple enzymes/processes</td>
<td>Loss of ionic homeostasis</td>
</tr>
<tr>
<td>Global stimulation of CNS</td>
<td>Activation of signaling cascades</td>
</tr>
<tr>
<td></td>
<td>Oxidative stress</td>
</tr>
<tr>
<td></td>
<td>Necrosis and/or apoptosis</td>
</tr>
</tbody>
</table>

CYANIDE ANTIDOTES

Different compounds have been used as cyanide antidotes (Marrs, 1988). These can be broadly classified into four groups based on their putative mechanisms of antagonisms. Sulfane sulfur compounds (prototype is sodium thiosulfate) are substrates
for rhodanese which converts cyanide to thiocyanate. Scavengers (e.g. nitrates) are compounds that inactivate cyanide by binding it or by forming methemoglobin, which in turn binds cyanide. Oxygen appears to be a physiological antagonist which may facilitate dissociation of cyanide from cytochrome c oxidase. The compounds classified as biochemical antidotes have largely unexplained mechanisms and their actions may be related to intracellular targets of cyanide other than cytochrome c oxidase.

**ACTIVATION OF NMDA (N-METHYL-D-ASPARTATE) RECEPTORS BY CYANIDE**

Potassium cyanide (KCN) can stimulate release of glutamate from intracellular stores resulting in elevation of cytosolic (Ca$^{2+}$) through NMDA receptor activation (Patel et al., 1992; 1994). Damage to neuronal cells of the retina has been attenuated with NMDA antagonists (Zeevalk and Nicklas, 1990). Cyanide may interact directly with the NMDA receptor to enhance NMDA receptor-mediated Ca$^{2+}$ influx (Patel et al., 1994; Sun et al., 1995). Oxidative stress also plays a role in cyanide neurotoxicity. Several antioxidant enzymes are inhibited by KCN and this contributes to oxidative damage (Ardelt et al., 1989; 1994). Johnson et al. (1997) proposed that increased intracellular Ca$^{2+}$ after KCN treatment generates reactive oxygen species (ROS) leading to lipid peroxidation and neuronal damage. Other oxidant species may be involved in the cytotoxic responses.

Nitric oxide (NO) is also generated during glutamate-mediated excitotoxicity associated with chemical hypoxia (Akira et al., 1994), and NO has been proposed to be a mediator of convulsions associated with cyanide toxicity (Gunasekar et al., 1996). It is possible that in cyanide toxicity, nitric oxide synthase (NOS) is activated by the increased intracellular Ca$^{2+}$ resulting in NO generation (Bredt and Snyder, 1992). Simultaneous production of NO and O$_2^-$ can give rise to the peroxynitrite anion which decomposes to hydroxyl radical (OH). OH$^-$ can then initiate lipid peroxidation and cause cellular damage (Beckman et al., 1990).

A better understanding of chemical mechanisms linked to outbreaks of konzo is needed to design protective programs. The hypothesis proposed in this dissertation is that a sulfur amino acids deficiency (as in malnourished populations) plays an important role in the metabolism of cyanide derived from cassava consumption. In well nourished individuals, cyanide is principally metabolized to non-neurotoxic SCN$^-$ which is excreted in urine. By contrast, in malnourished populations, cyanide detoxification may be impaired and cyanide may be preferentially metabolized to neurotoxic cyanate (Figure 1.9).
Figure 1.9 Hypothetical mechanism of cyanate formation from linamarin present in cassava (Manihot esculenta).

Linamarin is hydrolyzed to acetoxyanhydrin by the β-glucosidase (linamarase) when physical damage (bruising, crushing or gratting) occurs to the plant cell wall. High temperature (sundrying and heating) and low pH (soaking and fermentation) promote release of hydrogen cyanide into the air and may constitute a hazardous occupational exposure for cassava processors. The gut microflora β-glucosidase also releases cyanide from linamarin by hydrolysis.

Cyanide is absorbed gastrointestinaly and distributed systemically. In the blood, cyanide is sequestered to cyanomethemoglobin by cyanocobalamin (B₁₂) in a reversible reaction (methemoglobin trapping). Cyanide may also react directly with cysteine to form 2-amino thiazoline carboxylic acid, an excitant amino acid. In well-nourished subjects, cyanide is mainly detoxified to thiocyanate, a reaction that requires sulfane sulfur as sulfur amino acids (cysteine and methione) and is catalysed by rhodanese. However, in protein-malnourished populations, the sulfur amino acid deficiency may promote the formation of cyanate, a neurotoxic moiety. Impairment of the cysteine-dependent cyanase reaction (that converts cyanate to carbon dioxide and ammonia) (Anderson, 1980) will also exacerbate
accumulation of cyanate. Accumulation of cyanate in malnourished individuals may cause neuronal and/or axonal degeneration. Repeated daily exposure may lead to clinically evident neurodegenerative disease (TAN or konzo). The specific topic examined experimentally is conversion of cyanide to cyanate in normal and malnourished states.

**CYANATE NEUROTOXICITY**

Sodium cyanate (NaOCN) was used as an effective drug in the treatment of sickle cell anemia, but its use was discontinued due to its neurotoxic properties (Peterson et al., 1974). Prolonged treatment with NaOCN induces neuronal damage in cerebral cortex, basal ganglia, spinal cord and peripheral nerves of primates (Shaw, 1974), and a sensorimotor neuropathy in humans and rats (Peterson et al., 1974; Cerami et al., 1973). Exposure to cyanate has been considered an important risk factor for formation of cataracts in humans in India and England (Srivastava et al., 1993).

Chronic administration of OCN in high doses caused hind limb paralysis in rats (Alter et al., 1974) and spastic quadriplegia in monkeys (Shaw et al., 1974). Polyneuropathy manifested by severe motor weakness has been observed in sickle cell patients treated orally with OCN (Ohnishi et al., 1975). In addition to nerve fiber damage, OCN may inhibit nerve conduction velocity and elicit blockage of axonal transport by carbamylation of proteins (Samson and Hinkley, 1972). The neuromuscular system is specifically sensitive to cyanate toxicity. Prolonged administration of cyanate caused muscular damage and hind limb weakness (Cerami et al., 1973) in dogs. Crist et al. (1973) reported a dose-related decrease in learning ability in rats chronically treated with cyanate.

Cyanate is also etiologically implicated in a wide range of neurological diseases in protein-deficient subjects consuming the cyanophoric plant cassava as a staple food (Tylleskar 1994b). Cyanate carbamylates the amino group of N-terminal valine, inhibits cytochrome c oxidase (Cox) activity (Tor-Agbidye et al., 1995), uncouples oxidative phosphorylation in mitochondria (Cammer, 1982) and reacts readily with sulfhydryl (-SH) groups (Stark, 1963). However, the precise mechanisms of cyanate-induced neurotoxicity are unsolved.

Glutathione (GSH), α-L-glutamyl-L-cysteinylglycine is a tripeptide that plays a central role in the biotransformation and elimination of xenobiotics and the defense of cells
against oxidative stress (free-radical scavenger) (DeLeve and Kaplowitz, 1991; Orlowski and Karkowski, 1976). Glutathione (GSH) is a redox buffer present in all animal cells at high concentrations. It is derived from glycine, glutamate, and cysteine. The first step in glutathione synthesis is a condensation of the γ-carboxyl group of glutamate with the amino group of cysteine in an ATP-dependent reaction catalyzed by γ-glutamyl cysteine synthase (Figure 1.10a). The carboxyl group is first activated by ATP to form an acyl phosphate intermediate, which is then attacked by the cysteine amino group. The second step is similar with the carboxyl group of cysteine activated to an acyl phosphate to permit condensation with glycine. The chemical structure of GSH is that of the three constituent amino acids linked by peptide bonds (Figure 1.10b). The oxidized form of glutathione is basically two GSH molecules held together by a disulfide bond (Figure 1.10c).

![Figure 1.10](https://example.com/figure1.10.png)

Figure 1.10 Biosynthesis (a) and structure of reduced glutathione (b). The oxidized form of glutathione is shown (c).

GSH is the most abundant cellular low-molecular-mass thiol. Human erythrocytes contain 2 mM GSH, hepatocytes greater than 10 mM. Many of the reactions of GSH
involve the reactive sulfhydryl group, -SH. Cysteine is the key component and the rate-limiting amino acid in the GSH synthesis (Jain et al., 1995). Brain GSH arises by synthesis from its constituent amino acids (Jain et al., 1995). Cysteine is present in plasma and is transported to cells including the brain (Jain et al., 1995). Glutathione reductase (GR) and glutathione peroxidase (PD) are key NADPH-dependent enzymes that regulate GSH and GSSG homeostasis.

It is speculated that malnourished populations subsisting on cassava may have lower GSH levels than well nourished subjects since dietary intake of cysteine (cystine) will be reduced. Accumulation of cyanate following cyanide exposure may further reduce GSH levels in malnourished individuals. This thesis examined the effect of NaOCN on GSH concentrations in brain both in vitro and in vivo. The effect of NaOCN on glutathione reductase (GR) and glutathione peroxidase (GPx), enzymes which regulate GSH levels was studied. The impact of different doses of NaOCN on reduced and oxidized glutathione levels was also investigated in rat brain to elucidate the relationship between GSH homeostasis and the expression of cyanate neurotoxicity. Preliminary results of this study have been presented elsewhere (Sabri et al., 1996; Tor-Agbidye et al., 1995).

HYPOTHESES AND RATIONALE FOR EXPERIMENTAL STUDIES

Given that (a) the combination of cassava dependency and a protein-deficient diet is associated with slow onset of ataxic myeloneuropathy (Osuntokun, 1981), the subacute onset of spastic paraparesis (konzo) (Tylleskar et al., 1995; Essers et al., 1992) and the induction of neuronal/axonal disease in rats (Osuntokun 1970; Alter, 1974) and rabbits (Shenoy, Personal communication to Dr. Spencer), (b) non-detoxicated cassava generates cyanohydrins which form HCN, (c) KCN fails to induce neurological disease in rats, (d) NaOCN induces neuronal disease in rats, primates and humans (Tellez-Nagel et al., 1977; Ohnishi et al., 1975; Shaw et al., 1974), (e) use of incompletely detoxified bitter cassava precipitates sub-acute spastic paraparesis in young subjects (konzo), whereas dietary dependency of processed cassava is associated with an insidious onset of ataxic myeloneuropathy, and (f) primates receiving NaOCN (40 mg/kg/day i.m. for 6 weeks) develop spastic paraparesis and humans given NaOCN (44 mg/kg/day orally for 500 days) develop peripheral neuropathy, it follows hypothetically that:
(1) OCN⁻ is the proximate cause of neuronal/axonal disease (by an unknown mechanism which does not require conversion of OCN⁻ to CN⁻)

(2) decreased sulfur amino acids in the presence of KCN will increase OCN⁻

(3) blockade of CN⁻ to OCN⁻ conversion (via pyruvate, mercaptopyruvate etc.) will block neurological disease. Note this latter point is untestable because many factors affect CN⁻ conversion to OCN⁻.

The studies reported in this thesis were intended to test the hypothesis by comparing and contrasting the metabolic fate of potassium cyanide in rodents fed a sulfur-deficient diet or a sulfur-supplemented diet. Specific aims of this research are to:

(1) determine the fate of sulfur metabolism in animals maintained on a sulfur amino acid-free diet (Chapter 2)

(2) examine cyanide metabolism to cyanate in sulfur amino acid-deficient animals (Chapter 3)

(3) define the biochemical mechanisms involved with cyanate neurotoxicity using glutathione as a biomarker (Chapter 4)
CHAPTER 2: DIETARY DEFICIENCY OF CYSTINE AND METHIONE ALTERS SULFUR METABOLISM IN RATS

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SHORT TITLE: Dietary deficiency and sulfur metabolism

INDEXING KEY WORDS: rats, cassava cyanogens, glutathione, sulfur amino acids deficiency, urinary inorganic sulfate and thiocyanate.
ABSTRACT
Sulfur amino acid (SAA) deficiency has been proposed as a risk factor for human neurological diseases among protein-poor populations subsisting on the cyanophoric plant, cassava. Female Sprague-Dawley rats were used to develop and define a model of SAA deficiency for use in future studies examining cassava-related neurotoxicity. Rats were kept in metabolic cages for 7 to 21 days and fed a balanced diet (BD) of known composition or a diet selectively deficient in methionine and cystine (SAA-free diet). SAA-deficient rats lost significant body weight, failed to thrive, excreted porphyrinic materials, and showed a steep reduction of urinary inorganic sulfate which approached zero by day 3. By contrast, rats fed the BD gained weight, thrived, and maintained steady output of urinary inorganic sulfate. Urinary thiocyanate excretion did not differ between groups over the 3-week period, but plasma thiocyanate concentrations were twice as high in SAA-deficient rats on day 21. Increased plasma thiocyanate suggests mobilization of sulfur amino acids from endogenous sources. Brain glutathione (GSH) levels were increased in animals on BD by ~30%. In summary, a diet free of methionine and cystine results in increased retention of inorganic sulfur as thiocyanate and a near absence of inorganic sulfur excretion in urine.
INTRODUCTION

Cassava (*Manihot esculenta*) is consumed by several hundred million people in the tropics and sub-tropics (Rosling 1996), and some Hispanics and African-Americans in the continental United States. In Europe, imported cassava has been used in animal feed and food products. Neurologic disease, goiter and diabetes mellitus Type III (tropical diabetes Z-type) have been linked to high dietary intake of cassava (Moore, 1937, Bourdoux et al., 1978, McMillan and Greevarghese, 1979, Bell and Hockaday, 1996). Cassava contains cyanogenic glucosides which liberate hydrogen cyanide upon hydrolysis in the gastrointestinal tract (Montgomery, 1980; Essers et al., 1995). Ingestion of raw cassava precipitates headaches, nausea and malaise. Continued consumption of incompletely detoxified cassava materials is associated with outbreaks of spastic paraparesis (*konzo*, *Mantakassa*) (Essers et al., 1992, Tylleskar, 1994) and ataxic myeloneuropathy (Osuntokun, 1981; Roman et al., 1985) in protein-poor populations.

The mechanisms leading to neurologic deficits in cassava-consuming populations are unknown. One plausible hypothesis holds that the normal detoxification of dietary cyanide to thiocyanate is diverted to cyanate (a neurotoxic moiety) in sulfur amino acid (SAA)-deficient subjects (Tylleskar, 1994). The normal conversion of cyanide to thiocyanate is facilitated by the sulfur amino acids cysteine and methionine and catalyzed by the enzyme rhodanese (Sorbo, 1975). Thiocyanate levels in the urine and blood are used as a biomarker of cyanide exposure in humans (Lundquist et al., 1992). In malnourished subjects, in whom sulfur amino acid intake is deficient, cyanide conversion to thiocyanate may be impaired. In this setting, cyanide may be converted to cyanate, which causes neuronal degeneration in rodents, primates, and humans (Cerami et al., 1973; Alter et al., 1974; Tellez-Nagel et al., 1977, Shaw et al., 1974, Ohnishi et al., 1975).

SAA are the major precursors for the synthesis of key intracellular molecules, such as reduced glutathione (GSH). GSH, one of the most prevalent cellular thiols, sequesters free radicals and thus plays an important role in protection from reactive oxygen species (Ravindranath and Reed, 1990). Glutathione (GSH), a tripeptide (cysteine, glycine and glutamate) is involved with several reactions that protect and detoxify xenobiotics in the body. Since the concentration of cysteine is the rate-limiting step in GSH synthesis, a SAA-deficient diet will result in decreased levels of brain GSH.
The present study is designed to examine sulfur metabolism in rats maintained on a SAA-free diet accomplished by selective exclusion of cystine and methionine. These amino acids are rapidly depleted from tissue pools (Baker, 1987). Urinary inorganic sulfate was used as a biomarker of dietary intake of the sulfur amino acid pool. The effect of SAA-deficiency on brain GSH concentrations was investigated. Thiocyanate, the main end-product of cyanide metabolism, was determined in urine and plasma (Lundquist et al., 1995). GSH concentrations were determined in the brain and liver of rats. Preliminary results of this study have been presented elsewhere (Tor-Agbidye et al., 1995 and 1996; Sabri et al., 1996).

MATERIALS AND METHODS

Chemicals
Amberlyst A-21 (20-50) mesh, barium chloride, polyethylene glycol 600, sodium sulfate and sodium hypochlorite were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium thiocyanate was obtained from Baker (Phillipsburg, NJ), and sodium perchlorate was purchased from EM Science (Gibbstown, NJ). All other chemical reagents were analytical grade.

Animals and Diet
Fifty female Sprague-Dawley rats (250-300 g) were obtained from Bantin and Kingman, Seattle, WA, and used in these experiments. Female rats were selected because cassava-related disease has been reported mostly in women of child-bearing age. Rats were housed in standard cages in a temperature-controlled environment on a 12h/12h light-dark cycle, and given free access to rat chow and tap water. Rats were acclimated for 3 days, weighed and randomly assigned to one of two groups: (a) those receiving a balanced diet of known chemical composition (Table 2.1) containing all required nutrients (Harlan Teklad, Madison, WI) and (b) those on the same diet minus the sulfur amino acids, L-cystine and L-methionine (SAA-free diet) (Table 2.2). The vitamin composition of the diet is shown in (Table 2.3). The sulfur analysis of the diets (a) and (b) performed by Hazleton laboratories (Wisconsin) showed concentrations of 0.26% and 0.015% (w/w), respectively.
Experimental Protocol
Rats were weighed, individually kept in metabolic cages and fed either diet for 7 to 21 days. Animal behavior and body weights were recorded daily. Daily water intake and urine output were measured at a uniform time (10 00h.). Urine samples were coded and refrigerated prior to analysis for inorganic sulfate and thiocyanate. Plasma thiocyanate levels were measured in rats maintained for 21 days. Rats were anesthetized with isofluorane prior to the intracardiac collection of blood in sterile (Vacutainer) tubes containing sodium heparin (Becton Dickinson, Rutherford, NJ). Plasma was separated by centrifugation at 2500 x g for 10 min and stored at -84 °C until thiocyanate determination. Rats were perfused with 0.9% NaCl solution for 5 min to avoid blood contamination. The brain was removed and frozen immediately at -84 °C for GSH analysis.

Inorganic Sulfate Determination in Urine
Urinary inorganic sulfate levels were determined spectrophotometrically by the method of Lundquist and colleagues (1980). The method is based on a turbidimetric estimation of insoluble barium sulfate formed from the reaction between soluble barium chloride and urinary sulfate. Turbidity is measured at 600 nm. Aliquots of urine (100 μL) were diluted with double-distilled water to 3 mL, to which were added 1 mL HCl, 0.5 mol/L and 1 mL barium-polyethylene glycol mixture. A reagent blank was prepared in which urine was replaced with an equal volume of water. All assays were performed in duplicate without knowledge of the source of the sample. The amount of inorganic sulfate in the samples was read against a standard curve prepared with known quantities of sodium sulfate. Results were expressed as μmol inorganic sulfate excreted/(kg/24 h).

Thiocyanate Determination in Urine and Plasma
Thiocyanate (SCN⁻) was determined according to the improved spectrophotometric method described by Lundquist and colleagues (1995). A 500-μL aliquot of urine or plasma was diluted with 5.0 mL of 1.0 M NaOH and applied to a 2.5 x 0.7 cm column of Amberlyst-A21. The column was washed three times with 5-mL portions of water, and the thiocyanate eluted with 8.0 mL of 1 M sodium perchlorate. To a 4-mL aliquot of the eluate was added 0.2 mL of 0.35 M acetic acid and the solution mixed on a vortex mixer. The chlorinating reaction was performed by adding 0.1 mL of 50 mM sodium hypochlorite, and the sample was again mixed. Within 2 min, 0.6 mL of the color reagent (isonicotinic acid and 1,3-dimethyl-barbituric acid mixture) was added. The amount of thiocyanate in the
samples was read against a standard curve prepared with known quantities of sodium thiocyanate and the results expressed as μmol SCN\(^{-}\) excreted/(kg/24 h). This method affords a simple, rapid and sensitive assay for SCN\(^{-}\) and has a lower detection limit of 0.93 μmol/L (Lundquist et al., 1995).

**GSH Determination in Brain and Liver**

Brain tissue was homogenized in 0.25 M sucrose (20% w/v) using a Wheaton glass homogenizer (8 up and down strokes) on ice. A portion of the tissue homogenate was centrifuged at 1,000 x g for 5 min to remove the nuclear fraction and unhomogenized cell debris. The supernatant was centrifuged at 10,000 x g for 10 min to obtain a crude mitochondrial fraction (P-2 fraction). The P-2 fraction was resuspended in 0.25 M sucrose. Aliquots of the suspended P-2 fraction were mixed with an equal volume of cold 10% TCA to precipitate protein and the mixture placed on ice for 5 min. Precipitated protein was removed by centrifugation at 4,000 x g for 5 min. GSH was determined in the supernatant by a slight modification of the method of Ellman (1959). Briefly, 900 μL of 0.90 M, K\(_2\)HPO\(_4\) (pH 9.3) were added to the TCA supernatant (200μL), and 20 μL of DTNB reagent (36.9 mg DTNB in 10 ml of 0.1 M K\(_2\)HPO\(_4\), pH 7.0) were added. Absorbance of the yellow color was read at 412 nm in a Perkin-Elmer spectrophotometer. A standard curve of commercially available GSH (3-30 nmol) was generated under similar conditions to determine GSH concentrations in tissue samples. GSH levels were normalized to the tissue protein determined by the method of Lowry et al. (1951).

**Statistical Analysis**

Data are expressed as means ± SEM. Differences between two groups were considered significant at p < 0.05 using unpaired Student t-test.
RESULTS

**Body Weight Changes**

Body weight changes in BD and SAA-deficient animals was highly significant (p-value 0.001). Rats fed a balanced diet gained body weight throughout the experimental period of 3 weeks (Figure 2.1). In contrast, rats fed the SAA-free diet failed to gain, lost significant body weight. After one week on this diet, these rats had lost about 30 g body weight, and this downward trend continued up to 3 weeks when they had lost about 20% of their starting body weight. A brownish-yellow material collected around the eyes and on the fur of the neck, where hair loss (alopecia) was also prominent. Urine from rats fed the SAA-free diet was dark-red or 'port-wine' in appearance. Inspection of both urine and ocular secretions with UV radiation revealed a yellow fluorescence consistent with the presence of porphyrins (Meyer, 1988).

**Urinary Inorganic Sulfate**

Urinary inorganic sulfate excretion was measured daily for 7 days (Figure 2.2). Levels on day 1 (baseline) were similar in the two groups of rats (29.4 ± 5.1 and 32.6 ± 3.8 mmol inorganic sulfate/(kg/24 h) for balanced and SAA-free diets, respectively. On day 2, urinary inorganic sulfate levels in rats on the SAA-free diet showed a significant difference (p-value 0.001) and a precipitous drop while those on the balanced diet were close to the baseline level. The by day 3, mean inorganic sulfate excretion in the SAA-free group approached zero, a level that was maintained through day 7. The major group difference in the pattern of sulfate excretion seen at 7 days was maintained for 3 weeks (Figure 2.3). Water consumption and urine output did not differ in the two dietary groups (Table 2.4).

**Urinary Thiocyanate**

Urinary SCN\(^{-}\) levels in the two groups of rats after 7 days are shown (Figure 2.4).

Baseline SCN\(^{-}\) levels on day 1 were similar in both groups, with a mean value of 5.7 ± 1.0 and 6.5 ± 1.1 µmol/(kg/24 h) for balanced and SAA-free diets, respectively. Urinary SCN\(^{-}\) levels increased after day 1, peaked at day 3 and decreased thereafter in both groups. On day 7, urinary SCN\(^{-}\) levels in both groups decreased to 4.7 ± 1.5 and 4.3 ± 0.6 µmol/(kg/24 h) in balanced and SAA-free diets respectively. Some rats were also
maintained on either a SAA-free or balanced diet for 3 weeks. Urinary SCN⁻ excretion at 3 weeks was reduced to ~25% of baseline values in both groups of rats (Figure 2.5).

**Plasma Thiocyanate**

Plasma SCN⁻ levels were determined in rats maintained for 3 weeks on either the BD or SAA-free diet. The mean plasma SCN⁻ level in rats on BD was 464 ± 50 μmol/(kg/L), compared to 885 ± 60 μmol/(kg/L) in animals on the SAA-free diet.

**Brain GSH**

Brain GSH concentrations was determined in animals maintained for three weeks on either the BD or SAA-free diet (Table 2.5). A significant difference was observed in brain GSH concentrations between the groups (p-value < 0.0038). Mean GSH concentrations were 18.67 ± 0.7 and 13.24 ± 0.4 nmol GSH/mg protein in animals on BD and SAA-free diet, respectively.

**Liver GSH**

Liver GSH concentrations from animals maintained for three weeks on either the BD or SAA-free diet was determined. There was no difference in liver GSH concentrations between the two groups (Table 2.6). Mean GSH concentrations in liver were 29.00 ± 2.2 and 24.9 ± 2.5 nmol GSH/mg protein in animals on BD and SAA-free diet, respectively.
Figure 2.1  Body weight change in 3 weeks.

Female Sprague-Dawley rats were fed for 3 weeks either a balanced diet containing 0.26% sulfur or a SAA-free diet containing 0.015% sulfur (n=10 rats/group). Data are mean ± SEM.

* Significantly different from 0-day (p<0.001).
Female rats were fed for 7 days either a balanced diet or a SAA-free diet (n= 10 rats/group). Urinary inorganic sulfate was determined as described in Methods. Data are mean ± SEM.
Female rats were fed for 3 weeks either a balanced or a SAA-free diet. Urinary inorganic sulfate was determined as described in Methods. Data are expressed as % control with baseline values (week 0) taken as 100% (n=10 rats/group).

* Significantly different from week 0 (p< 0.003)
Figure 2.4  Urinary thiocyanate for 7 days

Female Sprague-Dawley rats were fed for 7 days either a balanced diet or a SAA-free diet (n= 10 rats/group as in Figure 2). Urinary thiocyanate was determined as described in Methods. Data are mean ± SEM.
Figure 2.5  Urinary thiocyanate for 3 weeks

Thiocyanate concentrations were determined from rats fed rat chow (these values were taken as baseline at 0 week i.e. before animals were switched to respective diet). Urinary thiocyanate was determined as described in Methods. Data are expressed as % control with baseline values (week 0) taken as 100% (n= 10 rats/group).

* Significantly different from week 0 (p < 0.003).
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>g/Kg *</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>12.1</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>6.0</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>40.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.3</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>4.5</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>11.1</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>18.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>7.5</td>
</tr>
<tr>
<td>L-Proline</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Serine</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>1.8</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>5.0</td>
</tr>
<tr>
<td>L-Valine</td>
<td>8.2</td>
</tr>
</tbody>
</table>

* Balanced diet has same amino acid composition plus 3.5 g/Kg of L-Cystine and 8.2 g/Kg of L-Methionine
Table 2.2  Dietary compositions of SAA-free

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>g/Kg *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>521.2002</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>150.0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>100.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>15.0</td>
</tr>
<tr>
<td>Vitamin Mix (Table 3)</td>
<td>10.0</td>
</tr>
<tr>
<td>Ethoxyquin (antioxidant)</td>
<td>0.02</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic (CaH₂PO₄)</td>
<td>22.0</td>
</tr>
<tr>
<td>Potassium Citrate, monohyd.</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>2.6</td>
</tr>
<tr>
<td>Magnesium Oxide (MgO)</td>
<td>0.85</td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>0.25</td>
</tr>
<tr>
<td>Manganese Carbonate</td>
<td>0.10</td>
</tr>
<tr>
<td>Zinc Carbonate</td>
<td>0.056</td>
</tr>
<tr>
<td>Cupric Carbonate</td>
<td>0.012</td>
</tr>
<tr>
<td>Chromium Chloride (CrCl₃·6H₂O)</td>
<td>0.011</td>
</tr>
<tr>
<td>Potassium Iodide (KI₃)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Sodium Selenite (Na₂SeO₃·5H₂O)</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

* Same for BD
Table 2.3  Vitamix composition of SAA-free

<table>
<thead>
<tr>
<th>Vitamin Mix</th>
<th>SAA-free diet (mg) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic Acid</td>
<td>110.10</td>
</tr>
<tr>
<td>Ascorbic Acid, coated (97.5%)</td>
<td>1016.60</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.44</td>
</tr>
<tr>
<td>Vitamin B$_{12}$ (0.1% tituration)</td>
<td>29.70</td>
</tr>
<tr>
<td>Calcium Pantothenate</td>
<td>66.10</td>
</tr>
<tr>
<td>Choline Dihydrogen Citrate</td>
<td>3496.90</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1.98</td>
</tr>
<tr>
<td>Inositol</td>
<td>110.10</td>
</tr>
<tr>
<td>Menadione (Vit K)</td>
<td>49.50</td>
</tr>
<tr>
<td>Niacin</td>
<td>99.10</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>22.00</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>22.00</td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>22.00</td>
</tr>
<tr>
<td>Dry Vitamin A Dry Palmitate</td>
<td>39.65</td>
</tr>
<tr>
<td>Dry Vitamin D$_{3}$ (500000 U/g)</td>
<td>4.40</td>
</tr>
<tr>
<td>Dry Vitamin E Acetate (500 U/g)</td>
<td>242.30</td>
</tr>
<tr>
<td>Corn Starch (diluent)</td>
<td>4666.90</td>
</tr>
</tbody>
</table>

* Same formula for BD
Table 2.4  Water intake and urine output in animals fed BD or SAA-free diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Water intake, (mL/24 h)</th>
<th>Urine output (mL/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced</td>
<td>30 ± 5.1</td>
<td>17 ± 3.5</td>
</tr>
<tr>
<td>SAA-Free</td>
<td>26 ± 5.1</td>
<td>16 ± 2.6</td>
</tr>
</tbody>
</table>

Rats were fed either a balanced diet or a SAA-free diet for 7 to 21 days as described in the Methods. Water intake and total urine were measured daily at a fixed daily time (10 a.m.). Data are mean ± SEM (n= 10) recorded over a 24-h period.

Table 2.5  Glutathione levels in brain of animals on BD or SAA-free diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>nmol GSH/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>18.67 ± 0.71</td>
</tr>
<tr>
<td>SAA-free</td>
<td>13.24 ± 0.44 *</td>
</tr>
</tbody>
</table>

Brain was removed and the homogenate was prepared, and GSH was determined as described in Methods. Data are mean ± SEM (n= 10).

* Significantly different (p= 0.0038) from animals on BD.

Table 2.6  Glutathione concentrations in liver of animals BD or SAA-free diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>nmol GSH/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>29.00 ± 2.4</td>
</tr>
<tr>
<td>SAA-free</td>
<td>24.89 ± 2.5</td>
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</tbody>
</table>

Liver was removed and the homogenate was prepared, GSH was determined as described in Methods. Data are mean ± SEM (n= 10). 10
DISCUSSION

A diet free of methionine and cystine reliably and reproducibly reduced inorganic sulfate excretion in urine. Inorganic sulfate excretion dropped precipitously and approached zero in rats within 3 days of commencing the SAA-free diet. The sharp and rapid decrease in urinary inorganic sulfate suggests the existence of only a small pool of available endogenous sulfur. These results are consistent with and extend the finding of Swenne and colleagues (1996) who showed lowered urinary inorganic sulfate excretion and weight loss in rats fed a low-protein diet. Inorganic sulfate is the major end product of sulfur metabolism and is excreted mainly via the kidney in mammals (Baker 1987). Inorganic sulfate, therefore, is an important contributor to the useful sulfane pool of a diet, and its urinary concentration reflects the dietary intake of sulfur amino acids.

A sulphane sulfur substrate (methionine or cysteine) is needed for the enzymatic detoxication of CN⁻ to SCN⁻. The sulfur donor is the rate-limiting factor for conversion of CN⁻ to SCN⁻ (Lundquist et al., 1980). While rats on a SAA-free diet predictably would have shown reduced SCN⁻ levels, at 3 weeks plasma SCN⁻ levels in these rats were double those on the balanced diet. Additionally, fluctuations of urinary SCN⁻ levels were observed across all three dietary groups for up to 7 days of treatment. Urinary SCN⁻ excretion increased at day 3, returned to baseline values on day 5, but continued to decrease progressively up to 3 weeks when excretion was only 25% of baseline in both groups. Thus, despite major reductions in sulfur intake, the critical metabolic process of sequestering toxic free CN⁻ in the form of SCN⁻ was maintained and even increased in the SAA-free group. Since the sole exogenous source of cyanide for both groups of rats is cyanocobalamin (vitamin B₁₂), which is present in equivalent concentrations in the two diets, the elevated level of plasma SCN⁻ in the SAA-free group is noteworthy and suggests increased endogenous mobilization of both thiol and CN⁻ moieties. Urinary SCN⁻ levels were unchanged in these rats. By contrast, in human populations reliant on cassava, SCN⁻ is markedly increased and plasma levels are somewhat increased (Ermans et al., 1980).

Rats maintained on the SAA-free diet significant body weight in contrast to the weight gain seen in rats on the balanced diet. Jain and associates (1995) have reported that rats fed a
SAA-deficient diet containing 0.25% L-methionine for 3 weeks failed to gain body weight. Loss of body weight is also reported in rats fed a low protein diet (~5% protein) for up to 9 weeks (Swenne et al., 1996). Feeding a low-protein diet has been shown to produce a marked decrease in body weight in males relative to that in females (Warnet et al., 1987). Protein breakdown due to increased proteolysis may explain the loss of body weight seen in rats fed SAA-free diet. The loss of body weight in SAA-fed rats may be directly attributable to the absence of methionine because this amino acid serves as the precursor for cysteine. However, excess dietary methionine is reported to modify lipid and lipoprotein metabolism, causing depressed growth, hemolytic anemia, pancreatic damage and many other disorders (Baker, 1987; Serougne et al., 1987; Sugiyama et al., 1997). Sulfur amino acids are required for the growth and maintenance of keratoid tissues such as skin and hair. Hair loss of the type seen in rats on the SAA-free regimen has been attributed to cystine deficiency (Baker, 1987).

SAA are the major precursors for the synthesis of key intracellular molecules, such as reduced glutathione (GSH). GSH, one of the most prevalent cellular thiols, sequesters free radicals and thus plays an important role in protection from reactive oxygen species (Ravindranath and Reed, 1990). We observed a significant difference in brain GSH concentrations between SAA-deficient animals and the group on BD (p-value < 0.0038). The higher concentration of brain GSH in animals on BD supports the role of cysteine concentrations as a limiting factor in GSH synthesis. Whereas animals on BD had adequate cysteine in the diet, SAA-deficient animals lack this amino acid resulting in lower brain GSH concentration in these animals. GSH concentrations in the liver however, did not differ between the two groups of animals. A higher GSH pool exists in the liver and would readily account for changes within the period that was under investigation.

While the red discoloration of urine, feces and body fur has been noticed as an incidental finding in rodent neurotoxicity and nutritional studies, including studies of rats on a SAA-deficient diet (Jain et al., 1995), the presence of porphyrins in the colored material has been poorly appreciated. A possible explanation for the excess porphyrins in SAA-deficient rats may be explained by deficient intake of cystine and methionine, both of which are normally used in GSH synthesis. If GSH synthesis is compromised by a lack of cystine, there may be a concomitant increase in glutamate and glycine since all three amino acids are used in the GSH tripeptide. Since glycine is a precursor in the synthesis of porphyrins (Lehninger et al., 1993) excess glycine may increase porphyrin synthesis in SAA-deficient rats.
In summary, a diet free of methionine and cystine results in increased retention of inorganic sulfur as thiocyanate and a near-total absence of inorganic sulfur excretion in urine. This rat model may prove useful to study pathophysiological changes resulting from selective SAA-malnutrition. It will be of particular value to test the hypothesis that cassava neurotoxicity in protein-poor populations results in people unable to detoxify CN\(^{-}\) via the SCN\(^{-}\) pathway.

Acknowledgements

The authors are grateful to Ms Barbara Lystrup for her technical assistance. The study was supported in part by the Du Pont Foundation.
REFERENCES


CHAPTER 3: CYANIDE METABOLISM IN SULFUR AMINO ACIDS DEFICIENT RATS

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ABSTRACT

Cyanide Metabolism in Sulfur-Deficient Rats: Relevance to Understanding Mechanisms of Neurological Disease in Humans Subsisting on Cassava.

Neurological disorders have been reported from parts of Africa with protein-deficient populations and attributed to dietary cyanide (CN\(^{-}\)) exposure from cassava roots. Cyanide is normally detoxified to thiocyanate (SCN\(^{-}\)). However, in protein-malnourished subjects where sulfur amino acids (SAA) are deficient, CN\(^{-}\) may be converted to cyanate (OCN\(^{-}\)), a neurotoxic moiety. This study investigates the metabolism of CN\(^{-}\) in rats fed for up to 4 weeks a balanced diet (BD) or a diet lacking SAA, L-cystine and L-methionine. In both groups, there is a time-dependent increase in plasma cyanate. Cyanate concentrations are markedly elevated in SAA-deficient rats. At four weeks, cyanate concentrations in the BD and SAA-free rats are 29.1 ± 6.71 and 113.4 ± 8.46nmol/L, respectively. A positive linear relationship between blood cyanide and plasma cyanate concentrations is observed (r = 0.93, 2-sided p-value << 0.001) in rats fed SAA-free diet. By contrast, such correlation is not observed in rats fed BD (r = 0.17, 2-sided p-value = 0.61). Urinary SCN\(^{-}\) levels rose ten-fold over baseline values in both groups within the first week of CN\(^{-}\) exposure and remain elevated up to 4 weeks. Urinary inorganic sulfate in SAA-deficient rats fell close to zero at weeks 3 and 4; while levels increased markedly in rats fed BD. Rats fed SAA-free diet consumed less food and progressively lost body weight. This rat model has provided data that would illuminate the mechanisms of neurological diseases.
INTRODUCTION

An estimated half-billion people in tropical and sub-tropical climes consume the processed roots and leaves of the cyanophoric plant cassava (*Manihot esculenta*) (Rosling, 1996). In certain parts of Africa, cassava is the main source of food for populations prone to protein-calorie malnutrition. The combination of dietary cyanide and protein-calorie deficiency has been implicated in outbreaks of neurological disease in these populations. One form of disease is a slowly developing ataxic neuropathy originally described in Nigeria (Osuntokun, 1968, 1973, 1981); the other is a sub-acute disease manifested principally by spastic paraparesis (Cliff et al., 1985, Howlett et al., 1990, Tylleskar et al., 1994; Rosling, 1996). The development of these syndromes is hypothesized to depend on (a) the amount and duration of exposure to dietary cyanide and (b) the ability of the body to detoxify cyanide, a function that may vary with nutritional status.

Free cyanide must be sequestered and metabolized to avoid blockade of mitochondrial electron transport and energy failure. Following an acute exposure, cyanide is first trapped by hemoglobin in the form of methemoglobin (Schultz, 1984). Cyanide is then converted to thiocyanate (SCN") by the major detoxification pathway, a reaction which requires sulfane sulfur as a rate-limiting cofactor for the enzyme rhodanese (Lundquist, 1992). The concentration of sulfane sulfur is dependent on the availability of sulfur amino acids (SAA) from dietary protein (Cliff et al., 1985; 1995). Even in severe protein malnutrition, available sulfur is preferentially utilized for cyanide detoxification (Swenne et al., 1996). Cyanide may also be sequestered by albumin or metabolized to aminothiazoline carboxylic acid (ATC) (Lundquist et al., 1995) or to OCN" which, in turn, is converted by the cystine-dependent enzyme cyanase to ammonia and carbon dioxide (Schultz, 1949). Since cyanate given exogenously induces neuropathy in humans and rodents, and spastic paraparesis in macaques (Ohnishi, 1975; Tellez-Nagel, 1977, 1979; Shaw, 1974), it is plausible that CN" must be converted to OCN" for neurological disease to develop.

We propose that formation of cyanate is promoted in SAA deficiency. The present study employs a rodent model of sulfur amino acid SAA deficiency to examine this question.
MATERIALS AND METHODS

Chemicals
Analytical-grade perchloric acid, sodium hydroxide, acid silver sulfate, barbituric acid, sulfuric acid and potassium cyanide; barium chloride, polyethylene glycol 600, sodium sulfate, sodium hypochlorite (Sigma, St. Louis, MO), potassium thiocyanate (Baker, Phillipsburg, MJ), and sodium perchlorate (EM Science, Gibbstown) were used.

Experimental design
The experiment was designed to assess the effect and possible interaction between diet (SAA-free & BD) and time (1, 2, 3 & 4 weeks). Four rats were randomly allocated to each of the eight possible treatment combinations (a 2x4 factorial experiment set off in a completely randomized design with replication). Analysis of variance was used on all responses as a first step, and if time could be modeled as a continuous covariate without disrupting the quality of their fit, multiple regression was then applied. All analysis were done using S-Plus 3.1 (Stat-Sci, 1995).

Rats diets and treatment
Female Sprague-Dawley rats (250-300g) three months old were obtained from Bantin and Kingman, Seattle, WA. Rats were housed in standard cages in a temperature-controlled environment on a 12h/12h light-dark cycle. Thirty six rats were allowed to acclimate (one rat per cage); after seven days of acclimatization on standard rat chow; 8 rats were randomly selected, placed individually in metabolic cages overnight, and 24-h urine collected to determine baseline values for the concentration of thiocyanate and inorganic sulfate. Blood was collected intracardially (n=4) and separated into 2 portions, one for determination of baseline values for cyanide and the second portion was centrifuged at 2500 xg rpm for 10 min at 4 °C, plasma was collected and baseline concentrations of cyanate determined accordingly.

Eight rats were randomly divided into two groups (4 rats per week for up to 4 weeks = 16 per group), kept individually in regular cages and fed one of two experimental diets (Harlan Teklad, Madison, WI). The diet for Group I rats had adequate levels of all required amino acids, while the diet for Group II rats lacked the sulfur amino acids L-cystine and L-methionine (Table 3.1). The composition of diet and vitamin mixture are given in Table
3.2 and Table 3.3, respectively. The concentration of cyanocobalamin (a cyanide source, not shown) was comparable (22 µg/kg) in the two experimental diets. One hundred mL of double distilled water containing potassium cyanide adjusted to a target dose of 50 mg/kg body weight was administered to all rats *ad libitum*; every 2 days, consumption was measured and a fresh solution of cyanide provided. Potassium cyanide solution was found to be stable up to 48 h.

Rats were maintained for up to 1-, 2-, 3-, or 4-week periods. The health of the rats was observed and their body weights recorded weekly. At the end of each week, 8 rats (4 from Group I and 4 from Group II) were transferred into individual metabolic cages. Urine samples were collected from each rat at a uniform time (10 00h), and refrigerated at -20 °C prior to analysis for inorganic sulfate and thiocyanate. Prior to animal termination at week 1 (n=8), week 2 (n=8), week 3 (n=8), and week 4 (n=8), blood samples were collected by cardiac puncture, and divided into two parts; one part was treated with acidified silver sulfate and frozen at -20 °C for cyanide analysis; the other was centrifuged at 2500 xg for 10 min and the plasma separated and frozen at -84 °C for cyanate analysis.

**Urinary inorganic sulfate**

Urinary inorganic sulfate was determined spectrophotometrically by the method of Lundquist et al. (1980). The method is based on a turbidity estimation of insoluble barium sulfate formed from the reaction between soluble barium chloride and urinary sulfate. Turbidity is measured spectrophotometrically at 600 nm. Aliquots of urine were diluted to 3 mL with double-distilled water; 1 mL HCl and 1 mL barium polyethylene glycol were added to the solution. A reagent blank was prepared in which urine was replaced with an equal volume of double-distilled water. All assays were performed in duplicate. The amount of inorganic sulfate in the samples was read against a standard curve with known concentrations of inorganic sulfate. Data were expressed in mmol inorganic sulfate/mL.

**Urinary thiocyanate**

Urinary thiocyanate levels were determined spectrophotometrically by the improved method of Lundquist et al. (1995). The method is based on a colorimetric estimation, the depth of blue color formed from the reaction between the color reagent (a mixture of isonicotinic acid and 1, 3-dimethyl-barbituric acid) and urinary thiocyanate. The intensity of the color is measured by absorbency at 607 nm. Briefly, aliquots of urine (500 µL) diluted with 5.0 mL of 1M NaOH were applied to columns (2.5 x 0.7 cm) of Amberlyst A-21. Columns
were washed 3 times with 5 mL of double-distilled water before eluting thiocyanate by the addition of 8 mL of 1M sodium perchlorate. Aliquots of 4 mL of eluate were acidified with 0.2 mL of 0.35M acetic acid, chlorinated for 2 min with 0.1 mL of 50mM sodium hypochlorite, and 0.6 mL of the color reagent added to the mixture. A reagent blank was prepared in which urine was replaced with double-distilled water. Assays were performed in duplicate. Thiocyanate concentrations in the samples were read against a standard curve with known concentrations of potassium thiocyanate. Data were expressed in nmol thiocyanate/mL.

**Blood cyanide**

Blood cyanide concentrations were estimated spectrophotometrically by the method of Lundquist et al. (1985). This method is based on the Konig reaction which produces a chromophore from cyanide. This method employs a simple aeration apparatus consisting of two side-arm tubes (150 x 25mm and 150 x 20mm fitted with a 1-hole rubber stopper carrying a 7-mm glass inlet tube) connected by a short length of rubber latex tubing. Aliquots of blood (1.0 mL) treated with 10 mL of 20mM acid silver sulfate were placed into the large side-arm tubes along with five drops of anti-foam agent. Four mL of 0.1M NaOH were placed in the smaller side-arm tube and 5 mL of 11M H2SO4 was added to the sample. The liberated HCN was transferred through a sodium hydroxide solution by nitrogen (purified by passage through a gas washing bottle containing 0.1M of NaOH) at a flow rate of 0.5 L/min. After 2 hours aeration, the nitrogen flow was turned off, stoppers were removed from the NaOH traps, and 0.4 mL of 2M acetic acid added and mixed briefly before the further addition of 0.1 mL of 5mM sodium hypochlorite. The solution was mixed again, and 0.5 mL of barbituric acid-pyridine reagent added within 1 min. Absorbency at 580 nm was determined 5-15 min. later. A blank (10 mL acid silver sulfate reagent) was prepared, and cyanide concentrations in the samples were read against a standard curve with known concentrations of potassium cyanide. Cyanide concentrations were expressed in μmol/L.

**Determination of cyanate**

Cyanate concentration in plasma was determined by the method of Lundquist et al. (1993). Cyanate was converted to compound Q (2,4 [1H, 3H]-quinazolinedione) and quantified by reverse-phase high-performance liquid-chromatography (HPLC). The authenticity of compound Q was verified spectrofluorometrically by excitation and emission wavelengths, retention times on HPLC (Figure 3.1), and by gas chromatography mass spectrometry (GC-MS) (Figure 3.2). The GC-MS of plasma from control animals
(i.e. maintained on chow diet) was determined as a positive control. One mL plasma from control animals was derivitized and subjected to GC-MS (Figure 3.3). Also, 1 mL plasma was spiked with 1 nmol potassium cyanate, derivatized to Compound Q and subjected to GC-MS (Figure 3.4). A typical chromatograph of the plasma sample from rats fed SAA-free diet for 3 weeks is shown (Figure 3.5) with a retention time for Compound Q comparable to that in Figure 3.1.

In order to derivatize cyanate to Compound Q, one mL of plasma was diluted with 0.5 mL of 0.2M sodium phosphate buffer (pH 7.4) and the pH adjusted to 4.7 with 4M acetic acid. This mixture was reacted with excess 40 mM 2-aminobenzoic acid (pH adjusted to 4.7 with few drops of sodium acetate) and incubated for 10 min at 40 °C. The reaction was stopped by cooling on ice. Cyclization of the intermediate product was performed by addition of 6M sulfuric acid and heating at 100 °C for 2 min followed by cooling on ice. Compound Q was extracted three times (3x) with ethyl acetate; the organic phases were pooled, and dried under nitrogen at 40 °C. The dried product was then reconstituted with 2 mL of HPLC-grade water, acidified with 60 µL 5.0M hydrochloric acid, and transferred onto PRS columns to remove excess 2-aminobenzoic acid. The column was washed two times with 2-mL portions of HPLC-grade water, 0.5 mL 0.1M sodium phosphate buffer (pH 7.4) added, pH was adjusted to 6-8 with 3M sodium hydroxide, and applied to a Sepak C-18 column. The column was washed with 3 mL HPLC-grade water, compound Q was eluted with 3 mL methanol, dried at 60 °C under nitrogen, dissolved in methanol:water (1:2) and subjected to HPLC. Plasma cyanate concentrations in the samples were calculated from a standard curve prepared with known concentrations of potassium cyanate. Cyanate concentrations were expressed in nmol/L.
RESULTS

Feed intake

Rats were fed two experimental diets for 1, 2, 3, or 4 weeks. Rats on the BD ate an average of 108 g/week (95% confidence interval from 95.6 to 120.3 g) while those rats on SAA-free diet consumed an average of 44.7 g less per week [t (28) = -5.26 2-sided p-value < 0.001] (Figure 3.6). At week 4, cumulative feed intake in rats on BD was 506.75 ± 13.7 while SAA-deficient rats consumed only 300.0 ± 20.5 g of diet. There was general weakness in rats on SAA-free diet. Rats in this group (SAA-free) excreted a brownish to yellow discoloration around their neck and were hyperreflexive to noise.

KCN intake

Total KCN intake was calculated at 9, 15, 23 and 25 days past the initiation of the experiment and can be modeled as a quadratic function of the elapsed time. For rats on a BD, the quadratic relationship is concave down (1-sided p-value = 0.0088) although the maximum value for the function is not observed within the first 25 days of the experiment. Rats on a SAA-free diet show a weak quadratic relationship that is concave up (1-sided p-value = 0.109). At nine days, there is no difference in mean amount of KCN accumulated up to that point (2-sided p-value = 0.681). At fifteen days, the rats on the balanced diet were estimated to be drinking an average of 110 mL more than those rats on SAAF (95% CI from 85.4 to 136 mL more). By 23 days after the starting date, the difference had lessened to only 77.4 mL (95% CI from 59.8 to 95.0 mL more). An additional two days saw the difference close to 33.6 mL which was not a substantial difference (2-sided p-value = 0.235). Total intake of KCN water for each diet is shown (Figure 3.7).

Body weight changes

A significant difference in mean rates of weight gain was found between rats on the two diets after accounting for baseline body weight [t(27) =8.49 2-sided p-value < 0.001] Figure 3.8. Rats on the balanced diet experienced an average increase of 11.8 g/week (95% confidence interval from 7.28 to 16.37 g) while those rats on a SAA-free diet lost body weight at a mean rate of 14.3 g/week (95% confidence interval from 9.93 to 18.7 g).
Urine output

Rats on the balanced diet had 3.31 mL greater urine output than rats fed a SAA-free diet (95% confidence interval from 2.63 to 3.99 mL). Urine output was not affected by duration of treatment (F=1.87 on 3 and 24 degrees of freedom, p-value= 0.162) but was highly influenced by the diet regimen (F=101.5 on 1 and 24 df, p-value << 0.001) (Table 3.4).

Inorganic sulfate

Urinary sulfate in rats fed balanced diet had more than doubled by week 1 and remained at this level up to week 4; the equivalent value for rats fed SAA-free diet decreased by 50% of the original value. Inorganic sulfate concentration continued to decline until close to zero at week 3 (Figure 3.9). Analysis of urinary sulfate showed that rats on the balanced diet maintained a constant median value of 752 mmol inorganic sulfate/mL over the four weeks. At the same time, those rats on SAAF diet exhibited a 48 percent decrease in median urinary sulfate for each additional week (95% CI is from 38.2 to 61.2% decrease).

Urinary thiocyanate

Urinary thiocyanate concentrations followed a similar temporal pattern in both groups of rats (Figure 3.10). Baseline values in both groups corresponded to 8 nmol/SCN/mL. Both displayed a marked increase in mean thiocyanate excretion coincident with the introduction of potassium cyanide in drinking water. Average urinary thiocyanate concentrations, which were approximately 5-times baseline values, were comparable to those at weeks 2, 3, and 4 in both groups. Analysis showed that urinary thiocyanate did not differ between the two diet groups, and there was no statistical difference between thiocyanate levels and cumulative potassium cyanide intake.

Blood cyanide

Blood cyanide levels (µmol/L) showed a definite increase with time (Table 3.5). Levels of the blood cyanide increased an average of 29.4 µmol/L per week (95% CI from 13.6 to 45.2 µmol/L). This rate of increase did not differ for the two diet types (2-sided p-value = 0.565) nor did the initial i.e. Week 2 levels of blood cyanide differ for the two diets (2-sided p-value = 0.506).
Plasma cyanate

Plasma cyanate levels exhibited a non-linear trend with respect to time for both treatment groups (Figure 3.11). Although it is clearly evident that cyanate levels in the SAA-deficient rats were significantly higher than those on balanced diet at weeks three and four, there was no difference in median levels at week one \( t (28) = -0.324 \), 2-sided p-value =0.75. Median levels of plasma cyanate at week two for the SAA-free group were estimated to be 44 percent higher compared to the balanced diet group (95 % confidence interval for this multiplicative effect was from 3.7 to 10 percent). Overall, median plasma cyanate levels for the BD group increased 1.79 times for each additional week the rats remained on the treatment (95 % confidence interval for this multiplicative effect was from 1.48 to 2.16). Similarly, the median plasma cyanate levels for the SAA-free group increased 2.80 times with each passing week (95 % confidence interval was 2.31 to 3.39 times).

Median cyanate levels for those rats on rat chow (5.0 ± 0.8 nmol/L) did not differ significantly from either treatment group (at week 1), 4.88 ± 1.5 and 4.91 ± 1.0 nmol/L for BD and SAA-free, respectively. However, both treatment groups were clearly greater than the control (chow diet) at weeks two, three and four. At week 2 plasma cyanate concentrations increased in the two groups of rats to 16.82 ± 5.4 and 19.23 ± 2.47 nmol/L in rats on BD and SAA-free diet respectively. By 3 weeks of treatment, plasma concentrations had risen in both groups of rats, with approximately two-fold higher concentrations in rats fed the SAA-free diet (2-sided p-value < 0.001) (Table 3.6). At four weeks, plasma concentrations were approximately 4-fold higher in the SAA-free group (2-sided p-value << 0.001).

Correlation analysis reveals that there is a substantial positive linear relationship between blood cyanide and plasma cyanate concentrations \( r = 0.927, 2\text{-sided p-value} << 0.001 \) for those rats placed on the SAA-free regimen (Figure 3.12). For those rats on a balanced diet, there was a weak, insignificant linear relationship between blood cyanide and plasma cyanate \( r = 0.166, 2\text{-sided p-value} = 0.61 \).
Compound Q was synthesized in our laboratory and subjected (10 pmol) to reverse phase high performance liquid chromatography (HPLC). The retention time for compound Q is 15.01 min.
Figure 3.2  Gas Chromatography-Mass Spectrophotometry (GC-MS) of compound Q

The mass spectrum of the compound Q shows 92, 119 and 162 mass ions. The molecular mass of compound Q was confirmed as 162 (a) and its chemical structure is shown in (b).
Figure 3.3 GC-MS of the plasma from control rats (chow diet)

One mL of plasma was derivatized to compound Q as described in Methods. The mass spectrum corresponding to the presence of mass ions at 92, 119 and 162 similar to those of authentic compound Q is not shown (i.e. normal cyanate is not detectable).
Figure 3.4  GC-MS of the plasma from control rats spiked with a known concentration of cyanate.

One mL of plasma was spiked with 1 nmol potassium cyanate and derivatized to compound Q as described in Methods. The mass spectrum corresponding to compound Q is shown by an arrow (a) and the respective mass ions are shown in (b). The mass spectrum shows the presence of mass ions at 92, 119 and 162 similar to those of authentic compound Q.
Figure 3.5  
A typical chromatograph of the plasma from SAA-deficient rats

One mL of plasma was derivatized to compound Q as described in Methods. The retention time (15.03 min) is comparable to that for authentic compound Q. Other small peaks were not identified and may represent plasma contaminants. Plasma cyanate concentrations were determined from a standard curve prepared as described in Methods.
At timepoint 0, rats were transferred from standard rat chow to one of two customized diets: a balanced diet (BD) and or SAA-free diet. Measurements of food intake for the four rats per group were taken approximately 1, 2, 3, or 4 weeks past timepoint 0. Values are the mean ± SEM.
Rats were fed BD and SAA-free diet; the dose of KCN administered via drinking water was adjusted for the number of rats at each timepoint: week 1, n=32; week 2, n=24; week 3, n=16, and week 4, n=8. Rats on a SAA-free diet show a relationship that is basically linear (1-sided p-value = 0.109, test of quadratic term). Values are the mean ± SEM.
Rats were maintained on experimental diets for 1-, 2-, 3- or 4-week periods. Positive and negative values reflect increased and decreased body weights relative to baseline values at time 0. The number of rats declined weekly from 32 (week 0) to 8 (week 4). Body weights of rats on BD and SAA-free diet differ significantly (2-sided p-value < 0.001). Values are the mean ± SEM.
Urine was collected over a 24-hour period once weekly from rats on BD and SAA-free diet (n=4/group). Rats received potassium cyanide for 1, 2, 3, or 4 weeks. Baseline value (n=8) is 330 ± 15 mmol inorganic sulfate/mL urine. At week 4 urinary inorganic sulfate concentrations in rats on BD were 757.4 ± 26 mmol inorganic sulfate/mL, while inorganic sulfate levels in urine of SAA-free rats dropped to 25.5 ± 14 mmol inorganic sulfate/mL urine. Urinary inorganic sulfate was determined as described in Methods. Values are the mean ± SEM.
Thiocyanate concentrations were determined as described in the Methods. Baseline mean value (n=8) is 4.0 ± 0.8 nmol/SCN/mL urine. At week 4 thiocyanate concentrations in the two groups were similar, (e.g. 42 ± 3 and 44 ± 6.0 nmol/SCN/mL in BD and SAA-free groups, respectively). Values are the mean ± SEM.
Rats were fed rat chow, balanced diet and SAA-free diet. Plasma cyanate concentrations were determined as described in the Methods. Median cyanate levels for those rats on chow diet (control) did not differ significantly from either treatment (BD or SAA-free) group at week one \( [F = 0.79 \text{ on } 31 \text{ df with two sided } p\text{-values } = 0.44 \text{ and } 0.72, \text{ respectively}] \). A significant difference between the two treatment groups was seen at weeks 2, 3 and 4 (2-sided \( p\)-value = 0.038, < 0.001, and << 0.001, respectively). Values are the median ± SE(median).
Figure 3.12  Concentration-response relationships of blood cyanide and plasma cyanate

Plasma cyanate and blood cyanide concentrations in rats on BD (n=16) and SAA-free diet (n=16) were analyzed by linear regression analysis. There is substantial positive linear relationship between blood cyanide and plasma cyanate concentrations for those rats placed on the SAA-free regimen ($r = 0.927$, 2-sided p-value $<< 0.001$). A non significant linear relationship is shown in rats on BD cyanate ($r = 0.166$, 2-sided p-value = 0.61).
Table 3.1  Amino acid composition of SAA-free diet

<table>
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<tr>
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<tr>
<td>L-Asparagine</td>
<td>6.0</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>40.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.3</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>4.5</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>11.1</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>18.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>7.5</td>
</tr>
<tr>
<td>L-Proline</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Serine</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>1.8</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>5.0</td>
</tr>
<tr>
<td>L-Valine</td>
<td>8.2</td>
</tr>
</tbody>
</table>

* In BD plus 3.5 g/Kg of L-Cystine and 8.2 g/Kg of L-Methionine
Table 3.2  Dietary compositions of SAA-free diet

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>g/Kg *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>521.20</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>150.0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>100.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>15.0</td>
</tr>
<tr>
<td>Vitamin Mix (Table 3)</td>
<td>10.0</td>
</tr>
<tr>
<td>Ethoxyquin (antioxidant)</td>
<td>0.02</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic (CaHPO₄)</td>
<td>22.0</td>
</tr>
<tr>
<td>Potassium Citrate, monohyd.</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>2.6</td>
</tr>
<tr>
<td>Magnesium Oxide (MgO)</td>
<td>0.85</td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>0.25</td>
</tr>
<tr>
<td>Manganous Carbonate</td>
<td>0.10</td>
</tr>
<tr>
<td>Zinc Carbonate</td>
<td>0.056</td>
</tr>
<tr>
<td>Cupric Carbonate</td>
<td>0.012</td>
</tr>
<tr>
<td>Chromium Chloride (CrCl₃,6H₂O)</td>
<td>0.011</td>
</tr>
<tr>
<td>Potassium Iodide (KI₃)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Sodium Selenite (Na₂SeO₃.5H₂O)</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

* Same formula for Balanced diet
### Table 3.3  Vitamix composition of SAA-free diet

<table>
<thead>
<tr>
<th>Vitamin Mix</th>
<th>SAA-free diet (mg) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminobenzoic Acid</td>
<td>110.10</td>
</tr>
<tr>
<td>Ascorbic Acid, coated (97.5%)</td>
<td>1016.60</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.44</td>
</tr>
<tr>
<td>Vitamin B₁₂ (0.1% titration)</td>
<td>29.70</td>
</tr>
<tr>
<td>Calcium Pantothenate</td>
<td>66.10</td>
</tr>
<tr>
<td>Choline Dihydrogen Citrate</td>
<td>34.96</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>1.98</td>
</tr>
<tr>
<td>Inositol</td>
<td>110.10</td>
</tr>
<tr>
<td>Menadione (Vit K)</td>
<td>49.50</td>
</tr>
<tr>
<td>Niacin</td>
<td>99.10</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>22.00</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>22.00</td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>22.00</td>
</tr>
<tr>
<td>Dry Vitamin A Dry Palmitate</td>
<td>39.65</td>
</tr>
<tr>
<td>Dry Vitamin D₃ (500,000 U/g)</td>
<td>4.40</td>
</tr>
<tr>
<td>Dry Vitamin E Acetate (500 U/g)</td>
<td>242.30</td>
</tr>
<tr>
<td>Corn Starch (diluent)</td>
<td>4666.90</td>
</tr>
</tbody>
</table>

* Same formula for Balanced diet
Table 3.4  Total urine output (mL/24h)

<table>
<thead>
<tr>
<th>Weeks on Diet</th>
<th>Balanced diet</th>
<th>SAA-free diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.8 ± 0.8</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>5.2 ± 0.3</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>6.8 ± 0.5</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>5.5 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

Rats were fed rat chow, a balanced diet and SAA-free diet. Urine was collected individually from 8 rats at a uniform time weekly as described in Methods. Values are means ± SEM (n=8).

Table 3.5  Blood cyanide (µmol/L)

<table>
<thead>
<tr>
<th>Weeks on diet</th>
<th>Balanced diet</th>
<th>SAA-free diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.0 ± 2.0</td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td>3</td>
<td>38.0 ± 12.0</td>
<td>40.0 ± 5.0</td>
</tr>
<tr>
<td>4</td>
<td>60.0 ± 21.0</td>
<td>81.0 ± 11.0</td>
</tr>
</tbody>
</table>

Blood was collected intracardially (n=8) from rats on chow diet (Week 0), BD and SAA-free (n=8) at Weeks 1, 2, 3 and 4 for determination of cyanide concentrations. Note values for week 1 are missing because the sample for this period was lost during assay procedures. Blood cyanide concentrations were determined as described in Methods. Values are means ± SEM.
Table 3.6  Plasma cyanate (nmol/L)

<table>
<thead>
<tr>
<th>Weeks on Diet</th>
<th>Balanced diet</th>
<th>SAA-free diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.9 ± 1.5</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>16.8 ± 5.4</td>
<td>19.2 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>20.7 ± 4.6</td>
<td>36.7 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>29.1 ± 6.7</td>
<td>113.4 ± 8.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Rats (n=4) fed BD and SAA-free diet were given KCN in drinking water for 1, 2, 3, or 4 weeks. Plasma was obtained as described in Methods. Plasma concentration in control rats (n= 8) maintained on chow diet and not exposed to cyanide was also determined. Cyanate concentration was assayed by reverse phase HPLC as described in Methods. Values are the median ± SE(median) [Standard error based on 100 Bootstrap replications ].

<sup>a</sup> p-value < 0.038  <sup>b</sup> p-value << 0.001  <sup>c</sup> p-value << 0.001.
DISCUSSION

The early and dramatic rise in urinary thiocyanate, a phenomenon equally apparent in both groups of rats, confirms that enzymatic conversion of cyanide to thiocyanate is a major method of cyanide detoxification in the rodent as in humans. This conversion is dependent on the availability of sulfur. The marked loss of body weight in cyanide-treated rats fed a SAA-free diet, a process concurrent with the dramatic rise in urinary thiocyanate, suggests the mobilization of endogenous sulfur principally from the breakdown of body proteins. Rats fed a balanced diet were able to increase their body weight presumably because dietary sulfur intake was sufficient to detoxify the cyanide as thiocyanate. The dramatic rise in urinary sulfate may in part reflect a higher concentration of dietary methionine in the balanced diet relative to that in the standard chow on which rats were acclimated. The marked decline of mean urinary sulfate concentrations in rats fed SAA-free diet by week 3 suggests the presence of a sulfur shortage and the possibility that mobilization of endogenous sulfur had begun. This interpretation is consistent with the similar mean body weights recorded at week 3. Sulfur shortage in rats fed a SAA-free diet was already evident at the end of week 1. However, even at week 3, when urinary sulfate was undetectable, thiocyanate levels were maintained at 5-times baseline values. The apparent modest decline in thiocyanate levels in both groups of rats at week 4 likely reflects the somewhat reduced cyanide intake between week 3 and week 4. Taken together, these observations strongly suggest that CN/SCN⁻ conversion is a priority detoxification pathway in the rat. This is consistent with observations in protein deficient rats treated orally with acetonitrile (Sweene et al., 1996).

The low level of blood cyanide in rats at week 2 with concomitant increase in thiocyanate levels, suggests that trapping of cyanide by conversion of methemoglobin to cyanomethemoglobin is not the priority detoxification pathway. This is contrary to the conventional view which holds that methemoglobin sequestration of cyanide is the more rapid detoxification method. While cyanomethemoglobin is a rapid buffer system in vitro (Lundquist et al., 1985), the present experiments suggest this mechanism comes into play only after CN/SCN⁻ conversion pathway has been saturated. The steady increase of total blood cyanide in both groups of rats suggests that the methemoglobin/cyanomethemoglobin conversion is an effective mechanism for the development of cyanide tolerance in rodents. The present experiment apparently was terminated before the limit of cyanide tolerance was
reached; the point at which this occurs would likely correspond to the sudden appearance of free plasma cyanide (Lundquist et al., 1985) and consequent effects on the central nervous system.

Based on the trend for higher total cyanide levels in rats fed a SAA-free diet, it seems likely these rats would have been the first to exhibit detectable free blood cyanide. A comparable process presumably occurs in cassava-dependent humans who have measurable free blood cyanide at the time of the sudden onset of leg weakness (konzo) (Tylleskar et al., 1992). By contrast, neurological deficits are not recognized in well-nourished populations which consume cassava as a regular dietary component.

The precise cause of konzo is unlikely to be free cyanide since this can be converted to two other neurotoxic compounds, aminothiazoline carboxylic acid and cyanate (Lundquist et al., 1993, 1995). Plasma cyanate increased in both groups of rats, but rats fed a SAA-free diet showed markedly higher levels of this neurotoxic species. The steep increase in plasma cyanate in SAA-deficient rats between weeks 3 and 4 is noteworthy; given that the onset of konzo in humans is sudden and non-progressive. It is plausible that the sudden onset of the disease may correspond to elevated plasma cyanate levels and subsequent neuronal damage via an excitotoxic mechanism. Repeated administration of the anti-sickling drug sodium cyanate to humans, primates and rodents results in spinal cord and peripheral nerve damage (Shaw et al., 1974, Tellez et al., 1977, 1979); and the carboxylic acid ATC acts as a glutamate-like excitatory amino acid which destroys nerve cells (Isom et al., 1995).

While levels of the amino acid ATC were not determined in this study, Swenne et al. (1996) reported the plasma concentration increased in rodents fed a protein deficient diet. It is noteworthy that lathyrism, a disease with clinical features closely similar to those of konzo, is attributed to an unusual excitatory amino acid (β-N-oxalylamino-L-alanine) found in the Grass pea (Lathyrus sativa), a protein-rich legume consumed by certain northern African populations (Abegaz et al., 1994).

Cassava leaves and roots harbor the cyanide-releasing glucoside linamarin. Research conducted in Nigeria examined the fate of pure linamarin (30g/100g body weight) administered in food to 4 groups of female and male Wistar rats: (a) rats deficient in dietary vitamin B₁₂, (b) rats with sufficient vitamin B₁₂, (c) malnourished rats, and (d) rats fed a nourishing diet (Philbrick et al., 1977, Umoh et al., 1986). Estimates were made of
cyanide, intact linamarin and thiocyanate in urine and feces at 0, 24, 48, and 72 h periods after linamarin administration. Results showed higher rates of excretion of cyanide, unmetabolized linamarin and thiocyanate in well-nourished rats at 24 hours. After 48 hours, the malnourished rats excreted higher concentrations of linamarin metabolites in urine relative to those from well-nourished rats. There were no detectable levels of linamarin in fecal or blood samples (drawn at 72 hours). In agreement with the present findings, the Nigerian study shows that protein-malnourished rats retain their ability to detoxify linamarin-generated cyanide to thiocyanate. Moreover, high levels of urinary thiocyanate were found in both malnourished and well-nourished rats, as in the present study. These findings support the view that thiocyanate excretion is a reasonable quantitative measure of cyanide exposure (Lundquist et al 1995). The present study shows that once a steady cyanide intake is established, this relationship holds true for at least several weeks of continuous potassium cyanide oral exposure.

Whereas our data demonstrate temporarily increasing blood cyanide concentrations in both groups given drinking water containing potassium cyanide, the Nigerian study was unable to detect cyanide levels in blood obtained 72 hours after the administration of a single dose of linamarin. This is consistent with the view that cyanide is rapidly converted in both well-nourished and malnourished rats to thiocyanate and not in the first instance to cyanomethemoglobin. We have demonstrated a significant increase in the plasma cyanate concentrations of SAA-free rats treated with cyanide for four weeks. This finding, supports our hypothesis that in SAA-deficient states (as in protein-calorie malnutrition), dietary cyanide exposure will culminate in higher cyanate concentrations leading to neurodegenerative disorders reported from parts of Africa.

**Acknowledgement**

The technical expertise and assistance of Juan Muniz with GC-MS analysis, is very much appreciated. This work was supported with NIH grant NS 19611.
REFERENCES


CHAPTER 4: STUDIES RELATING TO CASSAVA NEUROTOXICITY IN MALNOURISHED POPULATIONS: SODIUM CYANATE ALTERS GLUTATHIONE HOMEOSTASIS IN RODENT BRAIN

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E-mail: spencer@ohsu.edu
ABSTRACT

Sodium cyanate (NaOCN), an antisickling drug, is a neurotoxic agent in rodents, primates and humans (Gillette et al., 1974). Cyanate is also implicated in neurological disease in protein-deficient individuals subsisting on cassava, a cyanophoric plant. The mechanisms of cyanate-induced neuronal degeneration are unknown. To understand the possible mechanisms of cyanate-induced neurotoxicity, we investigated the effect of NaOCN on glutathione (GSH), an endogenous thiol involved in the detoxification of xenobiotics. Female CD-1 mice and Sprague-Dawley rats were administered purified NaOCN (i.p.). A time-dependent and dose-dependent decrease in brain GSH was observed in mice. The effect of NaOCN on glutathione reductase (GR) and glutathione peroxidase (Gpx), key enzymes in glutathione homeostasis was also studied. NaOCN inhibited GR activity in different brain regions; however, Gpx activity was refractory to even higher concentrations of NaOCN. Rats treated with 100 or 300 mg/kg NaOCN showed decreased levels of GSH. By contrast, oxidized glutathione (GSSG) levels were increased in selected brain regions (cerebellum, cortex, hippocampus and striatum) and in liver. GSH and GSSG levels in the liver of control rats were 37.30 ± 1.30 and 8.20 ± 0.60 nmol/mg protein, respectively. NaOCN induced a dose-related decrease in GSH/GSSG ratio both in liver and in selected brain regions. At 300 mg/kg, the striatum showed maximum decrease (~60%), followed by the cortex (~50%), cerebellum (~40%) and hippocampus (~30%). The ratio of GSH/GSSG in the liver showed a similar dose-related decrease (~65%). In summary, NaOCN reduces GSH levels and GSH/GSSG ratio in brain and liver, perhaps by inhibiting the activity of GR. Since GSH is involved in the detoxification of xenobiotics, we propose that cyanate neurotoxicity may be mediated in part by inhibition of GR activity.
INTRODUCTION

Sodium cyanate (NaOCN) was used as an effective drug in the treatment of sickle-cell anemia, but its use was discontinued because prolonged treatment with NaOCN induces neuronal damage in cerebral cortex, basal ganglia, spinal cord and peripheral nerves of Rhesus monkeys (Shaw et al. 1974) and sensorimotor neuropathy and cataracts in humans (Peterson et al. 1974; Srivastava et al., 1993). In rodents, treatment with low doses of cyanate causes weakness, spasticity of the hindlimbs, and seizures usually triggered by noise (Cerami et al., 1973). Chronic administration of high doses of NaOCN causes hindlimb paralysis in rats (Alter et al., 1974) and spastic quadriplegia in monkeys (Shaw et al., 1974). Crist and coworkers (1973) reported a dose-related decrease in learning ability of rats chronically treated with cyanate. Samson and Hinkley (1972) proposed that OCN-induced carbamylation of proteins causes reduction in nerve conduction velocity and blockade of axonal transport. Cerami et al.(1973) observed muscular tremors and weakness 6 months after cyanate administration to dogs.

Cyanate is also implicated in a number of neurological diseases, such as tropical ataxic neuropathy (TAN) and konzo, an upper motor neuron disease in protein-deficient subjects consuming the cyanophoric plant cassava (Manihot esculenta) (Osuntokun, 1981; Tylleskar, 1994; Rosling, 1996). Cyanate carbamylates the amino group of N-terminal valine, inhibits cytochrome c oxidase (Cox) activity (Tor-Agbidye et al., 1995), uncouples oxidative phosphorylation (Cammer, 1982) and reacts readily with sulfhydryl groups (Stark, 1963). It has been reported that cyanate reacts directly with glutathione and also inhibits the enzyme activity of ATPase, glucose-6-phosphate dehydrogenase and causes proteins to unfold (Harding, 1980).

We and others have postulated that in certain parts of Africa, where cassava (a carbohydrate-rich root tuber which harbors the cyanogenic glucoside, linamarin, and liberates hydrogen cyanide on hydrolysis) consumption is high and protein intake is poor, hydrogen cyanide is preferentially converted to cyanate, accumulation of which causes neuronal or axonal degeneration both in the central and/or peripheral nervous system (Tylleskar, 1994; Howllet et al., 1990, Tor-Agbidye et al., 1995, 1996). The mechanisms of sodium cyanate-induced neurotoxicity are unknown.
Glutathione (GSH), is a naturally occurring cysteine-containing tripeptide that plays an important role in the detoxification of xenobiotics (DeLeve and Kaplowiwzt, 1991; Orlowski and Karkowski, 1976). Since cysteine is the rate-limiting amino acid for the synthesis of GSH (Jain et al., 1995), the level of this important tripeptide in protein-malnutrition will be low (because of poor intake of methionine (cystine). The detoxification of cyanide to thiocyanate will be impaired under protein malnutrition because this reaction is dependent on dietary sulfur amino acids (cystine and methionine).

This study investigates the effect of NaOCN on: (i) GSH levels in brain and liver (ii) glutathione reductase (GR) and glutathione peroxidase (GPx), enzymes involved in the regulation of GSH concentrations and (iii) GSH and GSSG levels in rat brain were measured to examine the relationship between cyanate neurotoxicity and GSH homeostasis. Preliminary results of this study have been presented (Sabri et al., 1996; Tor-Agidye et al., 1995).
MATERIALS AND METHODS

Materials
Reduced (GSH) and oxidized (GSSG) glutathione, N-ethylmaleimide (NEM), o-
phthalaldehyde (OPT), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), t-butyl hydroperoxide,
ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide
phosphate(NADP), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) were
obtained from Sigma Chemical Company, St. Louis, MO. Sodium cyanate was purchased
from Fluka, Switzerland. All other reagents used were analytical grade.

Animals
Female CD-1 mice (25-35 g) and Sprague-Dawley rats (250-300g) were used. Female
animals were used in these studies because cassava-related disease (konzo) has been
reported mostly in women of child-bearing age. Rodents were obtained from Bantin and
Kingman, Seattle, WA, and housed in standard cages in a temperature-controlled room set
at a 12-h light:dark cycle. Food (Purina rat chow) and tap water were provided ad libitum.

Purification of Sodium Cyanate
Twenty grams of commercially available sodium cyanate were dissolved in 100 ml of
boiled distilled water and the solution filtered. An equal volume of 95% ethanol was added
to the filtrate and the mixture cooled on ice to about 5 °C. The precipitate was filtered and
rinsed with ice-cold ethanol. The purified sodium cyanate was dried in a desiccator oven at
60 °C. Percent yield of sodium cyanate was ~ 28% with purity of ~ 99.99% and found to
be free of cyanide.

Injection Procedure
Sodium cyanate solutions were made fresh in normal saline daily just before use and
administered intraperitoneally to rodents; controls were injected with an equal volume of
normal saline. Neurobehavioral changes were recorded for up to 30 min.

Perfusion and Tissue Preparation
Mice or rats were anesthetized with isoflurane and perfused intracardially for 1 min with
10-15 ml of ice-cold normal saline. This procedure was routinely done in all animal
experiments to remove blood from tissues. Whole brain was removed and selected brain
regions (cerebral cortex, cerebellum, mid brain, striatum, hippocampus and hypothalamus) were dissected on ice according to Glowinsky and Iverson (1966). The main lobe of the liver was also removed. Tissues were frozen on dry ice immediately and stored at -84 °C until analysis (within two weeks) for GSH and GSSG.

**GSH Assay**
Brain tissue was homogenized in 0.25 M sucrose (20% w/v) using a Wheaton glass homogenizer (8 up and down strokes) on ice. A portion of the tissue homogenate was centrifuged at 1,000 x g for 5 min to remove the nuclear fraction and unhomogenized cell debris. The supernatant was centrifuged at 10,000 x g for 10 min to obtain a crude mitochondrial fraction (P-2 fraction). The P-2 fraction was resuspended in 0.25 M sucrose. Aliquots of the suspended P-2 fraction were mixed with different concentrations of NaOCN (1, 5 and 10 mM final concentration) in 0.1M sodium phosphate buffer (pH 7.0) and incubated at 37 °C for 30 min. An equal volume of cold 10% TCA was added to precipitate protein and the mixture placed on ice for 5 min. Precipitated protein was removed by centrifugation at 4,000 x g for 5 min. GSH was determined in the supernatant by a slight modification of the method of Ellman (1959). Briefly, 900 μL of 0.90 M, K₂HPO₄ (pH 9.3) were added to the TCA supernatant (200μL), and 20 μL of DTNB reagent (36.9 mg DTNB in 10 ml of 0.1 M K₂HPO₄, pH 7.0) were added. Absorbance of the yellow color was read at 412 nm in a Perkin-Elmer spectrophotometer. A standard curve of commercially available GSH (3-30 nmol) was generated under similar conditions to determine GSH concentrations in tissue samples. GSH levels were normalized to the tissue protein determined by the method of Lowry et al. (1951).

For *in vivo* GSH studies, animals were first treated with different dosages of NaOCN, terminated after 30 min by intracardiac perfusion with saline, head decapitated, and brain regions excised. In some experiments selected brain regions were dissected, P-2 fractions were prepared and GSH concentrations assayed as described above.

**Glutathione Reductase (GR) Assay**
Brain tissue (100 mg) was homogenized with 1 ml 1.5% KCl and centrifuged at 15,000 x g for 5 min. GR activity was determined in the supernatant by the method of Carlberg and Mannervik (1985) as described by Rybak et al. (1995). Briefly, 25 μL of NADPH (2mM) in 10mM Tris-HCl buffer (pH 7.0), 25 μL of GSSG (20 mM) in phosphate buffer (0.05 M, pH 7.0 containing 0.1 mM EDTA), and 250 μL of phosphate buffer were incubated at 37 °C for 10 min. Two hundred μL of brain extract were added, and the change in
absorbance monitored at 340 nm for two min in a spectrophotometer. The millimolar extinction coefficient of 6.22 was used to determine GR activity expressed as nmoles NADPH oxidized/min/mg protein.

Glutathione Peroxidase (GPx) Assay
GPx activity was determined by the method of Rybak et al. (1995). Briefly, 400 µL of 0.05 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 100 µL of 0.01 M GSH, 100 µL of NADPH (1.5 mM), 100 µL of glutathione reductase (20 units/ml), and 100 µL of brain homogenate were added in a test tube and incubated for 10 min at 37 °C. Enzyme reaction was initiated by the addition of 100 µL of 0.1 % tetrabutyl hydroperoxide solution (prepared by diluting 1.5 µL of 70% tetrabutyl hydroperoxide to 1 mL with buffer). The rate of decrease of absorbance at 340 nm was monitored for 3 min and the activity of GPx expressed as nmol NADPH oxidized/min/mg protein.

GSH and GSSG Assay
GSH and GSSG content were determined essentially as described by Hissin and Hilf (1976). Brain tissue (50-200 mg) was homogenized in 3.7 ml of phosphate-EDTA buffer and 1 ml of 25% HPO₃ with the Polytron (Kinematica, Switzerland) on ice. The homogenate was centrifuged at 10,000 x g for 10 min at 4 °C and GSH and GSSG assayed in the supernatant. Briefly, 0.5 mL of the supernatant was mixed with 4.5 mL of the phosphate-EDTA buffer, pH 8.0. One hundred microliters of the diluted supernatant were mixed with 1.8 mL of phosphate-EDTA buffer and 100 µL of OPT (100 µg) solution added. The contents were mixed thoroughly and incubated for exactly 15 min at room temperature; fluorescence was determined at an excitation wavelength of 350nm and emission wavelength of 420 nm. For the GSSG assay a 0.5-ml portion of the supernatant was incubated with 200 µL of 0.04 M NEM for 30 min to block GSH in the sample. To this mixture, 4.3 ml of 0.1 N NaOH were added and GSSG was determined in 100-µL of this mixture by the procedure used for GSH assay. A standard curve of commercially available GSH (330-3330 pmol) and GSSG (660-6600 pmol) was generated under similar conditions to determine their levels in the samples. GSH and GSSG contents were normalized to the tissue protein determined by the method of Lowry et al. (1951).
Statistical Analysis

Data are expressed as means ± SEM. Differences between groups were considered significant at p < 0.05 by ANOVA and multiple range comparison by Fisher's protected least-significant procedure.
RESULTS

Effective dose of sodium cyanate
The effective dose of sodium cyanate (ED$_{50}$) was determined by assessing neurobehavioral changes in mice produced by different doses of the toxin. Mice dosed with 100 mg/kg sodium cyanate showed after five min signs of restlessness followed by a transient phase of weakness and palpebral closure. After 30 min all mice recovered and appeared normal. This dose of sodium cyanate was taken as a non-adverse effective dose.

Mice dosed with 200 mg/kg NaOCN exhibited reduced motor activity, ataxia and dysmetria five min after the toxin administration. Fifteen to twenty minutes later, some mice appeared sedated, stood stationary with an arched back, and were hyperreflexive to noise. In addition, mice failed to groom and scratched their cervical area with their forelimbs. At 20-30 min post injection, mice showed prominent dyspnea, hindlimb splay, prostration and palpebral closure. Weakness of hindlimbs and motor deficits in the upper extremities were also present. None of the animals developed convulsive seizures. The dose which produced adverse neurobehavioral changes without convulsive seizures in 50% of the mice was taken as effective dose (ED$_{50}$).

Mice injected with 300 mg/kg NaOCN appeared sedated and suddenly developed intense convulsive seizures often triggered by noise. One mouse (out of four) died within 15-30 min due to respiratory complications.

Effect of sodium cyanate on GSH in brain homogenate in vitro
Incubation of brain homogenate with sodium cyanate (1-10 mM final concentration) for 30 min produced a concentration-dependent loss of GSH. With 5 mM sodium cyanate, GSH was reduced to 5.3 ± 0.1 from a control value of 8.5 ± 1.1 nmol/ mg protein (Table 4.1); this represents a 64% decrease in GSH content. Increasing sodium cyanate concentration to 10 mM did not result in further loss of GSH.

Dose-effect of sodium cyanate on GSH in mouse brain
Administration of sodium cyanate to mice reduced GSH concentration of brain in a dose-dependent manner (Figure 4.1). GSH content in cyanate-treated samples was reduced to
18.3 ± 1.5, 15.8 ± 0.8 and 12.7 nmol/mg protein, with 100, 200, 300 mg/kg sodium cyanate, respectively (p < 0.05) as compared to a value of 25.5 nmol/mg protein in saline treated-mice. A 54% decrease in brain GSH occurred with 300 mg/kg sodium cyanate.

**Time-dependent decrease of GSH by sodium cyanate**
Mice treated intraperitoneally with sodium cyanate showed a time-dependent depletion of brain GSH; maximum depletion of GSH (~50%) occurred 30 min after 300 mg/kg sodium cyanate administration (Figure 4.2). GSH levels appeared to recover after 30 min however, remained significantly lower (p < 0.05) than in saline-treated controls up to 120 min after the toxin administration.

**Effect of sodium cyanate on GSH in selected brain regions**
Brain GSH levels were decreased in different brain regions following a single systemic injection of NaOCN (300 mg/kg i.p.) (Figure 4.3). Significant loss of GSH was found in the cerebellum (65%), hypothalamus (62%), midbrain (56%) and striatum (56 %), respectively, as compared to saline control. GSH levels in the cerebral cortex and hippocampus were reduced by 42 and 47 %, respectively.

**Effect of sodium cyanate on glutathione reductase activity**
Sodium cyanate administration (100 and 300 mg/kg) to mice inhibited glutathione reductase (GR) activity in selected brain regions (Figure 4.4). GR activity in both hippocampus and striatum was inhibited (~25% with 100 mg/kg and ~60% with 300 mg/kg) by sodium cyanate. Cerebral cortex and cerebellum showed relatively lower degrees of GR inhibition (~20% and ~40% respectively) than the striatum and hippocampus (~60% and 50% inhibition), respectively.

**Effect of sodium cyanate on glutathione peroxidase activity in vivo**
Brain Glutathione Peroxidase (GSH-Px) activity was not inhibited even with 300 mg/kg sodium cyanate. Infact, GSH-Px specific activity in saline control and sodium cyanate treated animals was almost identical (Table 4.2).

**Effect of sodium cyanate on GSH and GSSG in brain regions**
The effect of a single injection of either 100 and 300 mg/kg sodium cyanate on rat brain GSH and GSSG levels was investigated and the results are expressed as ratio of GSH/GSSG (Table 4.3). Sodium cyanate at 100 mg/kg had no significant effect on the GSH/GSSG ratio in the cerebellum, hippocampus and striatum but, in cerebral cortex, a
50% decrease was observed. Injection of 300 mg/kg sodium cyanate decreased GSH/GSSG ratio in cerebellum (~50%), cerebral cortex (~55%), and hippocampus (~40%). Maximum change in GSH/GSSG ratio was observed in the striatum (~60% decrease) by injection of 300mg/kg sodium cyanate.

**Effect of sodium cyanate on GSH and GSSG in rat liver**

The ratio of GSH/GSSG in the liver was decreased by sodium cyanate in a dose-dependent manner. (Table 4.4). The ratio of GSH/GSSG in rats injected with sodium cyanate100 mg/kg (60.5 %) and those administered 300 mg/kg (35.6 %) is significantly different (p < 0.01) from saline-treated rats (100 %). GSH and GSSG levels in the liver of control rats were 37.34 ± 1.3 and 8.15 ± 1.7 nmol/mg protein, respectively.
Figure 4.1  Effect of sodium cyanate on glutathione in mouse brain

Female CD-1 mice (25-30g), were injected (n=10/group) with saline and different doses of sodium cyanate (100, 200 and 300 mg/kg i.p.) in 0.5 mL normal saline. Animals were terminated after 30 min by intracardiac perfusion with cold saline. GSH content was determined in whole brain homogenate as described in Methods.

* Significantly different from the control (p<0.05) saline treated animals
Figure 4.2 Effect of a single injection of 300 mg/kg sodium cyanate on GSH in mouse brain.

Female CD-1 mice (25-30g), (=10/group) were dosed with sodium cyanate (200mg/kg) in 0.5 mL normal saline. The mice were terminated by intracardiac perfusion with cold normal saline at 15, 30, 60 and 120 min after toxin injection. Brain GSH levels were determined as described in Methods. GSH level in saline control was 18.7 ± 1.7 nmol/mg protein.

* Significantly different from time 0 min (p< 0.03) saline treated animals
Female CD-1 mice (25-30g) (n=10/group) were dosed with sodium cyanate and terminated after 30 min by perfusion with cold saline. Brains were excised and regions dissected according to Glowinski and Iversen (1966). GSH levels in saline treated control were: cerebral cortex (11.84 ± 1.70) hippocampus (11.02 ± 1.20), striatum (11.35 ± 1.0), hypothalamus (12.09 ± 1.2), midbrain (8.65 ± 1.5) and cerebellum (7.82 ± 1.7) nmol/mg protein. The values are plotted as % of control. * Significantly different from the control (p< 0.05) saline treated animals.
Mice (n=9-10/group) were injected with saline and different doses of sodium cyanate (100 and 300 mg/kg, i.p.). Glutathione reductase specific activities in brain regions of control animals was determined as described in Methods. Data are Means ± SEM.

* ** Significantly different from the control (p< 0.05 and 0.001 respectively) saline treated animals.

Figure 4.4 Effect of sodium cyanate on glutathione reductase activity in selected brain regions.
Table 4.1  Effect of sodium cyanate on GSH in brain homogenate \textit{in vitro}

<table>
<thead>
<tr>
<th>Sodium cyanate (mM)</th>
<th>nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.50 ± 1.10</td>
</tr>
<tr>
<td>1</td>
<td>8.20 ± 0.20</td>
</tr>
<tr>
<td>5</td>
<td>5.30 ± 0.10 a</td>
</tr>
<tr>
<td>10</td>
<td>5.30 ± 0.10</td>
</tr>
</tbody>
</table>

*a Statistically significant difference from control (p < 0.01)

Rat or mouse brain homogenate was incubated with different concentrations of sodium cyanate for 30 min. GSH activity was determined as described in the Methods.

Table 4.2  Effect of sodium cyanate on mouse brain glutathione peroxidase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nmoles NADPH oxidized/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>23.90 ± 1.50</td>
</tr>
<tr>
<td>300 mg/kg NaOCN</td>
<td>24.30 ± 0.90</td>
</tr>
</tbody>
</table>

Rats (n=9-10/group) were injected with either saline or sodium cyanate (300 mg/kg, i.p), terminated after 30 min by perfusion with cold saline and brain excised. Glutathione peroxidase activity was determined in whole brain homogenate as described in Methods.
Table 4.3  Effects of sodium cyanate on reduced and oxidized glutathione in rat brain regions.

<table>
<thead>
<tr>
<th>Sodium cyanate (mg/kg)</th>
<th>nmol/mg protein</th>
<th>nmol/mg protein</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH</td>
<td>GSSG</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.63 ± 0.11</td>
<td>0.26 ± 0.02</td>
<td>14.05 ±1.73</td>
</tr>
<tr>
<td>100</td>
<td>2.94 ± 0.10</td>
<td>0.39 ± 0.07</td>
<td>7.90 ±1.34*</td>
</tr>
<tr>
<td>300</td>
<td>2.49 ± 0.08</td>
<td>0.38 ± 0.03</td>
<td>6.57 ±0.37*</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.22 ± 0.11</td>
<td>0.65 ± 0.02</td>
<td>11.20 ±0.44</td>
</tr>
<tr>
<td>100</td>
<td>7.99 ± 0.05</td>
<td>0.58 ± 0.05</td>
<td>10.99 ±1.50</td>
</tr>
<tr>
<td>300</td>
<td>6.07 ± 1.13</td>
<td>1.27 ± 0.19</td>
<td>3.52 ± 0.44*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9.23 ± 0.88</td>
<td>1.19 ± 0.06</td>
<td>7.75 ± 0.40</td>
</tr>
<tr>
<td>100</td>
<td>6.15 ± 1.96</td>
<td>0.78 ± 0.13</td>
<td>7.60 ± 1.50</td>
</tr>
<tr>
<td>300</td>
<td>11.09 ± 0.46</td>
<td>2.12 ± 0.46</td>
<td>5.80 ± 1.20</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.53 ± 0.17</td>
<td>0.34 ± 0.01</td>
<td>10.29 ±0.22</td>
</tr>
<tr>
<td>100</td>
<td>3.61 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td>8.56 ± 0.24</td>
</tr>
<tr>
<td>300</td>
<td>3.21 ± 0.46</td>
<td>0.58 ± 0.06</td>
<td>5.66 ± 0.93*</td>
</tr>
</tbody>
</table>

Rats (n= 3/group) were injected with saline or sodium cyanate (100 and 300 mg/kg) and terminated after 30 min by intracardiac perfusion with normal saline. Rats were decapitated, brains excised and selected regions were dissected. GSH and GSSG concentrations were determined as in Methods.

* Statistically significant with respect to control, p < 0.05
Table 4.4 Effect of sodium cyanate on reduced and oxidized glutathione in rat liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSH/GSSG(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>37.34 ± 1.30</td>
<td>8.18 ± 1.70</td>
<td>100</td>
</tr>
<tr>
<td>100 mg/kg NaOCN</td>
<td>24.46 ± 2.10</td>
<td>9.64 ± 2.00</td>
<td>60.50 *</td>
</tr>
<tr>
<td>300 mg/kg NaOCN</td>
<td>35.56 ± 2.34</td>
<td>17.02 ± 2.60</td>
<td>35.60 *</td>
</tr>
</tbody>
</table>

* Statistically significant difference from control (p < 0.01).

Rats (n=3/group), were administered saline or sodium cyante (100 and 300 mg/kg, i.p.). Rats were perfused with cold saline, the liver removed, assayed for GSH and GSSG levels content as described in Methods. Data are expressed as the ratio of GSH/GSSG (%) by taking the ratio in saline control as 100%.
DISCUSSION

A single injection of sodium cyanate produces neurobehavioral changes in a dose-dependent manner in rodents. Based on neurobehavioral changes, 200 mg/kg NaOCN was taken as the ED_{50}. Mice appeared to be more sensitive to a single dose of 300 mg/kg cyanate than rats and developed convulsive seizures in ~80% of animals. Cerami and associates (1973) observed that mice injected with ~260 mg/kg of sodium cyanate were sedated and developed seizures triggered by noise. Cerami et al. (1973) reported that administration of NaOCN at 195 mg/kg/day resulted in seizures in 50% of rats and death by the fifth day. Gillette et al. (1974) reported that rats given 245 mg/kg NaOCN i.p, daily had 70% mortality by 48 h.

The biochemical mechanism by which sodium cyanate elicits neurotoxic effects is unknown. Orlowski and Karkowski (1979) reported that perturbation of brain GSH metabolism is associated with tremors, ataxia and limb paralysis in experimental animals. GSH is the most important thiol involved in the detoxification of xenobiotics by conjugation, a reaction catalyzed by the enzyme glutathione transferase (GSTs) (Coles, 1985). This important mechanism intercepts toxins and thereby prevents them from reacting with nucleophilic centers in key cellular macromolecules, such as proteins, RNA and DNA (Commandeur et al., 1995).

*In vitro* treatment of brain homogenate with sodium cyanate for 30 min reduces GSH content in a concentration-dependent manner. Administration of sodium cyanate (100, 200 or 300 mg/kg, i.p.) to adult mice causes rapid loss of brain GSH; GSH levels appeared to reverse 1 h after the toxin administration. The treatment of mice with 300 mg/kg NaOCN caused ~50% reduction in GSH content in brain as compared to saline-treated controls. Depletion of about 20-30% of GSH can be detrimental as reactive intermediates can disrupt vital cell functions and may cause cell death (Moldeus and Quanguan, 1987).

To our knowledge, this is the first report that shows that NaOCN decreases brain GSH concentration in vivo. Administration of diethyl maleate (DEM) to mice depleted GSH concentration in brain and liver in a dose-and time-dependent manner (Shivakumar et al., 1992). These investigators noted that hepatic GSH returned to normal levels 6 h after DEM administration, but brain GSH levels remained significantly lowered for up to 12 h.
GSH levels returned to normal levels in mice 48 h after DEM treatment (Shivakumar et al. 1992). This shows that GSH is rapidly turned over in the brain (Orlowski and Karkowski, 1976) and that GSH synthesis is not affected by DEM. Sodium cyanate has a similar effect on GSH concentration, suggesting thereby that the synthesis of GSH is not affected by NaOCN.

The toxicity of many xenobiotics is preceded by depletion of intracellular GSH. Any process which consumes GSH at a rate which exceeds the capacity of the cell to replenish its pool will cause GSH deficiency (Meister, 1991). Both conjugation of thiols with reactive electrophiles and autoxidation may cause depletion of intracellular GSH and lead to toxicity (Lomaestro and Malone, 1995).

Sodium cyanate produces a differential regional effect in GSH concentration in brain. Sodium cyanate produces a significant depletion of GSH in the hypothalamus, striatum and cerebellum, but levels were only slightly affected in cerebral cortex and hippocampus. A preferential depletion of GSH in striatum may be of interest because striatal damage has been reported in Rhesus monkey treated with NaOCN (Shaw et al., 1974). The selective depletion of GSH by NaOCN in brain regions may be due to differences in neuronal cell types. The physiological relevance of such depletion could only be understood by examining the GSH levels in individual cell types following cyanate treatment.

The mechanism by which sodium cyanate causes neurotoxicity is unknown. Carbamylation of the amino group of different enzymes, including cytochrome P-450, may be an important factor in NaOCN toxicity (Srivastava et al., 1993). GSH depletion causing mitochondrial disruption may be another mechanism associated with NaOCN neurotoxicity. GSH deficiency in newborn rodents is associated with cataract formation (Martensson et al., 1990).

Recent work has shown that the mitochondrial pool of GSH originates in the cytosol. An energy-dependent high-affinity transport system is required in the transportation of cytosolic GSH to the mitochondria (Martensson et al., 1990; Meister, 1995). Sodium cyanate may further reduce mitochondrial GSH by the direct inhibition of energy metabolism and blockade of energy-dependent GSH transport. Loss of mitochondrial GSH is implicated in neurodegenerative diseases in humans (Lomaestro and Malone, 1995). Depletion of nuclear GSH makes DNA repair less efficient, and sensitizes cells to DNA alkylating agents (Smith et al., 1996). Sodium cyanate inhibits the activity of GR, an
enzyme that regulates GSH levels in brain. Since GSH-Px activity is unaffected by cyanate, inhibition of GR might be responsible for regulating GSH level in brain.

There is little information on GSH homeostasis in the brain. Glutathione is present mainly in its reduced form. Brain GSH levels are quite high (0.5-3.3 µmol/g) (Martin and McIlwain, 1959; Orlowski and Karkowski 1976) relative to the much smaller concentrations (< 0.5 µmol/g) of brain GSSG (Folbergrova et al., 1979). An altered GSH/GSSG ratio has been reported in Parkinson’s disease (PD) (Sian et al. 1994). The percent ratio of GSH/GSSG in animals injected with 100 mg/kg sodium cyanate is 60.5 and those injected with 300 mg is 35.6 as compared to a ratio of 100 in control animals.

The concentration of GSSG in brain regions reported in this study is higher than those reported in the literature, this could be due to oxidation of GSH during various procedures (i.e. perfusion, dissection of brain regions and tissue extraction). These procedures are time consuming and considerable time (~ 30 min) is elapsed before freezing brain tissue in dry-ice. The ratio of GSSG/GSH is a sensitive index of oxidative stress (Toborek and Hennig, 1994; Hwang et al., 1992). Oxidized glutathione is rapidly reduced to GSH by a specific NADPH-dependent enzyme glutathione reductase.

In conclusion, sodium cyanate reduces brain GSH levels in a dose-dependent manner both in vitro and in vivo. GSH depletion is seen in all brain regions with significant reduction in hypothalamus and striatum. Sodium cyanate inhibits the activity of glutathione reductase, an enzyme that regulates GSH levels. These studies might be relevant to cassava-consuming populations where motor system disorders are common. While the etiology of neurological complications is not fully understood, we propose that in malnourished populations consuming cyanogenic cassava, cyanide may be preferentially converted to cyanate. Cyanate accumulation may then perturb glutathione homeostasis causing disruption of brain functions.

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REFERENCES


CHAPTER 5: SUMMARY

Data have been obtained to show that: (i) in SAA-deficient animals (protein malnutrition), CN⁻ conversion to OCN⁻ is markedly increased. A significant correlation (r = 0.93) was demonstrated between blood cyanide and plasma cyanate concentrations; (ii) cyanate is neurotoxic in the mouse, an effect which was exacerbated by pretreatment with diethylmaleate (DEM) (unpublished data) a well known depleter of GSH; (iii) GSH concentrations in brain are reduced by cyanate in a dose-dependent manner, perhaps by inhibiting glutathione reductase (GR) enzyme activity; (iv) urinary inorganic sulfate levels were decreased precipitously but thiocyanate levels were unaltered in SAA-deficient animals and (v) cyanate inhibits Cox activity and impairs oxidative phosphorylation (unpublished data). Taken together, these data support the hypothesis that a progressive accumulation of cyanate in protein-malnourished subjects is linked to neuronal dysfunction, a probable prelude to neuronal degeneration.

A low protein diet coupled with dietary cyanide exposure from excessive consumption of cyanogenic cassava has been linked with neurological disease in African populations. Although the cyanide hypothesis has not been tested in an animal model, there are strong reasons to support the proposal that konzo is caused by chronic cyanide exposure from cassava consumption and low dietary sulfur intake. Three biochemical findings support cyanide as a cause of this neurological disorder: high thiocyanate blood levels, decreased plasma hydroxocobalamin and absence of plasma cysteine and methionine, important sulfur donors for cyanide detoxification (Osuntokun, 1970, Osuntokun et al., 1970b). The main task of this thesis was to advance understanding of the mechanisms by which cyanide intake and sulfur amino acid deficiency may lead to neurological disease.

Studies were designed to (i) investigate sulfur metabolism in SAA-deficient animals; (ii) examine cyanide metabolism in SAA-deficient animals and (iii) elucidate the mechanisms of cyanate neurotoxicity in murine.

A diet free of methionine and cystine reliably and reproducibly reduced inorganic sulfate excretion in urine. Inorganic sulfate excretion dropped precipitously and approached zero in SAA-deficient animals. The sharp and rapid decrease in urinary inorganic sulfate suggests the existence of only a small pool of available endogenous sulfur. Inorganic sulfate is the major end product of sulfur metabolism and is excreted mainly via the kidney in mammals (Baker et al., 1987). Inorganic sulfate, therefore, is an
important contributor to the useful sulfane pool of a diet and its urinary concentration reflects the dietary intake of sulfur amino acids (Rosling, 1986).

Urinary SCN- levels were unchanged in these animals. These results are consistent with and extend the finding of Swenne et al. (1996) who reported lowered urinary inorganic sulfate excretion and weight loss in rats fed a low-protein diet. The results confirm the findings of Swenne et al. (1996) that even in severe protein malnutrition, available sulfur (supplemented with protein catabolism) will preferentially be used for cyanide detoxification, and most of the cyanide will be converted into SCN-. The elimination half-life of thiocyanate is estimated to be 2.7 days in healthy human subjects at physiological levels (Schulz, 1984). At a serum concentration above 250-450 µmol/L, thiocyanate is rapidly excreted into urine as the resorption in tubuli becomes saturated. This makes thiocyanate less valid as a biomarker for cyanide intake in nutritionally compromised subjects.

Rats maintained on the SAA-free diet lost body weight in contrast to the weight gain seen in animals on the balanced diet. Jain et al. (1995) reported that animals fed a SAA-deficient diet containing 0.25% L-methionine for 3 weeks failed to gain body weight. Loss of body weight is also reported in rats fed a low protein diet (~5% protein) for up to 9 weeks (Swenne et al., 1996). Subchronic feeding of a low-protein diet has been shown to produce a marked decrease in body weight in males relative to that in females (Warnet et al., 1987). Protein breakdown due to increased proteolysis may explain the loss of body weight seen in animals fed SAA-free diet. The loss of body weight in SAA-free animals may be directly attributable to the absence of methionine because this also serves as the precursor for cystine. However, excess dietary methionine is reported to modify lipid and lipoprotein metabolism, causing depressed growth, hemolytic anemia, pancreatic damage and many other disorders (Baker, 1987).

Cassava cyanogens: linamarin, cyanohydrin and HCN have different fates in the human body (Figure 5.1). Most ingested linamarin is hydrolyzed by β-glucosidase to cyanohydrin. Cyanohydrin is hydrolyzed by acetonitrile hydroxylase to HCN. HCN is absorbed through the gut wall and distributed systemically. Some linamarin may be absorbed intact but the fate of linamarin in the blood is not known; however 50% of ingested linamarin is excreted via urine (Carlsson et al., 1995, Banea, 1997).
Figure 5.1  Metabolism of cassava cyanogens and sulfur in human body.

The fate of the cyanogens and linamarin depend on microfloral activity in the gut and nutritional status. Soaking during fermentation and low pH promote breakdown of linamarin to cyanohydrin. The low pH favors further breakdown of cyanohydrin to HCN, which is released into the air.

It has been suggested that cyanide exposure from cassava may aggravate growth retardation in children by diverting SAAs from protein synthesis to cyanide detoxification, however there has been no epidemiological data to support this assumption. A mean urinary SCN/\text{SO}_4^{2-} ratio of 0.2 has been reported. This low value is consistent with the idea that cyanide exposure does not contribute to growth retardation in children (Banea, 1997). Experimental studies with rats orally treated with the cyanogen, acetonitrile, have shown that the body preferentially uses SAAs to convert cyanide to thiocyanate (Swenne, 1996). Sulfur is provided from dietary proteins containing the SAAs cysteine and methionine. In the human body, ingested SAAs and SAAs originating from endogenous protein breakdown are utilized for protein synthesis (Martensson, 1982; Mudd, 1995). Excess SAAs are oxidized to inorganic sulfate, which is rapidly excreted in urine.

The majority of ingested linamarin is absorbed from the gut and rapidly excreted intact in urine without causing any systemic cyanide exposure (Hernandez et al., 1995).
Ingested cyanohydrins are stable in the low pH of the stomach but are presumably completely hydrolyzed (cyanide) in the less acidic environment of the duodenum. Hydrogen cyanide formed is rapidly absorbed through the gastrointestinal tract. Since HCN formed during processing is rapidly lost before consumption, the dietary cyanide exposure from cassava will almost entirely result from cyanide released in the gut. Daily cyanide exposure thus depends on the amount and type of cyanogens remaining in the food after processing and the degree of cyanide released from linamarin in the gut (Rosling, 1994).

Rats fed a cyanogen, acetonitrile in drinking water have shown that the body preferentially uses SAA to convert CN⁻ to SCN⁻ (Swenne, 1996). The sulfur is provided in the body from dietary proteins containing the SAA methionine and cystine. In the body, the main part of ingested SAA as well as the SAA originating from endogenous protein breakdown are utilized for protein synthesis (Martensson, 1982; Mudd, 1995). The excess SAA are oxidized to inorganic sulfate, which is readily eliminated via urine.

These studies show an early and dramatic rise in urinary thiocyanate, a phenomenon equally apparent in both groups of animals, confirming that enzymatic conversion of cyanide to thiocyanate is a major method of cyanide detoxification in the rodent as in humans. This conversion is dependent on the availability of sulfur. The marked loss of mean body weight in cyanide-treated animals fed a sulfur-free diet, an event concurrent with the dramatic rise in urinary thiocyanate, suggests the mobilization of endogenous sulfur principally from the breakdown of body proteins (reported in Paper 1). Animals fed a balanced diet were able to increase their body weight presumably because dietary sulfur intake was sufficient to detoxify the cyanide to thiocyanate.

The dramatic rise in urinary sulfate in well fed animals may in part reflect a higher concentration of dietary methionine in the balanced diet relative to that in the standard chow on which animals were acclimated. The marked decline in urinary sulfate concentrations in SAA-deficient animals suggests a sulfur shortage and the possibility that mobilization of endogenous sulfur had begun. This interpretation is consistent with the similar mean body weights recorded at that time. Taken together, these observations strongly suggest that CN/SCN conversion is a priority detoxification pathway in the rat. This is consistent with observations in protein-deficient rats treated orally with acetonitrile (Swenne et al., 1996).

The low level of mean blood cyanide in all animals at week 2, following the five-fold increase in thiocyanate levels, suggests that trapping of cyanide by conversion of methemoglobin to cyano-methemoglobin is not the priority detoxification pathway. This is contrary to the conventional view which holds that methemoglobin sequestration of cyanide is the more rapid detoxification method. While cyanomethemoglobin is a rapid buffer
system in vitro (Lunquist et al., 1985), the present experiments suggest this mechanism comes into play only after the CN/SCN\(^{-}\) conversion pathway has been saturated. The steady increase of total blood cyanide in both groups of animals suggests that the methemoglobin/cyanomethemoglobin conversion is an effective mechanism for the development of cyanide tolerance in rodents.

The present experiment apparently was terminated before the limit of cyanide tolerance was reached; the point at which this occurs would likely correspond to the sudden appearance of free plasma cyanide (Lundquist et al., 1985) and consequent effects on neuronal function. Based on the trend for higher total cyanide levels in rats fed a sulfur-free diet, it seems likely these animals would have been the first to exhibit detectable free plasma cyanide had the experiment been prolonged. A comparable process presumably occurs in cassava-dependent humans who have measurable free plasma cyanide at the time of the sudden onset of leg weakness (konzo) after months of cassava consumption (Tylleskar et al., 1992b). By contrast, neurological deficits are not recognized in well-nourished populations which consume cassava as a regular dietary component.

The precise cause of konzo is unlikely to be free cyanide since this can be converted to two other potentially neurotoxic compounds, aminothiazoline carboxylic acid and cyanate (Lunquist et al., 1993; 1995). Plasma cyanate increased in both groups of animals, but animals fed a low SAA diet showed markedly higher levels of this neurotoxic species. The steep increase in plasma cyanate in SAA-deficient animals between weeks 3 and 4 is noteworthy, given that the disease (konzo) onset in humans is sudden but non-progressive. It is plausible that the sudden onset of the disease may be in response to elevated plasma cyanate levels and subsequent neuronal damage via an excitotoxic mechanism associated with cyanate-induced mitochondrial dysfunction. Additionally, the minor carboxylic acid (ATC), a metabolite of cyanide acts as a glutamate-like excitatory amino acid which destroys nerve cells (Bitner et al., 1995, Isom and Borowitz, 1995). While levels of this excitant amino acid were not determined, the plasma concentration increased in rodents fed a protein-deficient diet (Sweene et al., 1996). It is noteworthy that lathyrism, a disease with clinical features closely similar to those of konzo, is attributed to an unusual excitatory amino acid (\(\beta\)-N-oxalylamino-L-alanine) found in the Grass pea (Lathyrus sativa), a protein-rich legume consumed by certain north African populations (Abegaz et al, 1994).

In agreement with the present findings, a Nigerian study by Umoh (1986) shows that protein-malnourished rats retain their ability to detoxify linamarin-generated cyanide to thiocyanate. Moreover, high levels of urinary thiocyanate were found in both malnourished and well-nourished animals, as in the present study. These findings support the view that thiocyanate excretion is a reasonable quantitative measure of cyanide exposure.
in population studies (Rosling and Tyelleskar, in press). The present work shows that once a steady cyanide intake is established, this relationship holds true for at least several weeks of continuous potassium cyanide oral exposure.

Whereas our data demonstrate temporarily increasing blood cyanide concentrations in both animal groups given drinking water containing potassium cyanide, the Nigerian study was unable to detect cyanide levels in blood obtained 72 hours after the administration of a single dose of linamarin. This is consistent with the view that cyanide is rapidly converted to thiocyanate in both well-nourished and malnourished animals and not in the first instance to cyanomethemoglobin. We have demonstrated a significant increase in the plasma cyanate concentrations of SAA-deficient rats treated with cyanide for four weeks. This finding supports our hypothesis that in SAA-deficient states (as in protein-calorie malnutrition), dietary cyanide exposure will culminate in higher cyanate concentrations. Chronic and systemic administration of cyanate has been shown experimentally in macaques and rodents to induce neurodegenerative disease (Shaw et al., 1974; Alter et al., 1974). It is therefore plausible that cassava related neurodegenerative disorders are caused by chronic cyanate elevation.

Cyanate is a normal metabolite under physiological conditions and is detoxified to ammonia and carbon dioxide in the cysteine-dependent reaction catalyzed by cyanase. A cystine-deficient diet will reduce the activity of cyanase, thereby increasing cyanate concentrations.

Two interrelated mechanisms are discussed. The present results show that a single injection of sodium cyanate produces neurobehavioral changes in a dose-dependent manner. Administering sodium cyanate to animals fed rat chow produces dose-related signs of ataxia, convulsive seizure, functional hindlimb paralysis, palpebral closure and dyspnea. The biochemical basis of these changes is not understood, but could be related to mitochondrial dysfunction associated with toxic properties of cyanate. In preliminary studies, diethylmaleate (DEM), a well known glutathione depletor, was used as positive control for glutathione depletion in mouse brain. Similary, intraperinoneal injections of 100-300 mg/kg sodium cyanate dose-dependently decreased mouse brain GSH concentrations. Mice with brain GSH decreased by DEM showed an increased susceptibility to the biochemical and neurobehavioral effects of NaOCN. A substantial reduction in brain GSH was noted in mice pretreated with DEM + cyanate. These mice showed enchanced neurobehavioral changes.

GSH, a tripeptide, plays many biological protective and critical physiological functions in the cell. Since cysteine is a rate-limiting factor in GSH synthesis, absence or reduced dietary intake of SAAs may compromise GSH levels in the body.
The results of this study show that cyanate reduces GSH levels both in vitro and in vivo. Decreased GSH levels may be attributed to the inhibition of activity of glutathione reductase (GR) by NaOCN (Figure 5.2). Glutathione peroxidase (Gpx) activity was not affected. However, GSH-conjugation could be another contributing factor in cyanate neurotoxicity.

![Figure 5.2](image.png)

Figure 5.2 Inhibition of Glutathione by Sodium cyanate and other Chemicals. Modified (Harlan et al., 1984).

GSH concentration can be decreased by inhibition of glutamylcysteine synthase with buthionine sulfoximine (BSO), by conjugation of GSH transferases with diethyl maleate (DEM) or chlorodinitrobenzene (CDNB). This study shows that sodium cyanate (NaOCN) inhibits glutathione reductase activity and thereby decreases GSH concentrations in brain.

GSH is the most important thiol involved in the detoxification of xenobiotics by a conjugation reaction, catalyzed by the enzyme glutathione transferase (GSTs) (Coles, 1985). This important mechanism intercepts toxins and thereby prevents them from reacting with nucleophilic centers in key cellular macromolecules, such as proteins, RNA,
and DNA (Commandeur et al., 1995). The toxicity of many xenobiotics is preceded by depletion of intracellular GSH. Any process which consumes GSH at a rate which exceeds the capacity of the cell to replenish its thiol pool will cause GSH depletion (Meister, 1995). Both conjugation of thiols with reactive electrophiles and autoxidation may cause depletion of intracellular GSH and subsequent toxicity (Lomaestro and Malone, 1995).

Decreased GSH levels by inhibition of its synthesis with buthionine sulfoximine (BSO) or by conjugation of GSTs by diethylmaleate (DEM) or chloroditrobenzene (CDNB) has been reported (Meister, 1991, Harlan et al., 1984). This thesis has shown that NaOCN decreases GSH levels in rodent brain by inhibiting GR activity.

GSH is synthesized within the cell by two cytoplasmic ATP-dependent enzymes glutamylcysteine synthase and glutathione synthase. Since cyanate inhibits Cox activity (Tor-Agbidye et al., 1995) and blocks oxidative phosphorylation (Cammer, 1982), energy perturbation will be expected to result in decreased GSH production. Dietary amino acids cystine and methionine are other limiting factors in the synthesis of GSH. Considerable interest has been directed towards concentrations of GSH in the brain and other tissues mainly because the tripeptide is considered to have important functions in protecting cells against oxidative damage (Orlowiski and Karkowski, 1976). In this capacity, GSH acts as a free-radical scavenger and a substrate for glutathione peroxidase (Gpx) to quench peroxides.
These reactions lead to the conversion of GSH to GSSG, the latter being converted to GSH via GR (Figure 5.3).

![Glutathione redox cycle diagram](image)

Figure 5.3 Glutathione redox cycle. From (Reed, 1990)

Glutathione redox cycle is a system of GSH-related enzymes that is maintained by reducing equivalent agents such as NADPH. Hydrogen peroxide is reduced to water. Since glutathione redox cycle is ATP-dependent, energy perturbation and over production of \( \text{H}_2\text{O}_2 \) will result in accumulation of \( \text{H}_2\text{O}_2 \) and generation of hydroxyl radicals. This may damage mitochondria and cause cell death.

Both GR and Gpx are key enzymes in the regulation of GSH and GSSG concentrations. This study has shown that cyanate selectively inhibits GR activity. This may be a direct cause of GSH reduction in murine brain; however GSH conjugation by NaOCN cannot be ruled out. The molar concentration of GSSG is 1.5% of that of GSH in normal brain Martin and McIllwan (1959). Changes in the GSH/GSSG ratio were found in the brains of animals treated with cyanate.

In rats, NaOCN induced a dose-related decrease in GSH/GSSG ratio in both liver and selected brain regions. The striatum showed maximum change (~60% decrease), followed by the cortex (~50%), cerebellum (~40%) and hippocampus (~30%). The ratio of GSH/GSH in the liver showed a similar dose-related decrease (~65%), perhaps by inhibiting the activity of GR.

Depletion of GSH by sodium cyanate may also compromise mitochondrial integrity and generation of ATP leading to disruption of homeostasis and extracellular leakage of GSH (Personal communication with Dr. William Nicklas). Recent work has shown that the mitochondrial pool of GSH originates in the cytosol and is imported into mitochondria by an energy-dependent high-affinity transport system (Martensson et al., 1990; Meister, 1995). Sodium cyanate may reduce mitochondrial GSH by the direct inhibition of energy
metabolism and by blockade of energy-dependent GSH transport. Loss of mitochondrial GSH is implicated in human neurodegenerative diseases such as Parkinson disease (PD) and Huntington disease (HD) (Jain et al., 1991). Mitochondrial energy dysfunction (Beal et al., 1993) and mitochondrial DNA variants as "nuclear pseudogenes" abnormalities have been associated with neurodegenerative diseases (Wallace et al., 1997, Hirano et al., 1997). In a recent study, Smith et al., 1996 reported that depletion of nuclear GSH makes DNA repair less efficient and sensitizes cells to DNA alkylating agents.

There is little information on GSH homeostasis in the brain. Glutathione is present mainly in its reduced form. In the brain, the GSH level is quite high 0.5-3.3 μmol/g (Orlowski and Karkowski, 1976). By contrast, GSSG is present in much smaller concentrations< 0.5 μmol/g) of tissue (Folbergrova et al, 1979). The ratio of GSSG/GSH is a sensitive index of oxidative stress (Toborek and Hennig, 1994; Orlowski and Karkowski, 1976). In this study, the GSH/GSSG in animals treated with 100 mg/kg sodium cyanate was 60.5 and those injected with 300 mg was 35.6 as compared to a ratio of 100 in control animals. This result supports the hypothesis that oxidative stress plays an important role in the etiopathogenesis of neurodegenerative diseases.

The concentration of GSSG in brain regions was found to be higher than that reported in literature. This may be due to oxidation of GSH during various procedures (i.e. perfusion, dissection of brain regions and tissue extraction). These procedures are time consuming, and considerable time (~ 30 min) elapsed before freezing brain tissue in dry-ice. It is noteworthy that even under these conditions, sodium cyanate produced dose-related changes in GSH/GSSG ratio.

Oxidative stress refers to the cytopathologic consequences of a mismatch between the production of free radicals and the ability of the cell to defend against them. Experimental models and human brain studies suggest oxidative stress may play an important role in neuronal degeneration in diseases such as PD, AD, and amyotrophic lateral sclerosis (ALS). Mitochondrial oxidative metabolism, nitric oxide, phospholipid metabolism, and proteolytic pathways are potential sources of intracellular free-radicals. Alterations in free radical defense systems may also contribute to oxidative stress. Oxidative stress can occur when the production of free radicals increases, when scavenging of free radical or repair of oxidatively modified macromolecules decreases or both. A net increase in reactive oxygen species can produce damage to lipids, proteins, and DNA and induce necrosis and apoptosis. This imbalance results in a build-up of oxidatively damaged molecules that can cause cellular dysfunction and, and cell death for post mitotic cells such as neurons.
Clinical reports associate Parkinsonism with cyanide intoxication (Utti et al., 1985; Carella et al., 1988; Rosenberg et al., 1989). A high dietary intake of cyanogens from insufficiently processed cassava roots has almost exclusively been reported from poor populations which, during a period of food shortage, are saved from starvation by the availability of bitter cassava roots (Bokanga, 1993). These populations may either be those which normally do not eat bitter cassava and have no knowledge of effective processing, or populations that normally eat well-processed roots from toxic varieties but take short-cuts in processing as a desperate measure to cope with hunger (Rosling, 1996). Varying degrees of low dietary intake of cyanogens are reported in many cassava-eating populations that use ineffective processing methods (direct sun-drying).

Banea (1997) found that urinary linamarin concentrations were twice as high in konzo patients and their household members compared to control subjects. It is not possible to ascertain to what degree ingested linamarin is degraded in the gut, but recent experiments indicate that 50% or more linamarin pass the body in an unchanged form (Hernandez, 1995). The high urinary linamarin concentrations reflect a high dietary intake of the glucoside which, itself, might have an etiologic role in konzo, apart from being the source of cyanide. Linamarin absorbed into the blood might pass the blood-brain barrier and be directly toxic to neurons; or it might be metabolized by β-glucosidases in the central nervous system. This hypothesis would provide one explanation why other sources of cyanide exposure have not been linked to upper motor neuron damage.

In summary, these studies have shown that in sulfur amino acid-deficient rodents, cyanide is preferentially metabolized to cyanate. Accumulation of cyanate reduces brain GSH in vitro and in vivo in a concentration and dose-dependent manner by inhibiting GR activity. The site of lesions in TAN and konzo remains speculative (Figure 5.4).
In *konzo*, an upper motor neuron disease manifested by spastic paraparesis, the primary lesion may be the upper motor neuron or the corticospinal tracts. In TAN, lower motor neuron and peripheral nerves are also involved.

Cyanate is the proximate CNS toxin. However, we cannot exclude an additional role for the minor excitant amino acid ATC which has also been reported to increase in protein-deficient animals. Since neuronal cell death may involve disruption of energy metabolism, glutamate-mediated excitotoxicity and oxidative stress glutamate antagonists and antioxidants might be tested in therapeutic trials to arrest neuronal cell death.
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