FECAL THIAMINASE IN FEEDLOT CATTLE

by

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Introduction

Polioencephalomalacia (PEM), is a non-infectious disease of the central nervous system in ruminants. It is characterized by listlessness, circling, muscular incoordination, head pressing against solid objects, convulsions and death (Jubb and Kennedy, 1970). One cause of this disease is believed to be thiamin deficiency. The affected animals respond to parenteral thiamin, and the biochemical changes are indicative of a lack of thiamin.

The carcasses usually appear to be in good physical condition, but the pathological abnormalities are confined to the brain. The essential lesion is a laminar necrosis of the cerebral neurones, with associated perineuronal and peripapillary edema. There is a yellowish discoloration present with many of the cerebral gyri, swollen and soft to the touch (Jackman, 1985).

Cerebrocortical necrosis (CCN) is the term used in Great Britain for the same condition. For sake of uniformity, literature references to CCN have been changed to PEM throughout this thesis.

One cause of the thiamin deficiency may be the production of a thiaminase enzyme by bacteria in the rumen and intestines of affected animals. However, large quantities of the enzyme have been detected in clinically normal animals, and thiaminase- producing bacteria have been

introduced into rumens without producing PEM. The activities and pH optima of thiaminases vary with bacterial source, and it cannot be said with certainty whether ruminal pH or diets are related to PEM.

The question remains whether or not thiamin deficient animals reach their full growth potential, since thiamin must be supplied at a rate adequate for carbohydrate utilization. Estimates have been made of thiamin intake, generally based on the sum of dietary thiamin and the contribution from ruminal thiamin synthesis. These estimates may be erroneous when the effect of ruminal thiaminase activity is not taken into consideration. The primary focus of this study was to establish whether thiaminase enzyme activity is present in the gastrointestinal tracts of normal feedlot cattle.

LITERATURE REVIEW

Metabolic role of thiamin

Thiamin is an obligatory nutrient requirement for most forms of animal and plant life. Thiamin (B_1) in its active form, thiamin pyrophosphate (TPP) (also known as either cocarboxylase or diphosphothiamin), is an important coenzyme in several reactions in carbohydrate metabolism. In mammalian cells, TPP functions in several critical decarboxylation reactions, including oxidative decarboxylations of alpha-keto acids to carboxylic acids, pyruvic acid to acetyl CoA, and alpha-ketoglutaric acid to succinyl CoA. Secondly, TPP functions in the transketolase reaction of the pentose phosphate shunt; transfer of an alpha-keto group from xylulose-5-phosphate to ribose-5-phosphate to form sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate (Pike and Brown, 1984). These reactions catalyzed by the dehydrogenase complexes are necessary for the entry into the citric acid cycle of pyruvate as acetyl-CoA and alpha-ketoglutarate as succinyl-CoA. Transketolase is essential in the pentose phosphate pathway, which is the chief source of pentoses for the cell and a major source of NADPH for fatty acid and other biosyntheses.

It is believed that TPP, and(or) the closely related thiamin triphosphate, are essential for peripheral nervous system functioning. These forms of the vitamin occur in

nerve tissue and appear to play an essential role in transmission of nerve impulses (Pincus et al., 1973).

Thiamin in the Rumen

It has always been assumed that enough thiamin is synthesized by the rumen microorganisms to make ruminants independent of an extraneous thiamin supply. To estimate the thiamin requirements for ruminants, the following factors must be considered: dietary thiamin intake; microbial thiamin synthesis, as well as its enzymatic breakdown and(or) microbial utilization in the forestomach; thiamin absorption from different parts of the gut; and thiamin excretion (Breves et al., 1980). However, information on the magnitudes some of these factors is very limited. Kesler and Knodt (1952) found that concentrations of thiamin in the rumens of young calves were higher than those in the feed -- presumptive evidence for thiamin synthesis in the rumen. Edwin and Jackman (1982) stated that the thiamin requirement of a ruminant will be met by dietary intake and synthesis in the rumen, caecum and hind gut.

Thiamin intake varies, depending on the type of ration and supplements fed. Breves et al. (1980) found that on a diet where daily thiamin intake did not exceed .3 mg, with day-to-day variations of 10 to 25%, the average daily flow into the duodenum was between 1.5 and 3.5 mg -- 10 to 20 times the quantity of thiamin ingested with food. Steinberg and Kaufmann (1977) found in eight experiments that the

apparent thiamin synthesis of dairy cows averaged 32 mg per day. Breves et al. (1979) calculated that in sheep the daily flow of thiamin to the duodenum exceeded the daily intake by 18 to 24 times.

In clinical thiamin deficiency in sheep, reduction of thiamin content in the rumen was almost exclusively confined to the particle-free fluid phase (Naga et al., 1975). Gerboulet (1979) concluded that in the sheep rumen more than 90% of total thiamin is contained in the particle-free fluid and less than 10% is in the fraction made up of microbes and small feed particles. Buziassy and Tribe (1960) showed that when high-thiamin rations were fed, ruminal synthesis was very low, but when low thiamin rations were fed, synthesis was high. Naga et al. (1975) found that thiamin added to rumen contents in vitro almost entirely disappeared from the liquid phase. They speculated that this disappearance was at least partially due to uptake by microbes. The 25% loss of total thiamin during incubation was a result of microbial action, since it was not observed after pre-treatment of the samples with formaldehyde. These results indicate that either the rumen microbes utilized part of the soluble thiamin to satisfy their own needs, or they produced a thiamin-destroying enzyme (thiaminase).

Thiamin Deficiency and Polioencephalomalacia

Although the clinical signs of PEM and the biochemical functions of the vitamin are well defined, very little is

yet known of how disturbances in metabolic function caused by thiamin deficiency lead to the pathology so characteristic of the disease. Although PEM is not a simple thiamin deficiency, it may be caused by the effect of a thiamin-like metabolic inhibitor. Vorhees et al. (1975) suggested that neurological symptoms observed in thiamin deficiency might be due to decreased acetylcholine synthesis as a result of diminished acetyl-CoA production accompanying lessened activity of the thiamin dependent enzyme, pyruvate dehydrogenase.

Although several thiamin antagonists are known (Cerecedo, 1955), the two most commonly used in experimental studies are oxythiamin and pyrithiamin. Both possess potent antithiamin activity, but the mechanisms by which they oppose thiamin function differ (Rogers, 1970).

Oxythiamin is readily converted to the corresponding pyrophosphate which then competes with TPP in TPP-requiring enzyme systems. Oxythiamin depresses appetite, growth, and weight gain, and causes heart enlargement and increased blood pyruvate. No neurological symptoms have been demonstrated.

Pyrithiamin, on the other hand, exerts its antithiamin activity chiefly through its effects on thiamin kinase, the enzyme involved in formation of TPP. Pyrithiamin is not itself phosphorylated, but rather prevents conversion of thiamin to TPP. It has a marked effect on the central

nervous system and produces neurological symptoms (Rogers, 1970 and Cerecedo, 1955).

Amprolium, the 2-n-propyl pyrimidine analog of thiamin is used in the treatment of coccidiosis in chickens. When fed at high levels, it has antithiamin activity and has been used experimentally by Loew and Dunlop (1972) to produce a PEM-like condition, with reported anorexia in the terminal stages of the disease. Spicer and Horton (1981) noted significant weight losses in the weeks preceding clinical signs. Edwin et al. (1976) administered high levels of amproluim to calves and it resulted in brain lesions similar to those found in PEM cases.

The sulfate ion also appears to cause thiamin destruction in the rumen (Sadler et al., 1983, and Raisbeck, 1982). Sadler et al. (1983) produced PEM by incorporating 0.72% sulfate into the diets of steers. Raisbeck (1982) found that using gypsum ($CaSO_4 \cdot 2H_2O$) or other sulfate salts as feed intake regulators was closely related to occurrences of PEM. In the rumen, the sulfate ion is converted to sulfide. However, it must pass through sulfite during this conversion. The sulfite ion is known to destroy thiamin (Sadler et al., 1983). Brown (1979) fed gypsum on a daily basis at 0-30% of the concentrate mix. The gypsum caused variable reductions in both concentrate and hay intake. Rumen thiamin was within the normal range on day 1 of the

feeding trials, but dropped to near zero on day 2 and 3. However, no signs of PEM or thiamin deficiency developed.

High grain diets have been found to lower thiamin synthesis. Miller et al. (1983d) found that synthesis of thiamin was greatest (8 mg/day) in steers fed a gain sorghum diet, but apparent thiamin destruction varied from .5 mg/day on the corn diet to 8.3 mg/day on the wheat diet. However, Grigat and Mathison (1983) measured red blood cell transketolase and TPP effect on 239 feedlot steers at three locations and found no evidence of thiamin deficiency.

Substances with antithiamin activity occur naturally in some feeds. Originally the antithiamin factor was presumed to be an enzyme that splits the thiamin molecule thus rendering it inactive. This enzyme was referred to as thiaminase. It is now known that two types of thiaminase exist; thiaminase I and thiaminase II. These two types are discussed more fully in a later section. Thiaminase was discovered as a result of an outbreak of a disease that paralyzed silver foxes on a farm owned by J. S. Chastek. The disease, which became known as Chastek Paralysis, was traced to raw fish and was later characterized as a thiamin deficiency (Green et al., 1942). Thiaminase I occurs in a wide range of fish, shellfish and bacteria, whereas thiaminase II has so far been found only in culture fluids of various bacteria and yeastlike fungi (Whittliff and Airth, 1970).

Thiaminase I is also found in plants such as Nardoo, Braken and Rock ferns. These ferns have been associated with a PEM-like disease (McCleary and Chick, 1976).

Polioencephalomalacia

Polioencephalomalacia (PEM), also known as cerebrocortical necrosis (CCN) in Europe, has been described in many parts of the world. It occurs mainly in cattle and sheep, but it also can be found in other ruminants such as goats, deer, antelope and bison (Jackman, 1985). In cattle and sheep there is no breed or sex preference. Young animals between the ages of 2 to 7 months appear to be most susceptible, although cases outside this range occur (Edwin and Jackman, 1982).

The clinical signs of the disease involve impairment of the central nervous system and may include some but not necessarily all of the following: dullness, aimless wondering, circling, unsteadiness, incoordination, staggering, blindness, hyperaesthesia, clonic extensor spasms and lateral recumbency (Jackman, 1985). The clinical signs may last from two to six days, and if not treated, death will usually result (Edwin and Jackman, 1982).

In animals with PEM, irradiating the brain at autopsy with ultraviolet light produces fluorescence, a phenomenon first reported by Ziffo and Inglis (1974). This has been a helpful necropsy technique for confirming the diagnosis of PEM in cattle, sheep and goats (Anderson, 1984). This

characteristic autofluorescence is mainly confined to the cerebral cortex. However, within the cerebral cortex, fluorescence is scattered and almost always restricted to the superficial layers of the gray matter and follows linearly the convolutions of the gyri and sulci (Jackman, 1983). It has a yellowish or blue-green coloration depending on the intensity of reflected incident light and wavelength span of lamp used for excitation (Jackman, 1983 and Edwin et al., 1982).

In some PEM cases, intercranial pressure is so great that the cerebellum is herniated into the foramen magnum (Edwin et al., 1982 and Jackman, 1985).

The histopathological changes suggest that the predominant abnormality may be a focal or laminar necrosis of the cerebral cortex and its neurons, which in turn suggest localized anoxia (Pill et al., 1966).

Davies et al. (1965) suggested that PEM resulted from thiamin deficiency since affected animals, if treated early enough, responded dramatically to large intravenous doses of thiamin. Jackman (1985) reported that the clinical signs can be reversed rapidly by parenteral thiamin; a very useful diagnostic indication.

It has been shown that animals suffering from PEM have sub-normal tissue thiamin levels. Pill (1967) reported raised values for blood pyruvate as evidence for thiamin deficiency. Muellar and Asplund (1981), in two sheep fed

totally by an intravenous and intraruminal nutrient infusate without thiamin, found elevated blood levels of both pyruvate and lactate, which suggested the presence of a metabolic thiamin deficiency. Edwin and Jackman (1981) showed that blood levels of metabolic intermediates such as pyruvate, alpha-ketoglutarate and glyoxylate were significantly elevated in animals with PEM as compared to normal animals. Edwin et al. (1979) found tissue and brain thiamin levels in PEM animals to be approximately 20% of normal values. Thornber et al. (1980) found that the total thiamin in various regions of the brain in thiamin-deficient lambs dropped to 44 to 66% of normal values. The only deficiency sign displayed by these lambs was anorexia. This correlates with the findings in rats and pigeons by Dreyfus and Victor (1961), Ferrari et al. (1976) and McCandless et al. (1968).

Another evidence of thiamin deficiency in PEM is the lowered activity of thiamin-dependent enzymes. Pyruvate and alpha-ketoglutarate dehydrogenases were lowered in tissues (Guber, 1976), giving rise to increased pyruvate and other keto-acids in serum. Transketolase, which is also thiamin-dependent, showed a correlatable fall in activity. The lowered transketolase activity was accompanied by an increase in "percent TPP effect"; the percentage which addition of TPP to the reaction mixture increases transketolase activity. A high TPP effect inferres that the

enzyme under examination had been deficient in TPP. The estimation is usually carried out on an erythrocyte hemolysate. Figures in excess of 100 to 200 per cent have been found in animals with clinical signs of PEM (Edwin and Jackman, 1982; Thornber et al.,1981; Spicer and Horton, 1981; Mueller and Asplund, 1981; Pill, 1967; Edwin et al., 1967a; and Roberts and Boyd, 1974).

Thiaminases

Two thiaminases, designated as thiaminase I and thiaminase II, have been described. The two enzymes differ in their modes of action, although the end result is cleavage of thiamin at the methylene bridge and total loss of activity.

Work done by Edwin et al. (1968) showed that the thiamin deficiency in PEM was brought about by a thiamin-destroying enzyme in the rumen of affected animals and it became necessary to examine a large number of materials such as plants, bacterial cultures, rumen liquors and feces for the presence of thiaminases.

Thiaminase II is a hydrolase and adds the elements of water across the methylene bridge (Figure 1), but it will not hydrolyze thiamin diphosphate (Evans, 1975). Thiaminase II is inhibited by cosubstrates of thiaminase I and also by heavy metal ions (Edwin et al., 1982). Bacillus Aneurinolyticus, a spore-forming aerobe, produces thiaminase II (Edwin et al., 1982).

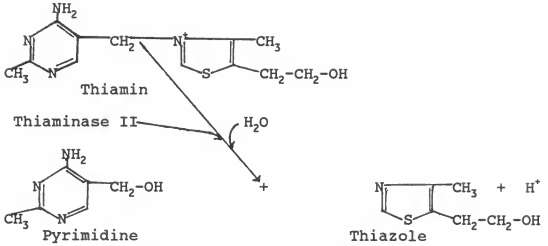

Figure 1. Cleavage of thiamin by thiaminase II.

Thiaminase I, on the other hand, is a methyl transferase that catalysis a base exchange reaction involving a nucleophilic displacement on the methylene group of the pyrimidine moiety. This reaction requires a cosubstrate, which is usually a nitrogen-containing organic base (Figure 2). Thiaminase I has been isolated from a

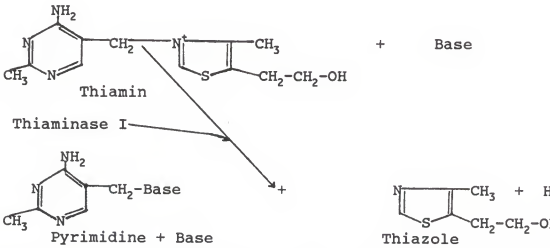

Figure 2. A base exchange of thiamin by thiaminase I.

number of bacteria, including Bacillus Thiaminolyticus, Clostridium Thiaminolyticus and Clostridium Sporogenes (Evans et al., 1975; and Shreeve and Edwin, 1974).

Haven et al. (1983) compared rumen contents of two heifers with acute PEM to rumen contents from a healthy steer fed a fibrous diet. In Animals with PEM, the net effect of thiamin metabolism by rumen bacteria was thiamin destruction by gram positive cocci, gram positive straight rods, gram negative cocci, gram negative coccobacilli and gram negative straight rods. Gram positive coccobacilli, gram positive curved rods and gram negative curved rods were absent from the population and thus had no apparent role in thiamin metabolism. The combined metabolic activities of all predominant morphological groups of bacteria in the rumens of animals with PEM was net thiamin destruction. Net thiamin production was seen by gram positive curved rods, gram negative straight rods and gram negative curved rods. In the control steers, the cumulative net effect of thiamin metabolism by the gram negative bacteria was substantial thiamin production, sufficient to negate the effects of thiamin destruction by gram positive bacteria and to result in net thiamin production.

Mann et al. (1983) isolated obligate and facultative anaerobes from rumen digesta and monitored thiaminase production. Of the organisms examined, none other than

Clostridium sp. or Bacillus sp. had high levels of thiaminase activity.

Attempts to introduce thiaminase-producing bacteria into the rumen of healthy calves or lambs have not been successful (Morgan and Lawson, 1974; and Cushine et al., 1979 and Brown, 1985). The conditions under which thiaminase-producing bacteria could survive and proliferate in a ruminal environment are not well understood, although Brent (1976) proposed that lactic acidosis might establish rumen conditions conducive to PEM development.

Murata (1982) explains that optimum pH of thiaminase I enzymes differ depending on its source and purity. Boyd and Walton (1977) found thiaminase I from rumen fluid to have two pH optima, at 4.2 and 7.6. Thiaminase from Clostridium sp. showed one pH optimum at 5.2; Bacillus Thiamininolyticus thiaminase had one pH optimum at 5.6. Kolb (1979) suggested that the optimum pH for thiaminase of Bacillus Thiaminolyticus was 5.8 to 6.8. Edwin and Jackman (1974), using nicotinic acid as the cosubstrate base, found a pH optimum of 6.4 for thiaminase I isolated from rumen fluid. Witliff and Airth (1968) reported little or no activity at pH below 4.2 and above 8.5.

The degree of activation of thiaminase I usually depends on the structure of the cosubstrate base as well as on the source of enzyme. Investigators have found a wide range of cosubstrate specificities for thiaminase I from

different sources. Mann and Smithies (1955) showed thiaminase I cosubstrates were formed during the metabolism by plant and bacterial enzymes of substances such as putrescine and cadaverine, or amino acids such as ornithine, proline, hydroxyproline and lysine. Boyd and Walton (1977) stated that aniline, nicotinic acid, pyridine, pyridoxine, histamine and imidazole were all active cosubstrates. Edwin et al. (1976) found that delta-1-pyrroline could serve as a cosubstrate for rumen thiaminase I from spontaneous PEM cases, and that the compound was present in rumen fluids and brains of animals affected with PEM. However, delta-1-pyrrolinium did not appear to possess antithiamin potential (Edwin et al., 1982).

Markson et al. (1972), used oxythiamine, a potent thiamin antagonist, and failed to reproduce the histopathology of PEM although the clinical and biochemical signs of the disease were present. Edwin et al. (1982) suggested that if the rate of thiamin depletion is very rapid, the animals could die before the encephalopathy of PEM developed. Evans et al. (1975) induced acute thiamin deficiency with PEM-like brain lesions in sheep by feeding large quantities of milled-pelleted braken rhizomes. Much evidence has shown that "thiaminase I" may be a rather broad classification of enzymes, and each individual thiaminase I may be unique in terms of cosubstrate affinity and(or) specificity.

Rumen and Fecal Thiaminases

In animals affected with PEM, thiaminase activity can be detected throughout the alimentary tract and in the feces. However, field surveys have shown that thiaminase excretion is not confined to clinically affected animals alone (Edwin and Jackman, 1982). A significant number of apparently healthy animals show fecal thiaminase activity. On some farms thiaminase excretion is persistent while on others it is sporadic (Roberts and Boyd,1974; and Edwin et al., 1976). In general, animals showing fecal thiaminase activity also show increased TPP-effects in erythrocyte hemolysates, leading to the conclusion that these animals must, to some extent, be deficient in thiamin. A survey on local farms (Edwin and Jackman, 1982) showed 25% of calves and 5% of diary cows excreted thiaminase to some degree.

Using levels of ruminal thiaminase commonly found in apparently healthy young calves, Jackman (1985) calculated that 1 mg thiamin can be destroyed per kg digesta per day. Both Roberts and Boyd (1974) and Edwin et al. (1976) established significant negative correlations between thiaminase in ruminal contents, feces and erythrocyte transketolase activity of affected animals. Spicer and Horton (1981) showed in their trials that all sheep showed negligible thiaminase activity when tested before, during and after recovery from PEM. However, most clinical submissions had quite high levels of thiaminase in feces.

Where thiaminase was present, added cosubstrate resulted in a 2 to 4 fold increase in activity indicating that a suitable cofactor as well as thiamin was limiting. High fecal thiaminase was also observed in about 5% of clinically normal animals from affected flocks. However, the sheep could be excreting high levels of thiaminase in one test, but be negative one week later. Linklater et al (1977) have also reported high levels of thiaminase in feces from clinically normal sheep in affected flocks.

Mann et al. (1983) found that when high levels of thiaminase were detected in the feces and rumen digesta, thiaminase-producing Clostridium sporogenies were in numbers of 10^2-10^3/g of feces, and thiaminase-producing Bacillus sp. were 0-10^6/g of feces. In their studies, high levels of fecal thiaminase were not always accompanied by high levels of rumen thiaminase.

Cushine et al. (1979) introduced Clostridium sporogenies into the rumens of conventional and gnotobiotic lambs. The conventional lambs failed to establish the inoculated organisms in the rumen and gut. However, the gnotobiotic lambs developed a population of Clostridium sporogenies in the rumen. Rumen and fecal thiaminase activity were consistently high throughout 86 days. Thiaminase activity was similar in both rumen and fecal samples.

Much of the inference from these experiments is that control mechanisms in the rumen prevent the proliferation of undesirable bacteria or enzymes. Outbreaks of PEM often seem to be preceded by some change in management such as movement to a new pasture, abrupt introduction of a new diet or possibly some antibiotic treatment(Edwin and Jackman, 1982).

Thiamin Absorption

Ruminants, whos' microbes synthesize thiamin, respond to thiamin when effected with PEM. This apparent discrepancy might be explained by a block in thiamin absorption from the gut. Rindi and Ventura (1972) concluded that the small intestine absorbs thiamin by two mechanisms. At high intraruminal concentrations, thiamin is absorbed passively, but at low intraruminal concentrations, thiamin is absorbed by an active process. Some doubt exists as to the exact nature of the active absorption mechanism.

Hoeller et al. (1977), using an in vitro system involving a sheep rumen wall, observed no appreciable thiamin absorption when thiamin levels were similar to those found in vivo. However, Miller et al. (1983) found that apparent intestinal absorption of thiamin was 91 and 75% of that flowing to the duodenum for steers fed high-grain and high-forage diets,respectively.

Edwin et al. (1968) established that there was no block in thiamin absorption in the alimentary tract of animals

with PEM. Even though these animals were recumbent, incoordinated and lapsing into spasms, they showed dramatic recovery, often within a half an hour after administration of thiamin by stomach tube.

Thiaminase Assays

Of the several methods of thiaminase assay available, the most convenient is the radioactive assay first proposed by Edwin and Jackman (1974). The substrate in this assay is [$^{14}C_2$-thiazole]-thiamin; the end product is [$^{14}C_2$-thiazole]. Therefore, the quantity of thiazole released is directly related to thiaminase activity. The procedure is based on the fact that the thiazole freed from thiamin during the thiaminase reaction is soluble in ethyl acetate, while thiamin itself is not. However, in their assay mixture the concentrations of both thiamin (2.2-2.9 μM) and the cosubstrate base, nicotinic acid (1.5 μM) were much less than saturating. McCleary and Chick (1976) modified the Edwin and Jackman procedure by replacing nicotinic acid with pyridine (50 mM). The rate of thiamin hydrolysis and thus the sensitivity of the assay was increased. One of the most troublesome aspects of the thiaminase assay is that both a substrate and a cosubstrate are involved. That makes the establishment of a Km difficult because changing the concentration of either substrate changes the Km of the other. In any case, many of the thiaminase values in the literature are seriously flawed because they were

established at substrate and cosubstrate concentration substantially below their Km values. Thus, many samples presumed to be negative for thiaminase were simply below the detection limit.

Brown and Brent (1985) varied thiamin, niacin (cosubstrate) and enzyme concentrations to establish an improved assay. At fixed niacin (7.4 mM) and enzyme concentrations, increasing thiamin (3.23 to 386 μM) yielded increased thiazole production until substrate saturation occurred, but additional thiamin was required to saturate the system at higher niacin concentrations. With fixed enzyme and thiamin (64.3 μM) concentrations, increasing niacin concentrations allowed increased thiazole production. Increasing the enzyme concentration increased thiazole production until substrate depletion resulted. This shows that fluctuations in sample endogenous thiamin and cosubstrates can greatly alter assays if thiaminase assays are done with low concentrations of cosubstrate and(or) thiamin.

Boyd's (1985) results with fecal thiaminase were consistent with a two step transfer mechanism. The affinity for one substrate varied with the concentration of the other substrate. When fecal thiaminase was saturated with aniline the apparent Km for thiamin was 176 μM and when it was saturated with thiamin the apparent Km for aniline was 3.19 mM. Substrate concentrations of eight times the Km values

are needed to saturate an enzyme sufficiently for it to achieve 89% of maximal activity (Boyd, 1985). Kazemi (1985) reported the Km for niacin was 5.6 mM and that for thiamin was 4.5 μM.

Much of the literature shows a much higher Km for cosubstrate than thiamin. Because of this disparity in Km values, one can expect cosubstrate concentration to be the limiting factor for thiaminase reaction. However, thiaminase from the rumen may have different enzymatic properties from the thiaminases in cultures of bacteria studied and thus may lead to underestimating thiaminase activities in ruminal fluid. If several cosubstrates are studied, the Km value for thiamin may be specific for each cosubstrate.

Edwin and Jackman (1976) assumed an extraction efficiency thiazole from an aqueous medium into ethyl acetate to be 80%; others found it to be less and dependent on the aqueous:ethyl acetate ratio. Kazemi (1985) found an extraction efficiency of 97% (slope of first extraction vs total extractions) for an ethyl acetate: aqueous ratio of 8:1. A 70% extraction efficiency was found for a 2:1 ratio.

A pre-extract readjustment of pH to 6.45 was suggested by Brown (1985), since he found an acidic pH drastically decreased the extraction efficiency. This was especially important when acidification was used to terminate enzyme action. However, Kazemi (1985) proposed a faster and more convenient method: ethyl acetate was simply added at the

appropriate time, and thiazole was extracted without stopping the reaction. His experiments showed that the presence of ethyl acetate over the reaction mixture had no effect on enzyme activity. A quick shake of the mixture extracts the thiazole released during the reaction period. As soon as the two phases are separated, small samples are taken from the top of EA phase for counting. To minimize differences in reaction time between samples, it is essential to separate reaction starts and subsequent samplings by 15 or 20 sec.

Introduction to Research

For years, nutritionists have assumed that the rumen microbial fermentation meets the requirements for B-complex vitamins. However, this assumption may need to be reexamined, especially in light of recent studies on PEM, and the destruction of thiamin by ruminal thiaminase.

Information is lacking on whether thiaminase is present in the gastrointestinal tracts of cattle handled under normal finishing conditions. The research that follows examines the fecal thiaminase incidence and levels in several pens of grain-fed cattle at several locations.

The thiaminase assay used in this study attempts to circumvent the problems with published assays, especially problems with low substrate and cosubstrate concentrations. These errors have led to the reporting of erroneously low thiaminase levels, and have led some workers to believe thiaminase was absent when, in fact, it was present at levels too low for their insensitive assays to measure.

Studies on the nature, etiology and distribution of PEM have revealed that it is a disease of cellular thiamin deficiency, probably due to the inhibitory effects of thiamin analogs on metabolic pathways. Little is known about thiamin dynamics in the gastrointestinal tract under conditions of intensive feeding.

While most of the thiamin research in ruminants has been directed toward PEM signs, it seems likely that even in

the absence of PEM, if thiaminase in the gastrointestinal tract lowers thiamin concentration below some critical level, performance might be degraded.

METHODS AND PROCEDURES

The thiaminase assay developed by Edwin and Jackman (1976) is probably the fastest and most convenient method currently available. It is based on the fact that thiazole freed from thiamin during the thiaminase reaction is soluble in ethyl acetate while thiamin itself is not. Thus, using [$^{14}C_2$-thiazole]-thiamin as the substrate allows calculation of thiaminase activity. Boyd (1985) modified that method by increasing substrate and cosubstrate concentrations. The assay used in the present study is a modification of Boyd's method. Verification of the method is discussed in a subsequent section.

Assay conditions

Temperature:	37 °C (in water bath)
pH:	5.0 (acetate buffer, 120 mM final concentration)
Reaction Time:	30 min
Substrate:	a.) [$^{14}C_2$-thiazole]-thiamin[1] (.1 μCi per assay)
	b.) unlabeled thiamin (2.7 mM final concentration)
Cosubstrate:	aniline (50 mM final concentration)
ethyl acetate:	to extract the freed thiazole
Scintillation fluid:	Fisher ScinitiVerse BD[2]

A stock solution of ^{14}C-thiamin was prepared by injecting 5 ml of 0.1N HCL into the capped serum vial that contained 50 μCi [$^{14}C_2$-thiazole]-thiamin and storing the

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[1]The radiothiamin had a specific activity of 26 mCi per mm, and was obtained from Amersham Corporation, 2636 South Clearbrook Drive, Arlington Heights, Illinois, 60005.

[2]Fisher Scientific, 1600 Parkway View Drive, Pittsburgh, Penns., 15205.

resulting solution at -20 °C. A 15 mM stock solution of nonradioactive thiamin was prepared by dissolving 5.059 g thiamin HCL in 1 l of deionized H_2O. Just prior to running assays, 1 part of the radiothiamin solution and 9 parts of the nonradioactive thiamin stock were mixed to yield a solution that was 13.54 μM in thiamin. The cosubstrate stock solution was prepared by diluting 23.28 ml aniline to 1 l with deionized H_2O. Thus, the stock cosubstrate solution was 250 mM in aniline. A 600 mM acetate buffer solution was prepared by dissolving 49.224 g sodium acetate in 900 ml deionized H_2O, titrating to pH 5.0 with 12 N HCL and bring the final volume to 1 l. The final volume of the assay volume was .5 ml, and the final assay solution contained .1 μCi labelled thiamin, and was 2.7 mM in unlabelled thiamin, 50 mM in aniline and 120 mM in acetate buffer.

The first addition into the reaction chamber was .1 ml buffer, followed by .1 ml cosubstrate solution and .1 ml substrate solution. After 5 min for the mixture to equilibrate to 37 °C, the reaction was started by adding the .2 ml of enzyme or sample solution. Incubation time was 30 min.

At the end of incubation, 4 ml of ethyl acetate (EA) was added to extract the thiazole, yielding an EA:aqueous ratio of 8:1 (0.5 ml aqueous phase and 4.0 ml EA). The thiazole was extracted without stopping the reaction. A quick and immediate shake of the mixture extracted the

released thiazole. As soon as the two phases are separated, 3 ml, of the EA was taken from the top of the EA phase and added to 10 ml of scintillation cocktail for counting. Kazemi (1986) showed that because of the fast separation of the aqueous and EA phases, stopping the reaction was not necessary, and any discrepancy between desired and actual reaction time was inconsequential.

Routine blanks with no enzyme were included in each assay to account for background activity and any radioactivity extractable into ethyl acetate in the absence of thiamin breakdown. A positive thiaminase control (.2 ml of Clostridium sporogenies culture at maximum crop) was included in each set to verify that the system was working normally.

This assay is based on Boyd's (1985) work in which substrate concentrations of eight times the Km values were needed to saturate the enzyme sufficiently for it to achieve 89% of maximal activity. Therefore, Boyd (1985) recommended that the initial concentrations of thiamin and aniline should be at least 1.5 and 25 mM respectively. The present study used 2.7 mM thiamin and 50 mM aniline.

Calculations

The following equations were used to calculate the rate of thiaminase activities, expressed as μmoles thiamin hydrolyzed per min per liter of test material.

$$\text{Thiaminase activity } (\mu m \cdot min^{-1} \cdot l^{-1}) = \frac{B_1 * cpm_2 * EARF * SDF_1 * SDF_2}{MRC * T}$$

where

B_1 = initial μmoles of thiamin per assay: (.00385 μm labelled thiamin plus 1.35 μm unlabelled thiamin = 1.3539).

cpm_p = cpm of test - cpm of blank

EARF = ethyl acetate (EA) recovery factor (EA added/EA counted) = 1.33

SDF_1 = sample dilution factor (.2 ml sample expressed as one liter) = 1000/.2 = 5000

SDF_2 = fresh feces sample dilution factor = 2 (1:1 ratio of feces and deionized H_2O). Use 1 for undiluted samples.

MRC = maximum recoverable counts (dpm of initial labelled thiamin (IDPM) * extraction efficiency * counting efficiency)

= IDPM * EE * CE = 183749 cpm

IDPM = initial dpm available = $3.7 * 10^4$ DPS/μCi * 60 sec/min * .1 μCi = 222000

EE = extraction efficiency determined for this system = .89

CE = counting efficiency determined for the counter = .93

T = incubation time in minutes = 30

Assay Verification

Clostridium sporogenies positive control

To measure the incidence of thiaminase in the feces of feedlot cattle, one must start from the null hypothesis that thiaminase is not present. That raises the necessity of being certain thiaminase can be detected when it is, in fact, present. To that end, cultures of Clostridium sporogenies were grown, their thiaminase level measured, and a sample of Clostridium sporogenies included with each fecal sample set to serve as a positive control. Clostridium sporogenies were grown as follows:

Source:	Clostridium Sporogenies from KSU food microbiology laboratory
Growth conditions:	pH 7.0, temp. 39 °C
Time:	24 hours
Growth medium:	Bacto, cooked meat medium (dehydrated) pH 7.2[1]

To rehydrate the medium, 1.25 grams of the medium was distributed into each of ten screw cap test tubes. Ten ml of cold distilled water was added to each test tube and mixed thoroughly. The test tubes were allowed to stand to insure thorough wetting of all particles, then sterilized in an autoclave for 15 min at 20 atmospheres and then allowed to cool to room temperature before inoculation of growth media.

To each tube of sterile growth media was added aseptically three drops of inoculant Clostridium sporogenies from a stored stock that had confirmed growth; then it was

[1]DIFCO Laboratories, Detroit MI.

incubated at 39 °C for 24 hours. The growth medium was examined for turbidity (indicating growth) and the specific offensive odor associated with Clostridium sporogenies. The tubes were stored refrigerated at 4 °C as stock.

Representative tubes were assayed for thiaminase activity; they were shown to hydrolyze thiamin at a rate of about 95 $\mu m \cdot min^{-1} \cdot l^{-1}$. That level of thiaminase activity proved relatively stable over a period of 90 days.

Thiamin level vs. measured thiaminase activity

To verify that a high level of thiamin was necessary for accurate thiaminase measurements, and to assure that sensitivity would not be excessively compromised by adding unlabelled thiamin, an experiment was conducted in which unlabelled thiamin was added to assay mixtures at concentrations of either .6 mM or 2.7 mM. The rate of thiamin breakdown in a Clostridium sporogenies sample increased from 39.8 $\mu m \cdot min^{-1} \cdot l^{-1}$ to 85.6 $\mu m \cdot min^{-1} \cdot l^{-1}$. However, cpm from radioactive thiazole decreased from 122053.9 to 53921.4 cpm, because a smaller proportion of the thiamin was radioactive. Thus, increasing unlabelled thiamin increases apparent detection limit, and at the same time, reduces sensitivity, but not seriously so.

Extraction efficiency

To determine the efficiency with which freed thiazole is extracted into ethyl acetate, an experiment was carried out in which thiazole present in an initial extract was

compared with the sum of thiazole present in three successive extracts. Three assays were ran, with Clostridium sporogenies as the enzyme source. At the end of the incubation period, 4 ml of EA was added and the assay mixture quickly shaken. After separation of the phases, 3 ml of EA was removed for counting and 3 ml of EA was quickly added back and shaken. This was done three consecutive times per assay.

The 3 ml of EA from the first extract yielded 59189 cpm, or 19730 cpm per ml. Three ml of EA from the second extract yielded 21518 cpm, or 7173 cpm per ml. Since 19730 cpm remained from the first extract, that number was subtracted from the second extract in order to find how much thiazole was actually removed by the second extraction. Thus, the first extraction removed (19730 x 4) = 78920 cpm. The second extraction removed (7173 x 4) - 19730 = 8962 cpm. Using the same reasoning, the third extract yielded 9855 cpm in 3 ml, or (3285 x 4) - 7173 = 5967 cpm. The blanks (1987 cpm) were considered constant, so 1987 was subtracted from each of the figures calculated above. The counts in the first extract (corrected for blank) was divided by the sum of the three extracts (each corrected for blank) The result was considered the extraction efficiency. Average extraction efficiency of the three assays was .89

Counting efficiency

Counting efficiency (ratio of cpm to dpm) was based upon external standardization. The known activity is not added to the sample, but is provided by an external radium source. The spectral index of external standard (SIE) is the index produced by analyzing the spectral distribution of the external standard (Packard, 1983). A relationship between the SIE and the measured efficiency in the sample region was established by measuring a series of quenched standards. The following formula was established for the counter used: CE = .8614 + .00014 * SIE. The SIE's reported averaged about 500. Since CE varies by only minor amounts with changes in SIE, a CE of .93 was assumed for all assays.

Partical purification of thiaminase

An attempt was made to prepare a semi-pure, stable thiaminase from Clostridium sporogenies that would relieve the necessity of using Clostridium sporogenies cultures for positive controls.

Eight hundred ml of the same growth medium used for producing Clostridium sporogenies positive control was divided into two 400 ml sealed serum bottles fitted with rubber septa. One ml of the stock culture from a 10 ml stock tube was injected aseptically into each bottle and incubated for 38 hours. At the time of inoculation, and at various times over the next 38 hours, samples were drawn aseptically

through the rubber septum and assayed for thiaminase activity. Results are shown in Figure 3.

Although Clostridium sporogenies is generally not considered an obligate anaerobe, several attempts to grow batches in non-sealed vessels produced only small amounts of growth and minimal levels of thiaminase activity.

Figure 3 demonstrates that to maximize production of thiaminase from Clostridium sporogenies, the culture medium needs to incubate for a 24 to 30 hour period under anaerobic conditions.

One bottle (400 ml) of maximum crop culture medium was centrifuged at 30000xg for 20 min, 4 °C in a refrigerated centrifuge to clarify the supernatant fluid. That supernatant fluid was used as the starting material for partial enzyme purification.

Ammonium sulfate precipitation was conducted immediately following clarification of the supernatant fluid. To achieve 75% saturation, 250 g of $(NH_4)_2\ SO_4$ was added to 400 ml of supernatant fluid (Kazemi 1985). The mixture was refrigerated at 4 °C for at least 12 hours, then centrifuged at 25000xg for 20 min in a refrigerated centrifuge. The supernatant fluid was discarded and the brownish pellet was dissolved in 50 ml of cold 0.1M pH 6.4 Na_xPo_4 buffer (prepared by titrating 0.1M Na_2HPo_4 with 0.1M NaH_2Po_4). The cloudy brown enzyme preparation was then

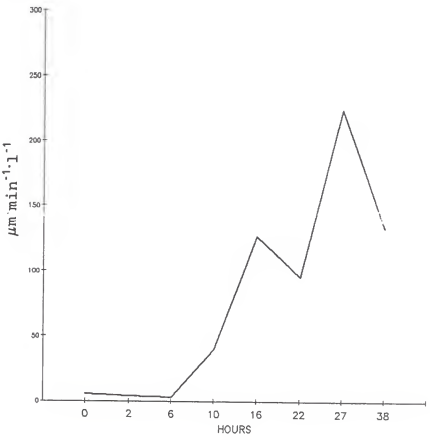

Figure 3. Anaerobic growth of Clostridium sporogenies in a 400 ml serum bottle at 39 °C.

clarified by spinning at 20000xg and 4 °C for 10 min to remove insoluble materials, and the clear but brownish supernatant fluid was stored frozen in convenient aliquotes at -20 °C

Stability of the partially purified enzyme

Assays where run at the completion of the ammonium sulfate precipitation to verify thiaminase activity (Table 1). However, over a 34 day period the thiaminase activity decreased dramatically. The #2 bottle of Clostridium sporogenies was incubated under the same conditions, but was stored at 4 °C for 80 days before enzyme purification was carried out.

At the time of thiaminase precipitation from the supernatant fluid, other enzymes may have been co-precipitated. Because Clostridium sporogenies is highly proteolytic (Brock, 1984), protease enzymes could be present in the salt precipitate. In a check of the purity of the enzyme preparation, enzyme activity without cosubstrate was 38% of that when cosubstrate was added, indicating that the preparation was contaminated with cosubstrate from the reaction mixture.

For future studies, further purification with gel filtration should be considered, to remove both contaminating proteins and cosubstrates.

Table 1. Thiaminase activity of partially purified enzyme from Clostridium sporogenies over period of time.

days[a]	$\mu m \cdot min^{-1} \cdot l^{-1}$[b]
from bottle #1	
1	254.4
10	45.4
34	12.2
36	10.3
from bottle #2	
81	57.9
82	24.6
84	24.4

[a]Enzyme purification was carried out on storage days 1 and 81 for bottles #1 and #2 respectively. Storage was at 4 °C.
[b]Thiaminase activity of Clostridium sporogenies at the end of incubation was 223.8 $\mu m \cdot min^{-1} \cdot l^{-1}$.

Stability of Clostridial thiaminase in fecal slurry

For thiaminase assays in feces to be valid, it is important to know if bacteria, bacterial enzymes, and(or) chemical entities present in the feces will deactivate the enzyme. Thus, an experiment was run in which Clostridium sporogenies was added to both fresh and autoclaved feces, and thiaminase was measured over time.

A similar experiment was run using the semi-purified enzyme, added to rumen fluid. However, because of the poor stability of the enzyme, results could not be interpreted.

Fecal samples were collected without regard to diet from 10 different penned steers and heifers at the KSU Beef Research Unit. The samples were mixed with an equal volume of deionized water and the resulting slurry was strained through one layer of cheese cloth. Five ml of the slurry

were placed into duplicate individual screw cap test tubes. The test tubes were sealed under N_2 and one set was autoclaved for 15 min at 20 atmospheres. Five ml of Clostridium sporogenies, from the stored bottle, were injected aseptically through each septum. Samples for assay were drawn at .5, 1, and 2 hours to assess changes in thiaminase activity. Results are shown in Tables 2 and 3 for fresh and autoclaved feces, respectively.

There was a statistical treatment x time interaction ($P < .01$) between the fresh and the autoclave feces (Appendix Table 1). Thiaminase appeared to decrease with time in fresh feces, and increase with time in autoclaved feces. There may be bacterial protease activity present in the fresh feces that could degrade thiaminase activity. However, it is questionable as to whether this statistical significance represents biological significance.

The thiaminase levels in autoclaved feces samples showed that there was no non-enzymatic effect from the feces on the thiaminase activity of the Clostridium sporogenies In fact, because thiaminase appeared to increase with time, Clostridia may have grown and produced additional thiaminase. These studies suggest that thiaminase I can survive in ruminant feces at least for several hours. Thus, there is probably no need to freeze samples or analyze them immediately for thiaminase.

Table 2. Thiaminase activity, *Clostridium sporogenies*[a] added to fresh feces extract, $\mu m \cdot min^{-1} \cdot l^{-1}$.

feces	hours				
	0[b]	.5	1	2	no Clost.[c]
1	87.3	66.1	76.8	76.5	3.9
2	88.1	72.4	81.6	83.4	5.4
3	88.4	90.7	73.1	80.6	6.1
4	95.9	81.6	81.5	88.9	21.0
5	87.4	83.8	74.9	68.8	4.1
6	122.1	77.5	84.7	70.5	73.4
7	91.5	87.5	80.9	75.8	12.3
8	88.3	83.1	75.4	71.0	5.8
9	88.1	92.2	78.9	78.7	5.4
10	87.2	75.7	64.4	74.0	3.6
$\bar{X}$	92.4	81.1	77.2	76.8	
SD	10.8	8.2	5.8	6.3	

[a] *Clostridium sporogenies* thiaminase activity = 170.8 $\mu m \cdot min^{-1} \cdot l^{-1}$.
[b] Calculated from Clostridial thiaminase and fecal thiaminase.
[c] SDF_2

Table 3. Thiaminase activity, *Clostridium sporogenies*[a] added to fresh autoclaved feces extract, $\mu m \cdot min^{-1} \cdot l^{-1}$.

feces	hours				
	0[b]	.5	1	2	no Clost.[c]
1	84.2	69.1	77.6	91.8	7.4
2	81.7	78.2	78.8	82.9	2.4
3	82.4	78.8	90.5	78.7	3.9
4	81.8	70.1	87.1	84.5	2.7
5	82.5	72.9	83.0	80.3	4.1
6	82.1	65.7	84.0	79.7	3.1
7	81.6	82.5	85.8	91.9	2.3
8	80.7	85.3	87.9	87.1	0.4
9	80.8	88.8	85.1	82.4	0.6
10	80.5	88.7	85.1	82.2	0.0
$\bar{X}$	81.8	78.0	84.5	84.1	
SD	1.1	8.3	3.9	4.7	

[a] *Clostridium sporogenies* thiaminase activity = 161 $\mu m \cdot min^{-1} \cdot l^{-1}$.
[b] Calculated from Clostridial thiaminase and fecal thiaminase.
[c] SDF_2

In Table 2, samples 4 and 6 show some natural thiaminase activity was present before the Clostridium sporogenies was added. When the same samples were autoclaved (Table 3), the activity has disappeared, suggesting that the thiamin destroying factor is enzymatic, and not chemical.

Fecal Thiaminase in Grain-fed Cattle

Experiment 1

Cattle from KSU Beef Research Unit

Fresh feces samples were collected at random from 37 cattle at the KSU Beef Research Unit. Diets were on an as-fed basis:

Pen I - 780 lb steers fed 24.8% corn silage, 69.9% milo and 5.3% supplement had been on this diet for 17 days.

Pen G - 545 lb heifers fed 40.2% milo silage, 40.2% corn silage, 16.2% milo and 3.4% supplement had been on this diet for 11 days.

Pen F - 625 lb heifers fed the same as Pen I had been on this diet for 14 days.

Pen S - 285 lb calves fed 57.9% milo, 34.4% whole plant silage and 7.7% supplement had been on this diet for 7 days.

Pen L - 750 lb Holstein steers fed the same as Pen I had been on this diet for 21 days.

The samples were made into a slurry by mixing a 1:1 ratio of feces and deionized water. The feces slurry was strained through one layer of cheese cloth and placed into screw cap test tubes. A subsample from each tube was autoclaved to check possible non-enzymatic thiamin degradation. Assays were run immediately after the feces slurry were made.

Results

Results from Table 4 show that there was substantial natural thiaminase activity present in many feces samples. The amount of activity varied between and within pens,

Table 4. Thiaminase activity per liter fresh feces in KSU Beef Research Unit cattle, $\mu m \cdot min^{-1} \cdot l^{-1}$.

feces	w/cosub	w/cosub[a]	w/o cosub	autoclaved
Pen I: 24.8% corn silage, 69.9% milo, 5.3% supplement				
1	10.1	8.4	5.4	4.7
2	11.5	16.8	4.1	7.2
3	0.7	--	--	--
4	146.3	131.2	6.4	1.9
5	5.7	--	--	--
6	25.6	25.5	6.2	5.2
7	3.6	--	--	--
8	29.0	28.2	5.6	2.7
9	160.5	144.4	3.3	5.1
10	10.5	12.1	4.1	3.5
11	36.5	31.5	3.1	3.4
Pen G: 40.2% milo silage, 40.2% corn silage, 16.2% milo, 3.4% supplement				
12	2.5	--	--	--
13	4.8	--	--	--
14	5.9	--	--	--
15	4.7	--	--	--
16	3.2	--	--	--
17	3.2	--	--	--
18	3.6	--	--	--
19	3.2	--	--	--
20	2.9	--	--	--
21	2.9	--	--	--
22	1.4	--	--	--
Pen F: fed same as Pen I				
23	96.9	83.8	1.5	3.3
24	94.0	79.5	3.7	2.2
25	34.1	25.2	2.2	2.8
26	6.4	--	--	--
27	5.3	--	--	--
28	6.3	--	--	--
29	7.8	8.3	1.3	2.4
30	4.7	--	--	--
31	121.4	84.3	6.8	3.7
Pen S: 34.4% whole plant silage, 57.9% milo, 7.7% supplement				
32	4.4	--	--	--
33	2.8	--	--	--
34	3.2	--	--	--
35	3.1	--	--	--
Pen L: fed same as Pen I				
36	51.7	43.7	3.6	2.9
37	0.6	--	--	--

[a]Samples set 24 hours sealed at room temperature before being re-assayed.

and appeared to be highest on the concentrate diet.

Those samples with thiaminase activity of 7.7 $\mu m \cdot min^{-1} \cdot l^{-1}$ or higher were re-assayed with and without cosubstrate, and following autoclaving. These samples set sealed at room temperature for 24 hours before being re-assayed.

Because of the high cost of [$^{14}C_2$-thiazole]-thiamin, none of the assays were run in duplicate. Comparing the re-assay data with the first assay data by a paired t-test (SAS), confirmed that changes in activity due to standing at room temperature for 24 hours (Appendix Table 2) were non - significant ($p > .8$).

A further comparison using a paired t-test (Appendix Table 3) showed there was no difference in thiaminase levels between autoclaved samples and those without cosubstrate ($p > .4$).

One problem with the assay used here is that anything that breaks the thiamin molecule at the methylene bridge will release thiazole to the ethyl acetate layer. Thus, the assay does not discriminate between chemical thiamin destruction due to the sulfite ion, hydrolysis due to thiaminase II (a simple cleavage enzyme requiring no cosubstrate), or activity of thiaminase I.

In all cases where samples were re-assayed, thiaminase levels following autoclaving or without cosubstrate approached zero. Lack of activity following autoclaving showed the activity was enzymatic and not chemical. Lack of

activity in the absence of cosubstrate showed that; 1) the cleavage was due to thiaminase I, and 2) there was little if any residual cosubstrate in the feces.

Experiment 2

Cattle from Fort Hays Experiment Station

The following experiment was performed to find if thiaminase is present in fresh feces at other locations, or under different management and feeding programs.

Fresh feces samples were collected at random from 82 cattle at the Fort Hays Experiment Station, as follows on an as fed basis:

Pen 1 and 2 - 1150 to 1200 lb steers fed 15.2% silage, 81.8% steamed flaked corn and 3% supplement.

Pen 3 and 4 - 1100 to 1200 lb steers fed 18.2% silage, 39.4% steamed flaked corn,39.4% ground milo and 3% supplement.

Pen 5 and 6 - 1125 to 1200 lb steers fed 21.2% silage, 75.8% ground milo and 3% supplement.

Pen 7 and 8 - 650 to 725 lb heifers fed 21.2% silage, 75.8% ground milo and 3% supplement.

Pen 9 - 575 lb heifers fed 21.2% silage, 75.8 % ground milo and 3% supplement.

After collection, the samples were mixed into a by 1:1 ratio of feces and deionized water. Each feces slurry was strained through one layer of cheese cloth and placed into a screw cap test tube. Assays were run immediately after the feces slurry were made.

Results

Results from Table 5 showed that considerable thiaminase was present in the feces samples. Out of the 82 samples collected, 25% of the samples had thiaminase activities of 12.7 $\mu m \cdot min^{-1} \cdot l^{-1}$ or higher. These samples

Table 5. Thiaminase activity per liter fresh feces, Fort Hays Experiment Station cattle, $\mu m \cdot min^{-1} \cdot l^{-1}$.

feces	w/cosub	w/cosub[a]	w/o cosub
pen 1		steam flaked corn	
1	42.7	61.8	2.2
2	4.9	--	--
3	3.8	--	--
4	165.1	217.1	0.2
5	3.1	--	--
6	2.0	--	--
7	251.3	331.2	1.8
8	9.5	--	--
9	23.1	21.7	0.3
10	155.5	151.9	2.3
11	116.8	121.2	2.7
12	13.6	14.8	2.1
pen 2			
13	301.5	312.3	1.7
14	39.1	37.2	0.6
15	6.2	--	--
16	6.1	--	--
17	3.5	--	--
18	5.0	--	--
19	104.6	122.8	2.5
20	7.0	--	--
21	7.2	--	--
22	361.4	406.2	7.6
pen 3		50 corn and 50 milo	
23	163.0	201.9	4.8
24	8.9	--	--
25	7.2	--	--
26	11.1	--	--
27	5.5	--	--
28	5.0	--	--
29	7.8	--	--
30	12.7	17.1	5.8
31	12.7	9.2	4.4
32	5.2	--	--
33	5.6	--	--
pen 4			
34	24.5	27.6	2.0
35	5.1	--	--
36	5.7	--	--
37	6.0	--	--
38	5.4	--	--
39	7.2	--	--
40	6.4	--	--
41	3.4	--	--
42	6.9	--	--

Table 5. Continued

feces	w/cosub	w/cosub[a]	w/o cosub
pen 5		ground milo	
43	7.5	--	--
44	6.1	--	--
45	9.0	--	--
46	20.7	20.7	0.0
47	8.9	--	--
48	3.2	--	--
49	5.1	--	--
50	4.7	--	--
51	4.6	--	--
52	4.1	--	--
53	13.3	14.4	1.0
54	4.0	--	--
55	10.7	--	--
56	7.3	--	--
57	5.4	--	--
pen 6			
58	24.4	20.4	0.0
59	6.4	--	--
60	9.1	--	--
61	6.3	--	--
62	4.6	--	--
63	4.1	--	--
64	3.2	--	--
65	6.9	--	--
66	7.1	--	--
pen 7			
67	4.8	--	--
68	11.2	--	--
69	8.5	--	--
70	10.9	--	--
pen 8			
71	1.7	--	--
72	5.2	--	--
73	28.1	27.0	0.0
74	3.6	--	--
75	3.4	--	--
pen 9			
76	8.0	--	--
77	5.4	--	--
78	3.7	--	--
79	5.1	--	--
80	430.0	490.8	14.8
81	10.5	--	--
82	33.0	36.8	2.1

[a]Samples set 24 hours sealed at room temperature before being re-assayed.

set sealed at room temperature for 24 hours before being re-assayed.

To reduce cost, it was decided to not assay the autoclaved samples unless there was a substantial amount of activity present in the samples re-assayed without cosubstrate. In only one case was the thiaminase level without cosubstrate higher than the 12.7 $\mu m \cdot min^{-1} \cdot l^{-1}$ that had been arbitiarely chosen as the cut off point for re-assay, and that sample contained the highest thiaminase level seen in the experiment.

This lack of activity without cosubstrate showed the activity was enzymatic and not chemical. The lack of activity in the absence of cosubstrate confirmed that thiaminase I was the active thiamin-hydrolyzing principal, and there was little if any residual cosubstrate in the feces.

The re-assay data were compared to the first assay data with a paired t-test to see if there was a possible change in activity from the 24 hour standing period. That test (Appendix Table 4) confirmed that the original and second assays were not different ($p > .5$). In fact, some samples increased in thiaminase activity. An increase in thiaminase during 24 hours of standing could represent active bacterial production of new thiaminase. A decrease in thiaminase under those conditions might mean a lack of bacterial growth, and

hydrolysis of the thiaminase already present by means of bacterial proteases.

This type of pattern might occur if thiaminase levels in feces cycle with time, increasing thiaminase during 24 hours of standing representing the increasing part of the cycle, and decreasing thiaminase representing the opposite. This hypothesis needs to be examined experimentally.

Experiment 3

Cattle from Ellis County Feeders

The following experiment was conducted to find the incidence of thiaminase activity under normal commercial feedlot conditions.

Fresh feces samples were collected at random from 33 cattle at Ellis County Feeders. Eighteen samples were collected from 670 lb cattle fed 87% roughage, 10% cracked corn and 3% supplement for 4 days. Fifteen samples were collected from 1100 lb cattle fed 7% chopped alfalfa, 90% cracked corn and 3% supplement for 21 days.

After collection, the samples were made into a slurry using the same procedure as in experiments 1 and 2. Assays were run immediately after the feces slurry were made.

Results

Results from Table 6 show the presence of thiaminase activity in the cattle on the starter ration. The cattle on the finishing ration had little thiaminase activity. Samples with 11.6 $\mu m \cdot min^{-1} \cdot l^{-1}$ thiaminase activity or greater were re-assayed after standing 24 hours, using the same procedure as in experiment 1 and 2. Results were consistent with earlier findings; there was little activity in the absence of cosubstrate.

Thiaminase levels on the high-concentrate diet seem inconsistent with data in Experiment 2, where high

Table 6. Thiaminase activity per liter fresh feces, commercial feedlot cattle, $\mu m \cdot min^{-1} \cdot l^{-1}$.

feces	w/cosub	w/cosub[a]	w/o cosub
pen 6-m: starting ration			
1	10.4	--	--
2	11.3	--	--
3	10.0	--	--
4	8.6	--	--
5	4.9	--	--
6	7.8	--	--
7	7.8	--	--
8	7.2	--	--
9	11.6	10.0	2.4
10	23.0	22.5	3.3
11	6.8	--	--
12	5.4	--	--
13	5.5	--	--
14	6.8	--	--
15	17.0	13.9	4.3
16	6.2	--	--
17	9.8	--	--
18	7.0	--	--
pen 3-m: finishing ration			
19	5.0	--	--
20	6.0	--	--
21	8.9	--	--
22	5.1	--	--
23	3.7	--	--
24	3.9	--	--
25	3.9	--	--
26	3.2	--	--
27	3.0	--	--
28	3.5	--	--
29	4.8	--	--
30	6.2	--	--
31	7.9	--	--
32	7.7	--	--
33	2.3	--	--

[a]Samples set 24 hours sealed at room temperature before being re-assayed

thiaminase levels were seen in eleven of 22 samples from corn-fed cattle. However, cattle in experiment 2 were fed steam-flaked corn, while those in experiment 3 received cracked corn.

The thiaminase levels on the starter diet may represent effects of an earlier diet, or the influence on the gut population of the recent (4-day) ration change. The influence of ration changes on thiaminase activity merits further research.

How Much Thiaminase is "Normal"

In an attempt to decide how much thiaminase activity is "normal," all data from the commercial feedlot, the Fort Hays Experiment Station and the KSU Beef Research Unit were combined together into one set of data. The data set was sorted by thiaminase level (the lowest to the highest), then re-assigned a serial number from 1 to 152. The thiaminase activity level was graphed with the thiaminase level on the y axis and the serial numbers on the x axis (Figure 4).

Results

Although not enough information is available to draw strong statistical conclusions, an attempt was made to evaluate the normal and abnormal ranges of thiaminase activity in the 152 samples.

A procedure was done by using a skeletal box plot to measure for outliers (Ott, 1984). By evaluation, the median was closer to the lower quartile than the upper quartile, and thus, nonsymmetrical. To include more information about extreme outliers in the tails of the distribution, a lower outer fence and an upper outer fence was calculated. This procedure defined the maximum "normal" level as 34 $\mu m \cdot min^{-1} \cdot l^{-1}$. However, inspection of Figure 4, shows a major break in thiaminase levels between 51 and 94 $\mu m \cdot min^{-1} \cdot l^{-1}$. The present study did not evaluate individual animals over time. Although no other biochemical or physiological measurements were made. No animals appeared physically abnormal.

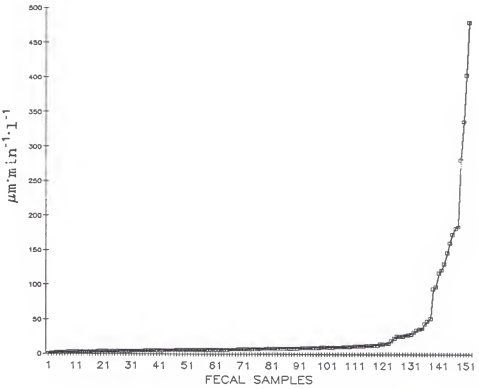

Figure 4. Thiaminase activity levels from three combined locations.

Discussion

Earlier assays of fecal thiaminase I by Edwin and Jackman (1974), failed to employ high enough concentrations of substrate and cosubstrate and under estimated the thiaminase activity in ruminant feces. There may have been thiaminase activity present which was not detected under the nonsaturating substrate and cosubstrate conditions they used. Adding unlabelled thiamin reduces cost without excessive reduction in sensitivity, and at the same time, yields enzyme saturating conditions so that even low levels of the enzyme will be detected when present.

According to the present study, fecal bacteria and (or) residual enzymes decreased only marginally when samples set at room temperature for 24 hours in sealed containers. Thus, one can collect feces samples and run "screening" assays to find which ones contain substantial levels of thiaminase activity. Then those samples can be re-assayed to confirm thiaminase activity and(or) ascertain the presence of cosubstrate or non-enzymatic thiamin destruction. By using this method, one can save assay time and money, and minimize the cost of labelled thiamin.

Thiaminase I has been found in Clostridium sporogenies and Bacillus Thiaminolyticus, but the role of thiaminase I in these microorganisms is not understood. Clostridium sporogenies and a few other species obtain their energy by fermenting amino acids or nitrogen containing compounds,

although there are strains that can ferment both sugars and amino acids. Since Clostridia have no cytochrome system, and thus cannot carry out oxidative phosphorylation, they obtain ATP only by substrate-level phosphorylation; one compound donates electrons and is oxidized, while another accepts electrons and is reduced (Brock, 1970). Thiamin, with its positive charge on the thiazole ring may be able to act as a reducing agent, oxidizing the pyrimidine ring.

Although most animals and man can synthesize both purine and pyrimidine rings, many microorganisms such as the lactic acid bacteria lack this ability. These organisms often derive their nutrients from other sources. Some soil bacteria such as certain Bacillus species produce enzymes that free pyrimidine bases from nucleic acids. These bases can be used directly as carbon, nitrogen, and energy sources or bases for other compounds. Some of these bacteria have extracellular nucleases that can act on nucleic acid at a distance from the organism (Brock 1970). Thiaminase I is believed to be an exoenzyme bound to the cell surface (Agee and Airth, 1973; Suzuki and Obida, 1973), and may operate in a similar manner.

It has been proposed (Rogers, 1962) that if a thiamin analog composed of the pyrimidine ring of the original thiamin and a cosubstrate is produced, the analog may inhibit thiamin-requiring metabolic reactions. However, If that theory is true, the inhibition must be reversible

because animals with PEM responded to large IV doses of thiamin. Thiaminase I apparently can utilize a variety of cosubstrates, so a variety of analogs should be produced, and it may be that PEM may result when a specific analog blocks a biochemical pathway. Therefore, PEM may be the result of a specific cosubstrate being present in the rumen (or lower in the GI tract), thus giving rise to a specific analog. Furthermore, because various bacteria may produce thiaminase I, only a few species may produce the specific "PEM" thiamin analogs. Thornber et al. (1979, 1981), however, proposed that thiamin analogs could not have been responsible for PEM in their experiments because of the absence of thiamin in their experiments from which to synthesize analogs.

T. Haven et al. (1983) found for PEM-affected heifers, the net effect of gram-positive cocci on thiamin metabolism was substantial destruction, with approximately 4 times as many thiamin bacteria degraders as producers. Although gram-positive straight rods comprised only about one-twentieth of the rumen population, they destroyed two-fifths of the total thiamin degraded. It seems unlikely that the population size is a critical factor, but rather what appears critical is the proportion of the total bacterial population involved in thiamin destruction, and the activity of particular components of the flora in thiamin destruction.

The present research has shown (Table 5) that a bacterial source is present in the gastrointestinal tracts of ruminants that produces thiaminase I. However, for the enzyme to become active, cosubstrate(s) need to be present, and in available form. Because several bacteria may be involved, there may be several different thiaminases involved, each with a different degree of cosubstrate specificity and thus requiring different cosubstrates. The Km values for known cosubstrates suggest that cosubstrates need to be present in relatively high concentrations. However, it is possible that the specific thiamin analog that causes PEM is produced from a cosubstrate with a low Km. In that case, only a minimal amount of the specific cosubstrate might be necessary.

Not all the cattle, even in the same pen, have high levels of fecal activity. The reason for the differences is open to question. However, for a bacteria to become established, it must first be introduced in some manner, then find an ecological niche into which it can fit. Clostridia are spore formers, so one could speculate that they could be transferred via soil of dust.

Another possibility is that bacterial thiaminase is present in all animals, but the cosubstrate (aniline) used in the present assay was not effective in activating the enzyme. However, aniline was chosen because most literature

shows it to be an effective cosubstrate for thiaminase I from a variety of sources.

The present study has shown that the thiamin-destroying principal in feces is enzymatic and corresponds in thiamin-hydrolyzing properties to thiaminase I. First, thiamin-hydrolyzing properties are lost when samples are autoclaved. Second, no thiamin hydrolysis occurs in the absence of a cosubstrate. Finally, little, if any cosubstrate is present in fecal samples, which is not surprising because these relatively small molecules have probably been absorbed further up the gut.

The lack of cosubstrate in the gut may explain why animals with high thiaminase I levels appear normal. Because Km values for known cosubstrates are high, and because the relatively small cosubstrate molecules should leave the gut fairly rapidly, one would expect the effective thiamin-hydrolyzing capability of gut contents to be minimal under normal circumstances. PEM, then, might be the result of a high level of cosubstrate. One condition conducive to PEM might be a situation in which gut microbes generate large amounts of amines from amino acids (Evans, 1975).

Based on results of the present study, additional research should be designed to address the following areas:

1) Confirmation of the metabolic thiamin status of animals with high levels of fecal thiaminase I, probably by measuring TPP effect on red blood cell transketolase.

2) Further examination of the relationship of gut thiaminase to dietary regimen.
3) The relationship between the presence of thiaminase in feces and the presence of thiaminase higher in the gut -- especially in the rumen.
4) Long term studies on individually fed animals to determine if thiaminase activity is cyclic.
5) Isolation and purification of fecal thiaminase to determine optimum pH and preferred cosubstrate(s) for the naturally -- occurring enzyme.
6) The effect of thiamin supplementation on gut thiaminase.

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APPENDIX

Appendix Table 1. Analysis of variance for Clostridium sp. added to fresh and autoclaved feces extract.

1 - The dependent variable activity:

source	d.f.	ss	ms	F-value
model	7	1861.2	265.9	5.83
error	72	3282.3	45.6	PR > F
c total	79	5143.5		.0001

R-square	c.v.	Root MSE	activity mean
.362	8.23	6.75	82.0

source	d.f.	Type III ss	F-value	P>F
TRT	1	1.13	0.02	0.875
Time	3	719.99	5.26	0.002
TRT x Time	3	1140.09	8.34	0.0001

Appendix Table 2. Analysis of variance from KSU Beef Research Unit comparing re-assay data to first assay data.

1 - The dependent variable μmoles:

source	d.f.	ss	ms	F-value
model	1	167.1	167.1	.06
error	26	67663.7	2602.4	PR > F
c total	27	67830.8		.802

R-square	c.v.	Root MSE	μmoles mean
.002	89.08	51.01	57.26

Appendix Table 3. Analysis of variance from KSU Beef Research Unit comparing the w/o cosubstrate assay to the autoclaved assay.

1 - The dependent variable µmoles:

source	d.f.	ss	ms	F-value
model	1	1.4	1.4	.54
error	26	68.5	2.6	PR > F
c total	27	69.9		.47

R-square	c.v.	Root MSE	µmoles mean
.020	41.97	1.62	3.87

Appendix Table 4. Analysis of variance from Fort Hays Experiment Station and Ellis County Feeders comparing re-assay data to first assay data.

1 - The dependent variable µmoles:

source	d.f.	ss	ms	F-value
model	1	5061.5	5061.5	.29
error	46	807900.5	17563.1	PR > F
c total	47	812962.0		.59

R-square	c.v.	Root MSE	µmoles mean
.006	129.07	132.5	102.67

FECAL THIAMINASE IN FEEDLOT CATTLE

by

Terry Hays

B.S., Oklahoma State University, 1986

AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

An improved radioactive assay of thiaminase activity included 2.7 mM unlabelled thiamin, .1 μCi [$^{14}C_2$-thiazole]-thiamin per assay and 50 mM aniline. This reduced cost without excessive reduction in sensitivity, and at the same time, gave saturation conditions so that low levels of the enzyme were detected. A sample of Clostridium sporogenies culture, at maximum crop, was included with each run to serve as a positive control.

Thiaminase assays were conducted on fresh feces from three independent locations to find if fecal thiaminase is present in ruminant feces under normal feeding conditions.

Thiaminase activity was present in all three locations and ranged from 0.6 to 479.4 $\mu m \cdot min^{-1} \cdot l^{-1}$. When substantial thiaminase levels were found, samples were re-assayed (after standing in sealed tubes for 24 hours).

The thiamin destroying principal in feces is shown to be enzymatic and corresponds in thiamin-hydrolyzing properties to thiaminase I. The thiamin-hydrolyzing properties were lost when the samples were autoclaved and in the absence of a cosubstrate. Natural cosubstrate appeared to be absent from feces. All cattle appeared to be normal even with the high thiaminase activities. Thus, PEM may depend more on the presence of a suitable cosubstrate than on thiaminase alone.

It was concluded that thiaminase is present in normal cattle under normal feeding condition, but further research

needs to be done to find why the enzyme develops and what levels of thiaminase should be considered physiologically abnormal.